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Influence of organic carbon sources and isotope exchange processes between water and nitrate on the fractionation of the stable isotopes $^{15}N/^{14}N$ and $^{18}O/^{16}O$ in dissolved nitrate during microbial denitrification in groundwater

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Zusammenfassung

Der Zugang zu sauberem Trinkwasser ist zu einem der wichtigsten Themen des 21. Jahrhunderts geworden. Der weltweite Rückgang verfügbarer Trinkwasserressourcen durch den steigenden pro-Kopf-Verbrauch einer wachsenden Weltbevölkerung wird durch den globalen Klimawandel noch weiter verstärkt. Dennoch wird die Qualität von Trinkwasser in zunehmendem Maße durch menschliche Aktivitäten wie die industrielle Landwirtschaft oder die Einleitung von Abwässern gestört. Liegen Nitratkonzentrationen von über 10 mg/l vor, deutet dies auf einen anthropogenen Eintrag hin und hat Auswirkungen auf die Gesundheit von Menschen sowie den Aufbau natürlicher Ökosysteme. Ökosysteme können jedoch auch sehr widerstandsfähig sein und ein hohes Selbstreinigungspotential besitzen, die dann in der Literatur als Ökosystemdienstleistung beschrieben werden. In diesem Kontext ist die mikrobielle Denitrifikation ein natürlicher Mechanismus, um erhöhte Stickstoffgehalte in Ökosystemen zu verringern und Wasser für den menschlichen Gebrauch wieder nutzbar zu machen.

Die Analyse stabiler Isotope im Nitrat wurde in den letzten Jahrzehnten häufig eingesetzt, um Quellen, Speicher und Senken von Nitrat in der Umwelt zu charakterisieren und Umwandlungsprozesse zu analysieren. Mariotti legte 1981 [91] die Grundlagen für die Untersuchung von Denitrifikations- und Nitrifikationsprozessen mittels stabiler Isotope. In der Folge erschienen zahlreiche isotopenchemische Studien über diese Prozesse in natürlichen und künstlichen Umgebungen. ^{15}N-gelabeltes Nitrat oder Nitrit wird darüber hinaus auch als Tracer zur Identifikation und Quantifikation biogeochemischer Prozesse verwendet. Seit einer Veröffentlichung von Kendall und McDonnell in 1998 [76] wurden natürliche isotopenchemische Zusammensetzungen von Nitrat vor allem dazu verwendet verschiedene Nitratquellen voneinander zu unterscheiden. Zu diesem Zweck wurde ein isotopenchemischer 2D-Fingerabdruck ($\delta^{18}O$ und $\delta^{15}N$) herangezogen, in dem unterscheidbare Nitratquellen in unterschiedlichen Feldern positioniert sind. Durch Kenntnis der Anreicherungsfaktoren ($\varepsilon^{18}O$ und $\varepsilon^{15}N$) aus Laborversuchen und der relativen Anreicherung der Isotope von Sauerstoff und Stickstoff ($\Delta\delta^{18}O/\Delta\delta^{15}N$) im Restnitrat bei der Denitrifikation wurde versucht, den mikrobiellen Abbau von Nitrat zu charakterisieren und zu quantifizieren. Hierdurch sollte festgestellt werden, ob gemessene Verringerungen der Nitratkonzentration im Wasser durch einen Nitratabbau verursacht werden. Um eine

derartige, genaue Bestimmung und Quantifizierung in der Umwelt zu ermöglichen, müssten die Anreicherungsfaktoren und/oder die relative Anreicherung der Isotope beider Elemente gegenüber Veränderungen der Umweltbedingungen sehr robust sein. In bisherigen Untersuchungen zeigte sich jedoch eine weite Spanne sowohl von Anreicherungsfaktoren als auch der relativen Anreicherung der beiden Isotope von Nitrat zueinander. Dadurch war eine genaue Quantifizierung nicht möglich. Es war auch unbekannt, welche Einflußfaktoren diese Variabilität hervorrufen.

Diese Einflußfaktoren waren der Fokus dieser Arbeit. Es wurden zwei Hypothesen formuliert, die als Erklärungsmodell für die starke Streuung der bisherigen Messungen und Berechnungen von Anreicherungsfaktoren im Restnitrat der mikrobiellen Denitrifikation dienen können: Erstens, könnten die Anreicherungsfaktoren der stabilen Isotope von Nitrat während der Nitratreduktion von der Art der vorhandenen Kohlenstoffquellen abhängen, indem diese die Zellphysiologie und dadurch die Isotopenfraktionierung von Nitrat verändern. Zweitens, könnte darüber hinaus ein Sauerstoffisotopenaustausch zwischen Nitrat und Wasser durch eine enzymatische Gleichgewichtsreaktion zwischen Nitrat und Nitrit in Mikroorganismen stattfinden. Da dieser Austausch nur den Sauerstoff im Nitrat betrifft, würde sich dadurch das Verhältnis der beobachteten Anreicherungsfaktoren der beiden Elemente verändern. Im Speziellen postuliert die Hypothese einen Unterschied zwischen „normalen“ denitrifizierenden Bakterien und denitrifizierenden Nitritoxidierern, wobei nur von Letzteren angenommen wird, dass sie einen derartigen Isotopenaustausch fördern. Um diese Hypothese zu testen wurden Reinkulturen von Denitrifizierern und Nitritoxidierern, sowie Sedimentproben anaerob inkubiert.

Für die Untersuchung des Verhaltens der regulären Denitrifizierer wurden anaeroben Reinkulturen von *Thauera aromatica* und “*Aromatoleum aromaticum”* (strain EbN1) drei verschiedene Kohlenstoffquellen und Nitrat zugegeben. Isotopenanalysen im Restnitrat der daraufhin auftretenden bakteriellen Nitratreduktion zeigten eine Abhängigkeit der Anreicherungsfaktoren ($\varepsilon^{18}O$ und $\varepsilon^{15}N$) der stabilen Isotope im Nitrat von den verwendeten Kohlenstoffquellen. Eine Hypothese wurde aufgestellt, die einen Bezug zwischen einer Veränderung der Zellphysiologie als Reaktion auf die Zugabe von Toluol und Benzoat und einer Transportlimitation von Nitrat in die Zelle herstellt, wodurch eine Veränderung der Anreicherungsfaktoren erklärt werden konnte. Das Wachstumsmedium dieser Kulturen

wurde darüber hinaus mit ^{18}O-markiertem Wasser angereichert ($\delta^{18}O$-H_2O~1700‰) bzw. es wurde ^{18}O-markiertes Nitrit zugegeben ($\delta^{18}O$-NO_2^- ~5200‰), um zu testen, ob es die postulierte Rückreaktion von Nitrit zu Nitrat oder einen Austausch von Sauerstoffisotopen zwischen Wasser und Nitrat gibt und ob dieser sich auf die im Nitrat beobachteten Anreicherungsfaktoren auswirkt. Sowohl *Thauera aromatica* als auch "*Aromatoleum aromaticum"* (strain EbN1) verfügen über die Nitratreduktase Nar als Enzym der Nitratreduktion. Beide zeigten keinerlei Beeinflussung der Anreicherungsfaktoren für Stickstoff und Sauerstoff im Restnitrat durch die isotopenchemisch markierten Substanzen. Man kann daher davon ausgehen, dass durch Bakterien, die dieses Enzym tragen, keine Beeinflussung der Isotopenzusammensetzung im Restnitrat durch Wasser oder Nitrit erfolgt.

Zur Untersuchung des Verhaltens von Nitrit oxidierenden Bakterien wurden Reinkulturen von *Nitrobacter vulgaris* in anoxischen Wachstumsmedien mit verschiedenen Isotopenzusammensetzungen für Sauerstoff im Wasser ($\delta^{18}O$-H_2O=-11‰ bis 396‰) und Nitrat als einzigem Elektronenakzeptor inkubiert. Die Messungen zeigten deutliche Hinweise auf einen Sauerstoffisotopenaustausch zwischen Wasser und Nitrat. Die Isotopenzusammensetzung für Sauerstoff im Nitrat zeigte eine lineare Abhängigkeit zu der verwendeten Isotopenzusammensetzung für Sauerstoff im umgebenden Wasser. In den Experimenten wurde ein Austausch von über 30% der Sauerstoffatome festgestellt. Daraufhin wurden weitere Inkubationen mit Sedimentproben aus natürlichen denitrifizierenden Umgebungen auf einen vergleichbaren Isotopenaustausch hin untersucht. Auch hier wurde die Isotopenzusammensetzung des Wassers verändert ($\delta^{18}O$-H_2O=-10‰ bis 1604‰), Nitrat zugegeben und die stabilen Isotope von Nitrat gemessen. Es war ein deutlicher — und eindeutig mikrobiell geförderter — Austausch von Sauerstoffisotopen zwischen Wasser und Nitrat zu beobachten. Bis zu 5.7±2.3% der Sauerstoffatome in Nitrat wurden durch diesen Austausch verändert, es wurde allerdings keine Limitation dieses Austausches festgestellt – ein deutlicherer Austausch ist bei einer längeren Verweilzeit sehr wahrscheinlich. Aerobe Inkubationen dieser Sedimenttypen mit Nitrit zeigten in allen Fällen ein Nitrifikationspotential, wodurch das natürliche Vorhandensein von Nitrit oxidierenden Bakterien im Sediment bestätigt wurde. Die Erklärungshypothese postuliert — auf der Grundlage eines bereits in der Literatur beschriebenen Austausches von Sauerstoffisotopen zwischen Nitrit mit Wasser — eine nachfolgende Rückreaktion zu Nitrat. Auf diese Weise wird dem entstehenden Nitrat ein weiteres Sauerstoffatom, das aus dem Wasser stammt, hinzugefügt.

Die beiden betrachteten Bakterientypen mit verschiedenen Enzymen unterscheiden sich dadurch, dass die mit der Nitritoxidoreduktase NXR ausgestatteten Bakterien einen solchen Austausch von Isotopen oder eine Rückreaktion von Nitrit zu Nitrat fördern, während jene mit der Nitratreduktase Nar keine solche Fähigkeit zeigen. Eine Mischung beider Typen von Bakterien in der Umwelt könnte daher den Sauerstoffisotopenaustausch zwischen Wasser und Nitrat in einem proportionalen Ausmaß fördern und so die Sauerstoffisotopen-zusammensetzung im Nitrat verändern. Auf diese Weise würde sowohl die relative Anreicherung der Isotope von Sauerstoff und Stickstoff ($\Delta\delta^{18}O/\Delta\delta^{15}N$) als auch die Gültigkeit des 2D-Fingerprintings beeinflusst.

Die Ergebnisse dieser Studie erklären die in der Literatur beschriebene Bandbreite an Anreicherungsfaktoren für stabile Isotope im Nitrat während der Denitrifikation, indem ein isotopenchemisches Modell vorgeschlagen wird, welches Transportprozesse und Veränderungen der Zellphysiologie als Reaktion auf veränderte Kohlenstoffquellen mit der Isotopenfraktionierung von Nitrat ($\varepsilon^{18}O$ and $\varepsilon^{15}N$) in Bezug setzt. Weiterhin wurde ein bakteriell geförderter Sauerstoffisotopenaustausch zwischen Wasser und darin gelöstem Nitrat nachgewiesen, wodurch sich die verschiedenen beschriebenen relativen Anreicherungsfaktoren der beiden Elemente von Nitrat ($\Delta\delta^{18}O/\Delta\delta^{15}N$) während der Nitratreduktion erklären lassen. Darüber hinaus zeigt der Nachweis dieses Isotopen-austausches, dass die stabilen Isotope von Sauerstoff in Nitrat nicht immer für eine eindeutige Quellenbestimmung des Nitrats oder den sicheren Nachweis einer Denitrifikation verwendet werden können.

Abstract

The access to clean groundwater for drinking and agricultural use has become a prime issue of the 21st century. An increasing demand and scarcity is to be expected in the face of growth in human population and consumption as well as reduced supply due to global climate change. Yet, the quality of groundwater is further disturbed by agricultural activities and introduction of wastewaters. Excess nitrate in the groundwater at concentrations of more than 10 mg/l is mostly of anthropogenic origin and affects human as well as ecosystem health. But resilient ecosystems also show considerable self purification strategies. By that, they provide valuable Ecosystem services, for example by purifying water. In the case of excess nitrogen, microbial denitrification is a major natural mechanism reducing the load of nitrate in the aquatic environment and providing clean water for human consumption.

Stable isotopes of nitrogen and oxygen have been widely used in ecosystem studies to determine sources, pools and processes of the nitrogen cycle, including the parameters concerning nitrate in the subsurface. Mariotti described in 1981 [91] the fundamentals of the behavior of stable isotopes in nitrate during denitrification and nitrification and since then numerous studies have used natural and artificially created isotope compositions of nitrate to describe transformation processes involving nitrate in the environment (denitrification, nitrification, DNRA, Anammox,…). Stable isotopes of nitrate have also been widely applied to distinguish sources of nitrate in water following a publication by Kendall and McDonnell in 1998 [76]. To describe sources and microbial reduction of nitrate in the environment, not only 2D-Fingerprinting of nitrate ($\delta^{18}O$ and $\delta^{15}N$), but also the relative increase of these two isotopic parameters during nitrate reduction ($\Delta\delta^{18}O/\Delta\delta^{15}N$) as well as the isotope enrichment factors of both elements of nitrate ($\varepsilon^{18}O$ and $\varepsilon^{15}N$) have been used. The enrichment factors were considered to be tools to assess and quantify denitrification in the environment. For this purpose, the enrichment factors would have to be robust under varying environmental conditions. However, a wide range of enrichment factors and the relationship between the enrichment of the two involved elements were found in field studies as well as laboratory experiments.

To explore the reasons behind this variability in the isotope enrichment factors ($\varepsilon^{18}O$ and $\varepsilon^{15}N$) of nitrate during bacterial nitrate reduction and the variability in the relative enrichment of the two involved elements, nitrogen and oxygen ($\Delta\delta^{18}O/\Delta\delta^{15}N$), two

hypotheses were formed in this work. Carbon sources were hypothesized to have an influence on cell physiology and correspondingly on the observed isotope enrichment factors for residual nitrate during denitrification. Also, an isotope exchange of oxygen between water and nitrate under certain conditions was hypothesized to change the isotopic composition of nitrate in only one of its two elements. Hence it would change the ratio of relative enrichment of both elements during nitrate reduction. The hypothesis was that nitrite oxidizing bacteria can promote an isotope exchange between water and nitrate under anoxic conditions, while regular denitrifying bacteria do not catalyze such an exchange. To test this hypothesis, anoxic incubations of pure cultures of denitrifying and nitrifying bacteria as well as sediment samples were conducted.

By adding three different carbon sources and nitrate as only electron acceptor to pure anaerobe cultures of the regular denitrifying bacteria *Thauera aromatica* and "*Aromatoleum aromaticum"* (strain EbN1), a dependency of nitrate stable isotope enrichment on the carbon source was found. An explanatory model for this effect was devised, involving changes in the transport kinetics of nitrate from the growth medium to the nitrate reductase enzyme depending on an adaptation in the cells physiology to toluene and benzoate as carbon sources. Growing the same cultures in strongly ^{18}O-labeled water with a $\delta^{18}O\text{-}H_2O$ of ~1700‰ or adding strongly ^{18}O-labeled nitrite with a $\delta^{18}O\text{-}NO_2^-$ of ~5200‰ to the cultures served as a test for a possible oxygen isotope exchange between water and nitrate. For *Thauera aromatica* and "*Aromatoleum aromaticum"* (strain EbN1), this test was negative and no oxygen exchange with water, reoxidation of nitrite or influence of either of them on the enrichment factors in the residual nitrate was observed. Both strains of bacteria carry the Nar nitrate reductase. The nitrate reducing step in bacteria carrying the nitrate reductase enzyme Nar is thus considered to be irreversible.

Batch incubations of nitrite oxidizing bacteria (*Nitrobacter vulgaris*) in anoxic growth medium with various isotopic composition of water ($\delta^{18}O\text{-}H_2O$=-11‰ to 396‰) and nitrate as only electron acceptor provided strong evidence of a microbially catalyzed oxygen isotope exchange between water and nitrate. The isotopic composition of oxygen in dissolved nitrate changed in a linear dependency to the isotopic composition of oxygen in water. An exchange of more than 30% of the oxygen atoms was observed in this case. Further batch incubations were conducted with three natural sediments from denitrifying environments to

determine if the isotope exchange effects observed for nitrite oxidizing bacteria are applicable to the field. Again, the isotopic composition of oxygen in water was changed ($\delta^{18}O$-H_2O=-10‰ to 1604‰), nitrate was added and nitrate stable isotopes were measured. A clearly observable oxygen isotope exchange between nitrate and water occurred in the microbially active incubations of all three sediment types. The maximum observed isotope exchange affected 5.7±2.3% of the oxygen atoms in the dissolved nitrate, but there was no plateau observed, leaving the possibility of a more extensive exchange given more time. Using additional incubations of these sediment types with nitrite under oxic conditions allowed the determination of a presence of nitrite oxidizing bacteria in all of them by observing nitrite oxidation. A hypothesis was formed to explain the mechanism of the oxygen isotope exchange observed: Nitrite is known to exchange oxygen atoms with water and thus adopt the oxygen isotopic composition of water to a high degree. A reversal of the nitrate reducing step under anoxic conditions would transport this isotopic signature to the dissolved nitrate and additionally add another oxygen atom from water to the nitrate produced. The difference between the two enzymes studied reflects itself in their ability to promote this process. The nitrate reductase enzyme Nar which is part of most regular denitrifying bacteria is incapable of such a reverse step, while the nitrite oxidoreductase NXR involved in nitrification and denitrification by nitrite oxidizing bacteria is inherently reversible and can promote nitrite oxidation under anoxic conditions. A mixture of both types of bacteria in a microbial community can thus promote an oxygen exchange between ambient water and dissolved nitrate to various degrees. As a result, the oxygen isotope composition of nitrate is not stable and 2D-fingerprinting of nitrate may be influenced on the $\delta^{18}O$-axis by the described oxygen isotope exchange. The enrichment factor for oxygen in residual nitrate during denitrification may be influenced as well, creating a different ratio in the relative enrichment of $\delta^{18}O$ over $\delta^{15}N$.

The results of this study thus provide an explanation for the variable enrichment factors previously observed for nitrate reduction by proposing a model involving carbon sources and cell physiology as a major influence on the isotopic enrichment factors ($\varepsilon^{18}O$ and $\varepsilon^{15}N$) observed in residual dissolved nitrate during bacterial nitrate reduction. An exchange of stable oxygen isotopes between ambient water and the residual dissolved nitrate during denitrification can have a strong influence on the relative enrichment of the isotopes of the two elements of nitrate, nitrogen and oxygen. As nitrogen is not affected, the relative

enrichment ($\Delta\delta^{18}O/\Delta\delta^{15}N$) changes in the way as was observed in previous studies. Additionally, the oxygen isotope exchange between nitrate and water can interfere with the determination of sources of nitrate in the environment when it heavily relies on oxygen isotope values of dissolved nitrate.

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Publications and clarification of contribution, authorship and copyright

Parts of this manuscript as well as some of the figures and data herein have been submitted or accepted for publication in scientific journals:

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The PhD candidate has designed, planned and conducted the experiments and prepared and analyzed all samples independently. The PhD candidate also measured all sample values except the isotopic composition of water, which was done by Harald Lowag (technician in the work group of Dr. Martin Elsner). The PhD candidate processed the data, applied statistical analysis and finally prepared the figures and manuscript texts. Prof. Dr. Rainer U. Meckenstock and Prof. Dr. Florian Einsiedl provided the idea and general concept of the project and organized funding by the DFG and access to the laboratories of the Helmholtz Center Munich and supervised the work of the PhD candidate. They also discussed results, suggested modifications in the concept and revised the manuscripts. Prof. Dr. Florian Einsiedl (TU Munich), Prof. Dr. Michael Böttcher (Leibniz-Institute for Baltic Sea Research) as well as Dr. Emanuel Braig, Uta Gruenert and Nicolas Eckert (TU Munich) provided samples of sediment and water for some of the experiments in chapter 3.

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1 Introduction

1.1 Stable isotopes in aquatic biogeochemistry

Most of the chemical elements involved in biogeochemical cycles on earth exist as different isotopes. These isotopes have a different number of neutrons, but share the same number of protons and electrons which determine their chemical properties. The notion for describing isotopes is ^{m}X with m equaling the mass number of the isotope and X being the designation of the chemical element. The mass number is the sum of protons and neutrons in the nucleus. Examples are ^{15}N and ^{14}N which describe two isotopes of nitrogen (N), both with 7 protons, but with 8 and 7 neutrons, respectively.

In contrast to radioactive isotopes, stable isotopes are rarely created or destroyed, but are rather enriched or depleted in biogeochemical pools by various processes such as transport and physical or chemical reactions [53]. These processes often either prefer light over heavy isotopes or vice versa, leaving the residual pool enriched in one of the isotopes compared to the product pool of the process. This is called an isotope effect or isotope fractionation [136]. It has a variety of applications in identifying pools and flow-paths of elements in nature and its observation is for example used to study ecological patterns, food-webs, mass fluxes and biochemical processes [53].

As usually one isotope of an element is the dominant one and others occur only in minor fractions, the ratio between them (Eq. 1.1) changes only by a small fraction due to isotope effects. For that reason, the δ-notion (Eq. 1.2) is commonly used in stable isotope geochemistry [91]:

$$R = \frac{^{h}X}{^{l}X} \qquad \text{Eq. 1.1}$$

$$\delta^{h}X = \left(\frac{R_{sample}}{R_{standard}} - 1\right) \qquad \text{Eq. 1.2}$$

R is the Ratio of a heavy (h) over a light (l) isotope of a certain element (X) in a sample. In an IRMS, samples are not analyzed as absolute ratios but in comparison to standards with a known value for δ to eliminate influences of the instrument on the measurement. Also, by standardizing all measurements in respect to a common reference material and using the δ-notation, the small changes in isotopic composition become more apparent.

Table 1.1: Commonly studied stable isotopes, their respective isotope standards and isotopic properties [5; 53].

Element	High mass	Low mass	$\frac{\text{High mass}}{\text{Low mass}}$	Aver. % high mass	Aver. % low mass	Standard (name)	$\frac{h_X}{l_X}$ of Std.	Common range of δ
hydrogen	^{2}H	^{1}H	2.00	0.02	99.98	SMOW	0.0001558	±700‰
oxygen	^{18}O	^{16}O	1.13	0.20	99.76	SMOW	0.0020052	±100‰
						VPDB	0.0020672	
nitrogen	^{15}N	^{14}N	1.07	0.36	99.64	AIR	0.0036765	±90‰
carbon	^{13}C	^{12}C	1.08	1.11	98.89	VPDB	0.0111800	±110‰
sulfur	^{34}S	^{32}S	1.06	4.21	95.02	VCDT	0.0441626	±150‰

Values for δ are often in a range of -100‰ to +100‰. By using this notation, a valid comparison between laboratories is facilitated as well. Positive δ-values represent materials that contain more of the heavy isotopes than the reference material. In contrast, negative δ-values represent materials that are lighter. The standards in table 1.1 are usually used to measure some commonly studied pairs of stable isotopes of hydrogen, oxygen, nitrogen, carbon and sulfur.

Isotope effects are described by a fractionation factor α which depends on the kinetics of a process involving two stable isotopes of the same element (Eq. 1.3):

$$\alpha = \frac{^{H}k}{^{L}k} \qquad \text{Eq. 1.3}$$

In this case, ^{H}k is the kinetic constant of a process for molecules containing the heavy isotope, while ^{L}k is the kinetic constant of the same process for molecules containing the light isotope. As α differs only slightly from 1, it is common to describe isotope effects in terms of an enrichment factor ε (Eq. 1.4).

$$\varepsilon = (\alpha - 1) \qquad \text{Eq. 1.4}$$

As with the δ-notion, this expression allows for a better inter-laboratory comparison of results and permits working with numbers in the same range as δ. A process favoring light isotopes over heavy isotopes has an isotopic enrichment factor ε<0 (α<1), a process that is indiscriminate of the two isotopes has an ε=0 (α=1) and one that favors heavy isotopes over light ones has an ε>0 (α>1).

In general, two types of isotope effects are distinguished, depending on the kinetics involved. An equilibrium isotope effect describes the enrichment of one pool for the element in relation to another pool when these pools are connected by a process that is fully reversible with equal forward and backward reactions. An example would be the headspace and liquid phase in a closed bottle after a steady state has been reached. The kinetic isotope effect describes the fractionation which happens during a predominantly unidirectional process that has a source and a product and that has not reached equilibrium. This is typically the case for open systems in which the products are removed from the scope of observation, for example evaporation of surface water. It is also typically the case for ongoing chemical reactions that transform the chemical compounds containing the observed isotopes such as the oxidation of a hydrocarbon compound to CO_2 and H_2O. In a closed system, kinetic isotope effects eventually stop influencing the system once the system has reached a steady state, usually after all substrate has been depleted. Batch cultures in the laboratory are an example of a closed system. In how far processes in the environment can be approximated by this model as well, for example in the case of biodegradation in a contaminant plume, was discussed by Abe and Hunkeler [1]. Closed systems are described by the "Rayleigh equation" (Eq. 1.5) which correlates a decrease in the concentrations of the source compound to the change in isotopic composition of that compound as a result of isotope fractionation with the fractionation factor α.

$$\propto\times \ln(\frac{L_t}{L_0}) = \ln(\frac{H_t}{H_0}) \qquad \text{Eq. 1.5}$$

Here, H_t and H_0 denote the concentrations of the heavy isotopes, L_t and L_0 that of the light isotopes in the substrate at times t and the start of the observation, respectively. Given that the usual analytical methods provide the researcher with isotope ratios R_t and R_0 (Eq. 1.1) and total concentrations of the substance C_t and C_0, this formula has to be rearranged accordingly to allow determination of α from samples. Using an approximation of H<<L and thus C~L, the formula can be transformed according to (Eq. 1.6) [91]:

$$ln\left(\frac{R_t}{R_0}\right) = (\propto -1) \times ln\left(\frac{C_t}{C_0}\right) = (\propto -1) \times ln(f) \qquad \text{Eq. 1.6}$$

R_t and R_0 are the stable isotope ratios at times t and the beginning of the observation. C_t and C_0 are the respective concentrations of the observed compound and α is the kinetic fractionation factor. Using this formula, (α-1) and consequently ε can be calculated from a

double-logarithmic plot of $\frac{R_t}{R_0}$ versus $\frac{C_t}{C_0}$ by linear regression [33]. This approximation is valid for samples with a much higher abundance of light isotopes compared to heavy isotopes as well as for samples that show only a small change in the absolute isotope ratio R, which is true for most applications. It cannot be used for samples of strongly labeled substances that deviate from natural distributions by several atom-percent [72].

A way to describe multi step kinetic processes is to look at the apparent kinetic isotope effect (AKIE). It describes the isotopic fractionation observed in the residual substance which is depleted by the multistep process as a combination of the kinetic and equilibrium isotope effects of each step in combination with the dynamics of the respective forward and backward processes. The last step of such a chain is irreversible. All processes influencing the isotope composition afterwards cannot reflect back to the source. An example for a two-step process is the transport model for nitrate from the periplasm into the cytoplasm of a bacterial cell and subsequent irreversible reduction of nitrate to nitrite via the nitrate reductase (Nar) enzyme (Fig. 1.1)[c].

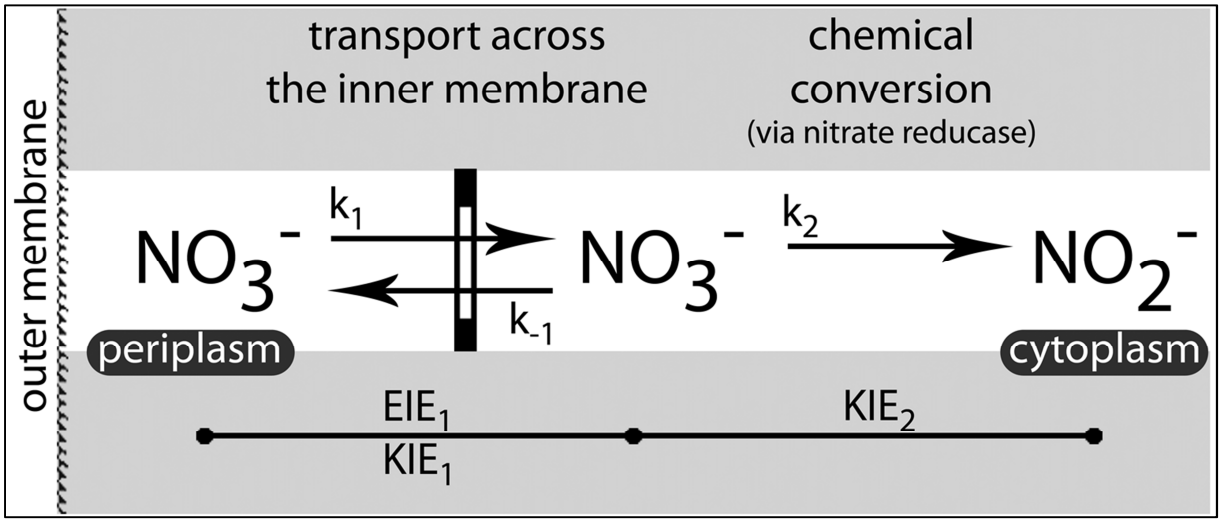

Fig. 1.1: Simple example of a two-step process involving isotope fractionation in both steps. k1 , k2 and k-1 are the forward/backward rate constants of the two steps, EIE1 , KIE1 and KIE2 are the associated equilibrium and kinetic isotope effects.

The description of AKIE in this case has two factors (Eq. 1.7 as described by Elsner [45])

$$AKIE = \frac{k_{-1}}{k_{-1} + k_2} \times (EIE_1 KIE_2) + \frac{k_2}{k_{-1} + k_2} \times KIE_1 \qquad \text{Eq. 1.7}$$

[c] In chapter 2, this process will be explored in more detail and expanded to a more realistic three step process.

Here, k_1 and k_{-1} are the forward and backward rate constants of the transport of nitrate across the inner cell membrane into the cell. The parameter k_2 is the forward rate constant of the nitrate reduction at the Nar enzyme. EIE_1 and KIE_1 are the equilibrium and kinetic isotope effects associated with the transport respectively. KIE_2 is the kinetic isotope effect of the irreversible nitrate reduction step. Depending on the dynamics of the reaction steps in comparison to each other, the observed isotope effect AKIE may reflect a corresponding mixture of the equilibrium and kinetic isotope effects of both steps involved.

1.2 The nitrogen cycle and nitrate in the groundwater

Nitrogen is the most abundant element in the atmosphere of earth and plays an important role in life on this planet. It is one of the most abundant elements in living organisms. Plants, animals and microbes depend on it for the formation of their bodies and for respiration. It enters the biotic environment mostly by nitrogen fixation, a microbial process that reduces nitrogen gas to ammonia which is then available for organisms. Within and between ecosystems, nitrogen cycles in various forms like nitrate, nitrous oxide, nitrogen gas, organic matter and ammonia. These substances can be transferred from the soil to the groundwater and from there on to fluvial systems and into the oceans. A decomposition and reduction of these substances can release nitrogen back to the atmosphere (Fig. 1.2).

Groundwater naturally contains nitrogen in various forms. With the advent of industrial agriculture and artificial fertilizers as well as the growth in human population, the amount of nitrate and ammonia from mineralization of fertilizer and manure increased in soils, groundwater systems and rivers [37; 48; 74]. The production of artificial fertilizer captures nitrogen from the air and binds it in solid or liquid form under investment of energy.

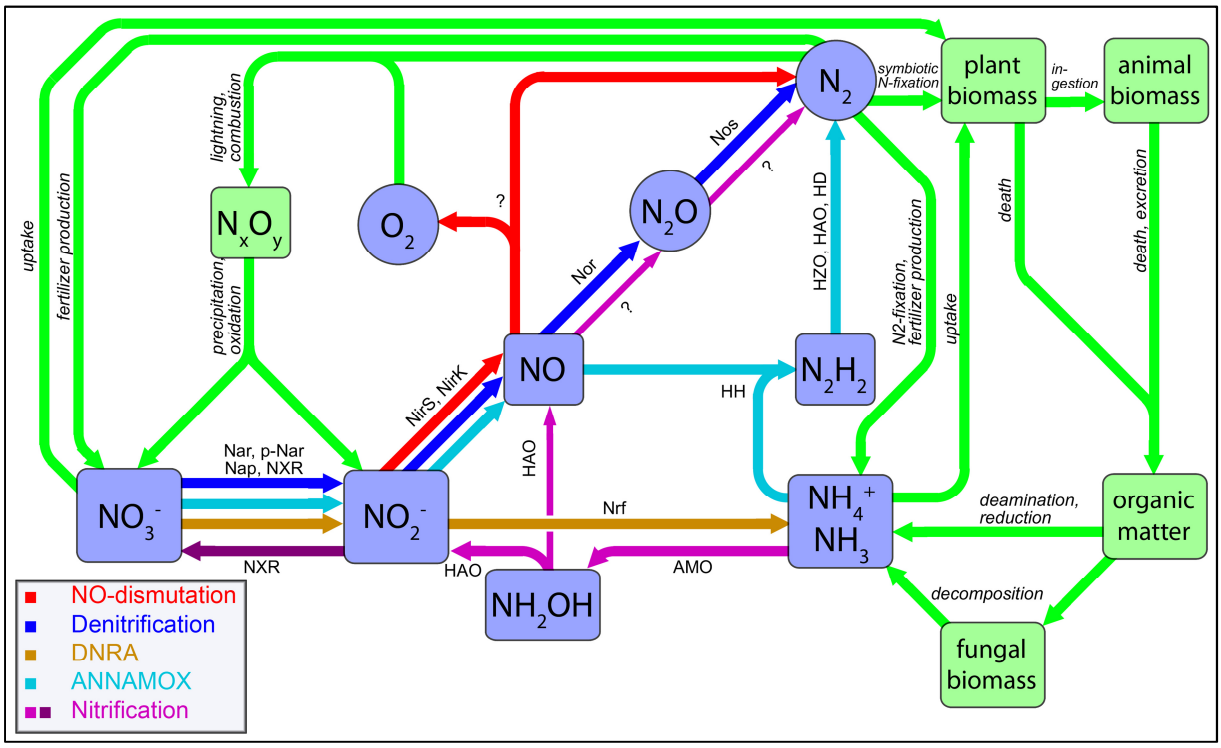

Fig. 1.2: Major chemical forms and pathways of transformation of nitrogen in the biosphere. The pools of interest for this study are colored blue; the according pathways are color coded. For these pathways, the enzyme(s) catalyzing each reaction are given (references from section 1.2). All other pools and pathways that are not considered in detail in this study are colored green and described only generally. Not part of this figure is eukaryotic and fungal denitrification as well as microbial biomass (which takes up nitrogen from many of the depicted nitrogen pools).

The biotic systems fertilized that way cannot retain all this nitrogen, so about 30% of the applied nitrogen ends up in aquifers and surface waters [115]. Additionally, nitrogen accumulates in wastewaters of cities or farms from which it also can leach into the environment. This has negative effects on the quality of the water for human consumption and ecosystem health. For small children, even very low concentrations of nitrate can be harmful [36]. The WHO has set 50 mg/l nitrate as the limit for water that is considered safe for drinking. Various countries and water utility companies adopted much lower limits for providing premium quality water. Aquatic life can be harmed by an excess of nitrate or ammonia [113] and an excess runoff into lakes or the ocean causes algal blooms and hypoxic conditions in anthropogenic "dead zones". Overall, the effects of anthropogenic nitrogen fixation and utilization are vast and have led to massive global changes [46]. It is mainly microbial processes that return excess nitrogen to the atmosphere and thus remediate the anthropogenic imbalance in the nitrogen cycle. Nitrate reduction processes can also play a role in the degradation of organic contaminants in groundwater by serving as electron acceptors for the metabolism of microbial degrader communities [128]. The pathways involved in the transformation of nitrogen in the environment have been studied extensively and there are a number of review papers to be found in literature on this topic [29; 83; 142; 149]. The major processes involving the inorganic nitrogen compounds are described in the following sections.

1.2.1 Nitrification

The nitrification of ammonia to nitrate is a two step process (Fig. 1.3). First, autotrophic proteobacteria oxidize ammonia to nitrite, but are not able to further oxidize nitrite [67]. Ammonia is oxidized to hydroxylamine by ammonia monooxygenase AMO [140] which then is further oxidized to nitrite by hydroxylamine oxidoreductase HAO [95]. A byproduct of hydroxylamine oxidation can be NO, N_2O and N_2 [107]. In a second step, nitrite is oxidized to nitrate by nitrite oxidizing bacteria [13].

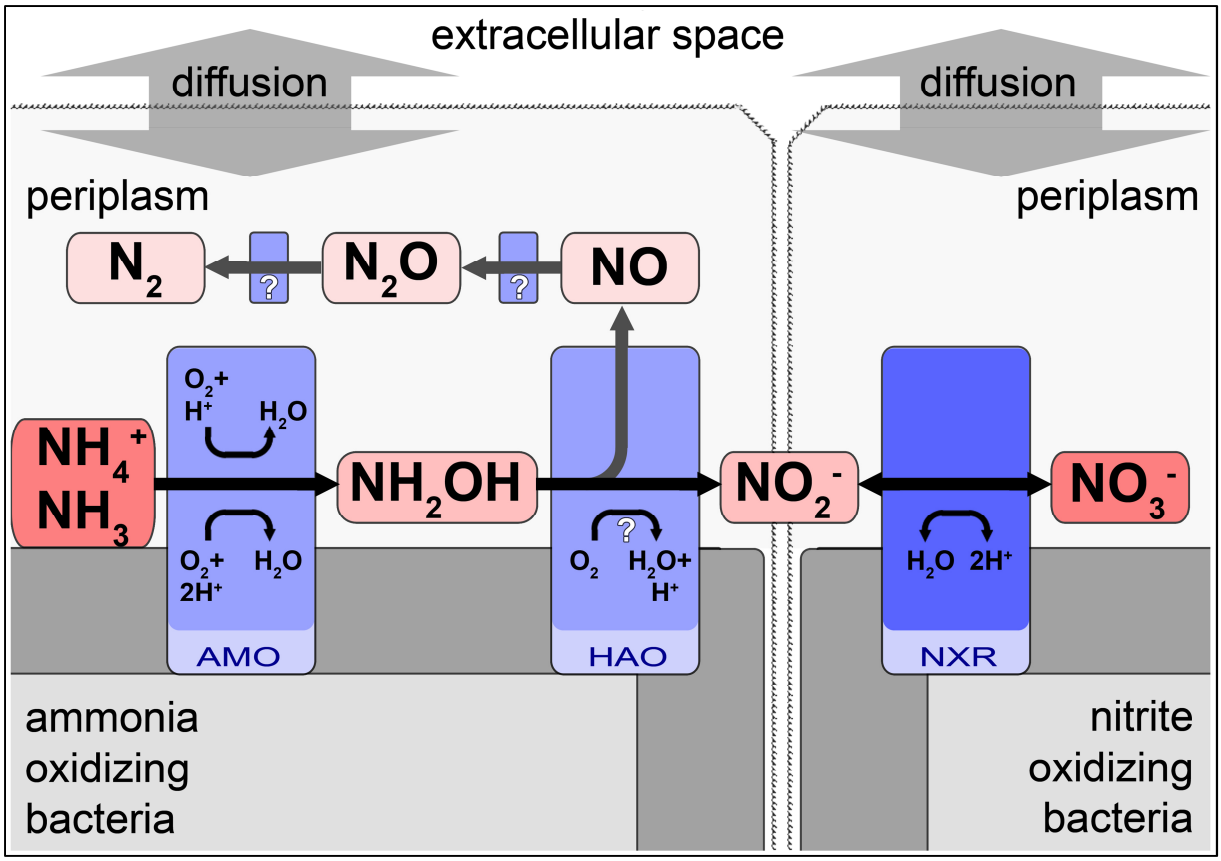

Fig. 1.3: The steps of nitrification with the corresponding nitrogen pools (red) and enzymes (blue). References are described in section 1.2.1. Two different bacterial species are always involved in the complete process. During ammonia oxidation, small amounts of NO, N_2O and N_2 can be produced as byproducts. All substances are subject to potential diffusion into and from the extracellular space.

These are phylogenetically diverse [133] and use a nitrite oxidoreductase NXR[d] for the oxidation of nitrite. Normally it is a membrane bound enzyme [132], but periplasmic variations exist [88].

1.2.2 Denitrification

Most of the denitrifying organisms described up to now are facultative anaerobic chemoheterotrophic proteobacteria that use reduced organic compounds, minerals (e.g. iron, pyrite) or methane as electron donors and nitrogen oxides (nitrate, nitrite, nitric oxide and nitrous oxide) as terminal electron acceptors [7; 49; 119]. During complete bacterial denitrification, nitrate is reduced to N_2 in several steps with a number of nitrogen oxides as intermediary substances (Fig. 1.4). For these bacteria, the key steps of denitrification are the following [65; 81; 83]: After entering the cell through diffusion across the outer cell membrane, nitrate is reduced via the cytoplasmic membrane bound nitrate reductase Nar [10] or one of the periplasmic nitrate reductases pNar [93] or Nap [59].

[d] In some literature, nitrite oxidoreductase is referred to as "NOR", which leads however to confusion with nitric oxide reductase, so the acronym "NXR" is used in this study.

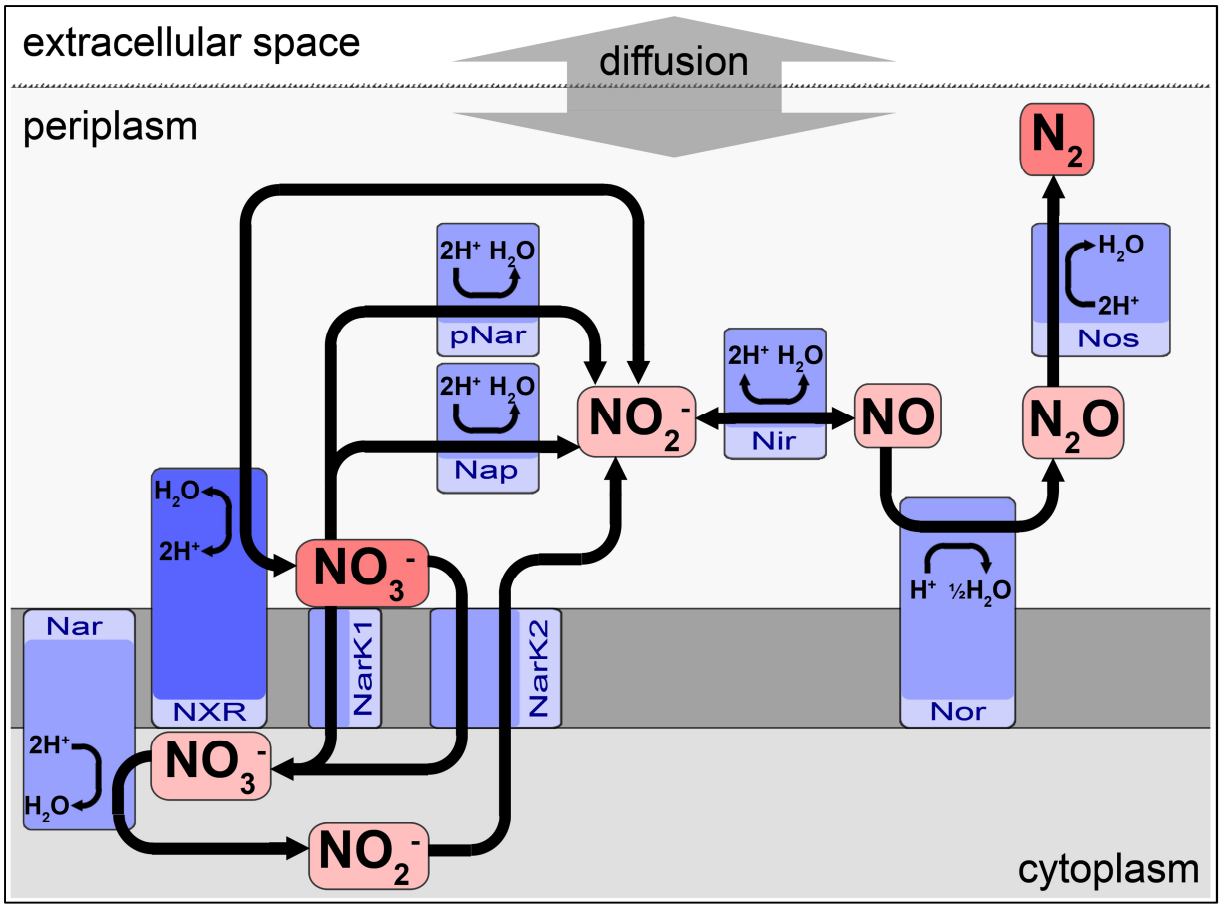

Fig. 1.4: Possible pathways of denitrification with the involved nitrogen pools (red) and enzymes (blue) with their location in the cell. Individual species of denitrifying bacteria possess only a selection of these enzymes. Prior to the steps in this diagram, nitrate has to pass through the outer cell membrane by diffusion to enter the periplasm. All intermediates including the final product dinitrogen gas can pass the outer cell membrane as well. References are given in section 1.2.2

In the first case, nitrate is first imported into the cytoplasm via the NarK1 transporter or the NarK2 nitrate/nitrite antiporter [103] and after the reduction step, nitrite is exported to the periplasm via NarK2. It was reported that Nap does not involve a significant energy gain for the organism but rather may have a regulatory application [56]. Nitrite oxidizing bacteria also can use their nitrite oxidoreductase enzyme NXR to reduce nitrate [50] (see also section 1.2.3). All of the reduction steps following the formation of periplasmic nitrite occur in the periplasmic space or at the outer side of the inner cell membrane. The next step is nitrite reduction to nitric oxide via the heme-type NirS or copper-type NirK nitrite reductases [104; 149]. This step is reversible [56; 81]. Nitric oxide is then further reduced to nitrous oxide via a nitric oxide reductase NOR and finally to N_2 via a nitrous oxide reductase NOS [149]. All substances present in the periplasm are subject to diffusion transport across the outer cell membrane.

Not all of the mentioned steps always occur in the reaction pathway: denitrification can for example end with N_2O or start with NO_2^-, but technically only the inclusion of a gas

producing step allows the use of the term “denitrification” [57]. While normally restricted to anoxic conditions, denitrification can occur in the presence of low oxygen concentrations, mainly in environments with changing redox conditions [11; 51; 109].

1.2.3 Denitrification by nitrite oxidizing bacteria

Nitrite oxidizing bacteria of the genus *Nitrobacter* have been described to be able to perform the last step of nitrification as well as the first step of the denitrification pathway. The reason for this flexibility is the nitrite oxidoreductase enzyme NXR which is a reversible enzyme that can perform nitrite oxidation to nitrate as well as nitrate reduction to nitrite depending on the redox conditions [14; 50]. NXR has been described in more detail by Meincke et al. [98], Kirstein and Bock [77]. There is evidence of further steps of denitrification in these bacteria, as a nitrite reductase enzyme was detected [2].

1.2.4 Dissimilatory nitrate reduction to ammonium (DNRA)

During DNRA, nitrate is reduced to ammonium in two steps [35]. First, nitrate is reduced to nitrite by the Nap or the Nar enzyme [110] and then further reduced to ammonium by the Nrf nitrite reductase enzyme [44]. The first step is largely carried out by enzymes similar to those involved in denitrification. The second step may involve several sub-steps that are however bound to the respective enzyme [38]. There is some evidence of a release of N_2O as an intermediate [138]. DNRA occurs in many groups of the phylogenetic tree of bacteria. In some special environments it can be a more important process of nitrate reduction than denitrification [124].

1.2.5 Anaerobic ammonia oxidation (Anammox)

Anammox is carried out by bacteria that are able to gain energy from the reduction of nitrite in conjunction with the oxidation of ammonium to form nitrogen gas and water [73; 130]. It is described mainly for marine environments, some lakes and some contaminated aquifers, but is thought to exist in a variety of environments [69]. The proposed pathway is a reduction of nitrite to nitric oxide by a Nir enzyme and a subsequent reaction of nitrous oxide with ammonium to form hydrazine via the enzyme hydrazine hydrolase (HH) [137]. Hydrazine then is transformed into nitrogen gas by a hydroxylamine oxidoreductase (HAO) [114], a hydrazine-oxidizing enzyme (HZO) [117] or a hydrazine dehydrogenase (HD) [131]. Anammox bacteria can also be able to reduce nitrate to nitrite by using the Nar enzyme [63] also present in the denitrification pathway.

1.2.6 Other processes

Beyond the scope of this chapter there are several other processes involved in the formation and depletion of nitrate in the aquatic environment. For example, methane oxidizing bacteria have recently found to reduce nitrite and to split nitrous oxide into N_2 and O_2 in a process called NO-dismutation [143]. Heterotrophic bacteria and methanotrophs are also able to oxidize ammonia to nitrate [67]. Furthermore, eukaryotes and fungi have been described that show denitrifying properties [26; 80; 111]. Another denitrification process is nitrifier denitrification which oxidizes ammonia to nitrite and then combines nitrite and ammonia to nitrous oxide or N_2 [142] and H_2O similar to Anammox. Yet another way for microorganisms to reduce nitrate is by the assimilatory nitrate reductase Nas. It was however reported that Nas is inhibited by ammonia, which is regularly produced by catabolism in the cell [62], making this process relatively less significant than dissimilatory nitrate reduction.

1.3 Stable isotopes of nitrogen and oxygen in nitrate during dissimilatory nitrate reduction and nitrification

1.3.1 Dissimilatory nitrate reduction

A major focus of research about nitrogen in the groundwater is centered on dissolved nitrate and ways it is reduced to nitrite and eventually to ammonia, N_2O or N_2. Ammonia is a dissolved substance that retains the nitrogen in the aquatic system and N_2O is a potent greenhouse gas – only N_2 is regarded as an environmentally neutral product of nitrate reduction. Of interest for environmental research are ways to determine sources of nitrate in the environment and to determine if nitrate is reduced and what the products of this reduction are.

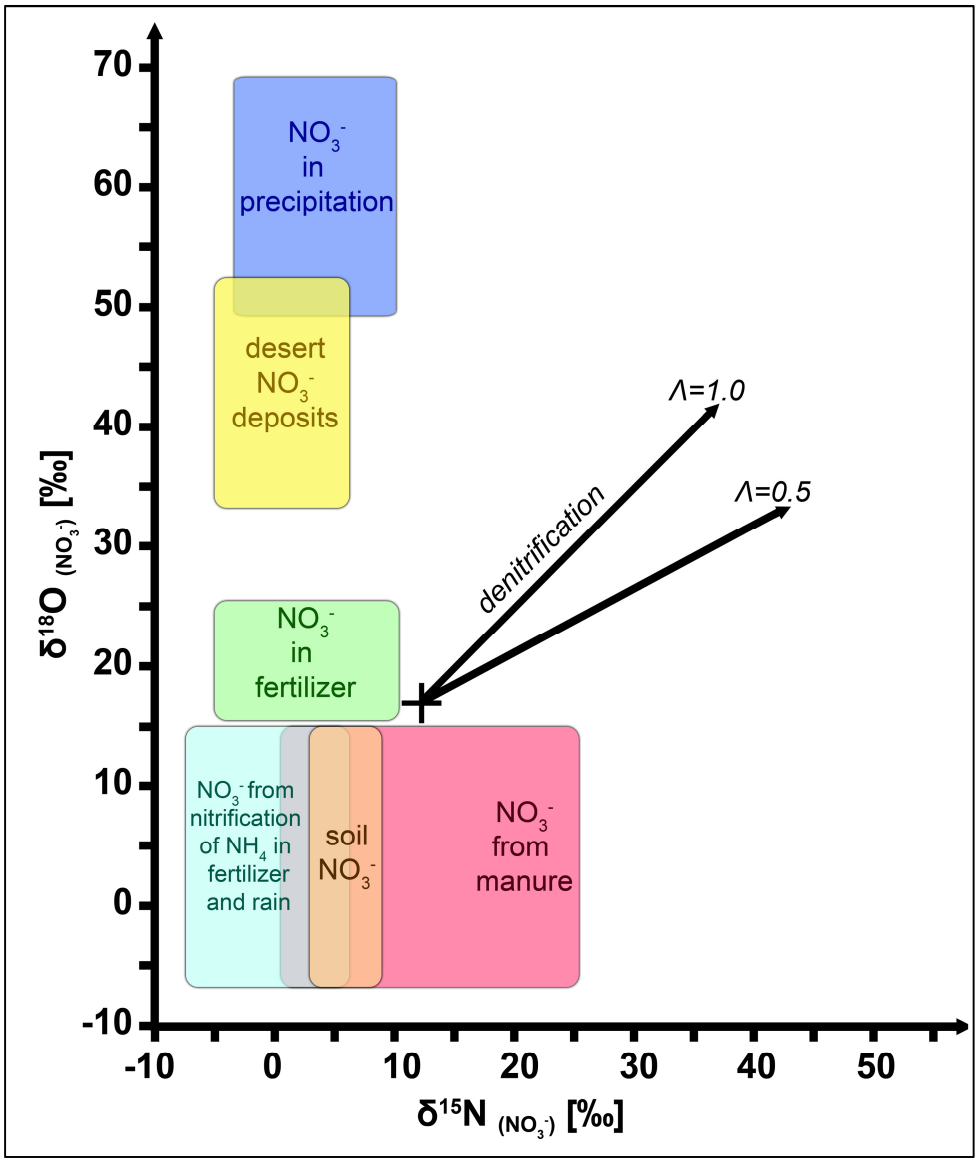

Fig. 1.5: Typical dual isotope plot depicting sources of nitrate that can be identified by their isotopic signature. Data from Durka et al. [42]; Kendall and McDonnell [76]; Mengis et al. [101] and references therein. Two exemplary trends for the enrichment of both isotopes during denitrification are given as examples

A combination of nitrogen and oxygen isotope measurements in nitrate (2D fingerprint) in a dual isotope plot (Fig 1.5) plus determination of ion concentrations and trace elements can help determine the origin of nitrate [76; 101]. Adding boron isotope data can further elucidate the origin and fate of dissolved nitrate [116; 145]. This is possible because nitrate sources represent isotopic pools with a distinct fingerprint stemming from the mechanism by which the nitrate in these pools is formed.

Measuring stable isotope enrichment can provide evidence whether decreasing concentrations of nitrate represent an elimination of nitrogen from the system or are caused by dilution. Microbial nitrate reduction (and a subsequent process of denitrification, DNRA or Anammox) can shift the isotope values of nitrogen and oxygen in nitrate towards more positive values, as nitrate with lighter isotopes is consumed by microorganisms more rapidly, thus enriching the residual dissolved nitrate in heavy isotopes of nitrogen as well as oxygen. The enrichment factor ε is thus negative for this process according to the definition of Eq. 1.4. Several attempts have been made to determine a robust enrichment factor for microbial denitrification in aquatic systems. If the enrichment factors would be known and stable under different environmental conditions, a combination of Eq. 1.4 and Eq. 1.6 would enable the calculation of the fraction of reduced nitrate from observed changes in isotopic composition of nitrate and thus quantify nitrate reduction. However, the observations for the enrichment factors in literature vary widely. Values of -40‰ to 0‰ for $\varepsilon^{15}N$ and -24‰ to -2‰ for $\varepsilon^{18}O$ have been reported (Table 1.2 and references therein).

The reason for this wide range of enrichment factors may be unknown and quantification of microbial nitrate reduction by application of $\varepsilon^{15}N$ and $\varepsilon^{18}O$ of nitrate is elusive. To qualitatively describe the presence or absence of the process of microbial nitrate reduction, the correlation between the enrichment in ^{18}O versus the enrichment in ^{15}N of the residual dissolved nitrate was used (Eq. 1.8).

$$\Lambda = \frac{\Delta\delta^{18}O}{\Delta\delta^{15}N} = \frac{\delta^{18}O_t - \delta^{18}O_0}{\delta^{15}N_t - \delta^{15}N_0} \qquad \text{Eq. 1.8}$$

Lambda (Λ) is the ratio of the relative increase of the δ-values of the two isotopes ^{18}O and ^{15}N of nitrate at time t in relation to t=0. It was originally proposed that Λ=0.5 for nitrate reduction and that denitrification can be identified if such a linear shift in both isotopes can be observed in the environment [4; 17; 106].

Table 1.2: Selected literature data on stable isotopes of nitrate during denitrification. FW=freshwater, SW=saltwater

Reference	$\delta^{18}O$ [‰]	$\delta^{15}N$ [‰]	$\varepsilon^{15}N$ [‰]	$\varepsilon^{18}O$ [‰]	Λ	Salinity	Method according to	Max. c(NO3) [mg/l]	Probable NO_3^--source	Experimental conditions
Amberger and Schmidt [4]	-3 to 46	-14 to 13	n.a.	n.a.	n.a.	FW	Amberger and Schmidt [4]	2652	artificial	peat incubation
Aravena and Robertson [8]	2 to 17	6 to 58	-22.9	-11	0.48	FW	Amberger and Schmidt [4]	349	septic tank	sandy aquifer
Barford et al. [9]	n.a.	n.a.	-28.6	n.a.	n.a.	FW	Sigman et al. [118]	1860	artificial	pure culture of *Paracoccus denitrificans*
Blackmer and Bremner [12]	n.a.	$\Delta\delta^{15}N$ <20	-11 to -17	n.a.	n.a.	FW	Bremner and Keeney [21]	1667	artificial	soil incubations with glucose
Böttcher et al. [17]	10 to 43	9 to 80	-15.9	-8	0.47	FW	Amberger and Schmidt [4]	160	agriculture	sandy aquifer
Brandes and Devol [20]	n.a.	0 to 2	0 to -3	n.a.	n.a.	SW	?	n.a.	?	marine sediments
Cey et al. [32]	-1 to 12	5 to 25	n.a.	n.a.	0.59	FW	Silva et al. [123]	130	?	sandy aquifer plus stream
Cline and Kaplan [34]	n.a.	5 to 19	-30 to -40	n.a.	n.a.	SW	own	<1	?	open marine water
Delwiche and Steyn [39]	n.a.	?	-13.4 to -20.8	n.a.	n.a.	FW	own	n.a.	artificial	pure culture of *Pseudomonas denitrificans* with glucose
Meincke et al. [98]	0 to 15	3 to 15	-5.9	-2	0.40	FW	Silva et al. [123]	99	agriculture	tile drainage of agricultural field
Devito et al. [40]	n.a.	n.a.	n.a.	n.a.	0.56	FW	?	50	agriculture	sandy aquifer
Fukada et al. [55]	9 to 17	9 to 21	-13.62	-9.80	0.77	FW	Silva et al. [123]	22	agriculture	river bank infiltration
Fukada et al. [54]	8 to 30	9 to 42	-14	-7	0.53	FW	Silva et al. [123]	30	sewage	sandy urban aquifer
Granger et al. [60]	n.a.	n.a.	6 to 20	5-21	0.9 to 1.0	SW	Casciotti et al. [30]; Sigman et al. [119]	10	artificial	cultures of algae
Granger et al. [62]	$\Delta\delta^{18}O$ <80	$\Delta\delta^{15}N$ <80	5 to 27	5-23	0.9 to 1.0 (<0.7 for R. spher.)	FW+ SW	Casciotti et al. [30]; Sigman et al. [119]	186	artificial	pure cultures of *Ochrobactrum sp., Pseudomonas chlororaphis, Paracoccus denitrificans, Pseudomonas stutzeri, Rhodobacter spheroides*
Lehmann et al. [87]	5 to 16	8 to 30	11 to 20	7 to 11	0.57	FW	Silva et al. [123]	1	?	open lake water
Mariotti et al. [91]	n.a.	n.a.	-24.6 to -29.4	n.a.	n.a.	FW	own	884	artificial	soil incubation
Mengis et al. [100]	0 to 17	4 to 50	-27.6	-18.3	0.67	FW	Silva et al. [123]	53	agriculture	mixed aquifer plus stream
Olleros [106]	27	16	-30	-15	0.5	?	?	n.a.	artificial	laboratory experiment
Seiler [116]	8 to 19	4 to 16	n.a.	n.a.	n.a.	FW	Silva et al. [123]	28	?	mixed aquifer
Sigman et al. [122]	1 to 12	5 to 16	n.a.	n.a.	1	SW	?	n.a.	?	open marine water
Sigman et al. [120]	0 to 12	6 to 16	n.a.	n.a.	up to 1.25	SW	Casciotti et al. [30]; Sigman et al. [119]	0	?	open marine water
Wunderlich et al. [144]	n.a.	n.a.	-17.3 to -23.5	-15.9 to -23.7	0.91 to 0.97	FW	Silva et al. [123]	620	artificial	pure cultures of *Thauera aromatica* and *„Aromatoleum aromaticum“* strain EbN1
Burns et al. [25]	-10 to 100	7 to 17	n.a.	n.a.	0.37 to 0.51	FW	Casciotti et al. [30]; Sigman et al. [119]	31	?	rivers
Hatzinger et al. [64]	n.a.	n.a.	n.a.	n.a.	0.8	FW	?	n.a.	artificial	in situ experiment with injections

Since then, several values for Λ during denitrification have been observed (Table 1.2), ranging from Λ=0.4 to 0.8 for terrestrial environments and Λ=1.0 to 1.3 for marine environments. In the laboratory, pure denitrifying cultures showed Λ=0.5 to 1.0, though values close to 1.0 are the most common ones and are presently thought to be representative for nitrate reduction with the Nar enzyme. Nitrate reduction by eukaryotes (diatoms and molds) was shown in cell extracts to have Λ=0.96-1.09 [75]. As other processes involving nitrate reduction, like DNRA or Anammox, rely on the same types of nitrate reductase enzymes for nitrate reduction as denitrification, one can assume that they show similar isotopic patterns in the residual nitrate. In summary it can be concluded that the field samples show a much higher variability in Λ than laboratory experiments and while in all cases residual nitrate seems to have been enriched in both heavy isotopes by nitrate reduction, no single value for Λ could be found that can serve as a clear indicator of denitrification. However it still is assumed that a linear dependency of the enrichment of the isotopes of both elements of nitrate exists, even though Λ may vary depending on the environment. Hence researchers are usually looking for linear patterns with a positive value of Λ in dual isotope plots of nitrate to identify microbial nitrate reduction and thus denitrification.

1.3.2 Nitrification

Nitrate derived from nitrification of ammonia tends to have a $\delta^{18}O\text{-}NO_3^-$ reflecting a mixture between the isotopic composition of oxygen in air and water at a ratio of 2:1 to 1:2, depending on how many oxygen atoms originate from atmospheric oxygen ($\delta^{18}O$~23‰) and how many are derived from ambient water ($\delta^{18}O$~0‰ for marine environments and $\delta^{18}O$<0‰ for groundwater). This ratio is possibly linked to the acidity of water [84; 94]. During microbial nitrite oxidation, nitrite is oxidized to nitrate by incorporation of one oxygen atom from the ambient water [3; 13; 66; 84]. As under nitrite oxidizing conditions an isotopic exchange between water and nitrate was observed [41; 82] which has the potential to shift $\delta^{18}O\text{-}NO_3^-$ closer to $\delta^{18}O\text{-}H_2O$, this exchange process might also play a role in the apparent ratio of oxygen atoms in nitrate derived from air and water. However, as groundwater usually has a negative $\delta^{18}O\text{-}H_2O$ but nitrate found in groundwater has a value for $\delta^{18}O\text{-}NO_3^-$ between -5 and 15‰ even after a long residence time (Fig. 1.5), it may be that an equilibrium isotope effect for the exchange process exists. The generally accepted explanation for the values of

$\delta^{18}O\text{-}NO_3^-$ in groundwater however involves the uptake/reduction and release/oxidation processes of nitrate by soil organisms under oxic conditions.

For nitrogen, an enrichment of $\varepsilon^{15}N$=-34.7(±)2.5‰ was observed for ammonia oxidation [91] and an inverse isotope enrichment of $\varepsilon^{15}N$=11.8 to 13.3‰ was observed [31] for nitrite oxidation.

1.3.3 Explanatory models for the variability in the (relative) enrichment of N and O in NO_3^- during denitrification

1.3.3.1 Influence of the carbon source on $\varepsilon^{15}N$ and $\varepsilon^{18}O$ during denitrification

Changes in the enrichment factors $\varepsilon^{15}N$ and $\varepsilon^{18}O$ of nitrate during denitrification can have a variety of reasons. Presumably, the nitrate reduction rate could have an influence [87; 91; 92], as well as salinity, pH, transport limitation or the concentration/type of carbon source. Granger et al. [62] showed that salinity and pH did have no significant influence on the enrichment factors of nitrate. They hypothesized however that the dynamics of nitrate transport into the cell could have an effect. They assumed such a transport would have only small isotope effects and thus have the potential to shift the enrichment factors if transport becomes rate limiting. Previous studies on sulfate reducing bacteria showed a dependence of electron acceptor isotope enrichment factors on the carbon source [16; 78; 125]. Therefore it is a possibility that the enrichment factors of nitrate during denitrification are not constant because they depend on the carbon sources in their environment.

1.3.3.2 Isotopic exchange between oxygen in nitrate and water

The problems with the application of nitrate dual isotope analysis to assess denitrification, which were mentioned in sections 1.3.1 and 1.3.2, have another analogy in bacterial sulfate reduction. The slope of the enrichment of sulfur versus oxygen was not equal in all environments. A major influence on the relative isotope enrichment ratio for microbial sulfate reduction was found to be an exchange of oxygen isotopes between water and intermediates of the reduction process, coupled with a reoxidation of these compounds to sulfate [43; 89; 90]. The ratio of $\Delta\delta^{18}O$ to $\Delta\delta^{34}S$ in bacterial sulfate reduction is analogous to the ratio of $\Delta\delta^{18}O$ to $\Delta\delta^{15}N$ described as Λ in Eq. 1.8 for microbial nitrate reduction. However, no process similar to the oxygen isotope exchange between sulfate and water was described until now but its absence was not proven either. It is known that intermediates of denitrification can exchange oxygen isotopes with water and particularly the reduction of

NO_2^- to NO has a reverse pathway, which transfers oxygen atoms from water into the pool of NO_2^-. This exchange of oxygen appears to depend on the type of respiratory nitrite reductase involved. The two main distinctions are into heme-type (NirS) or copper-type (NirK) enzymes, which exclude the presence of each other within one bacterium [58] and it is proposed that this distinction already reflects the amount of oxygen exchange of nitrite with water with NirS catalyzing this oxygen exchange to a higher degree on average [146; 147]. The variability in oxygen exchange between different bacteria of the same Nir type is however high and extreme exchange rates (high and low) have been determined for both types [34; 56; 81; 127]. A reverse reaction of the nitrate reducing step would hold the possibility of isotopic exchange of oxygen between nitrate and water. In addition to the influence of water oxygen isotopic composition on the oxygen isotopic composition of nitrite by previously described exchange processes, another oxygen atom derived from water would be involved in the reoxidation process to nitrate as well (Fig. 1.6).

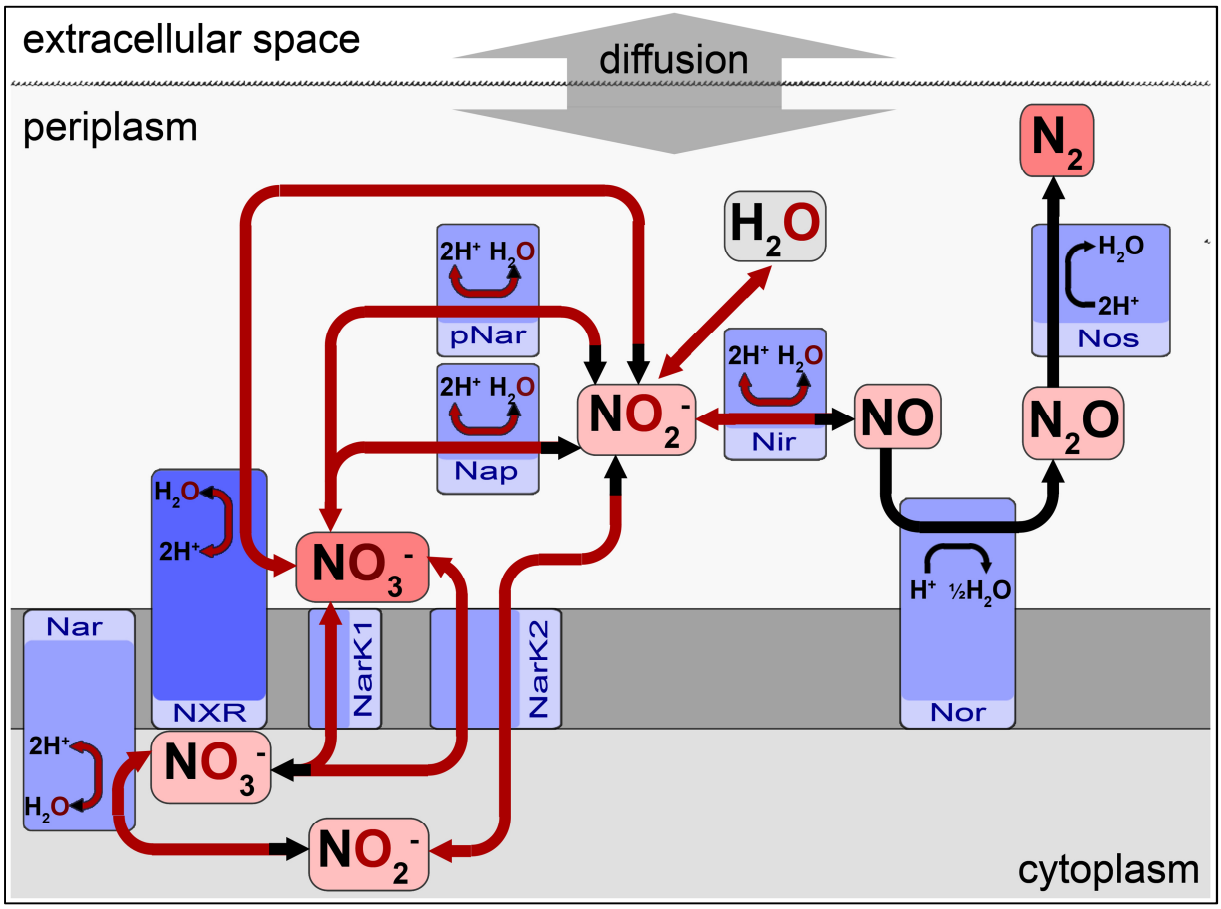

Fig 1.6: Pathways that have to be considered as possibilities for incorporating oxygen atoms from ambient water into residual nitrate. Nitrogen pools (red) and enzymes (blue) are identical to Fig. 1.4. Oxygen atoms and pathways in question are marked in dark red. Additionally, nitrite can also abiotically exchange oxygen atoms with intracellular water (grey). The nitrate with the changed oxygen isotope composition would subsequently also have to pass the outer cell membrane by diffusion to be measureable in the extracellular space.

A hypothetical model of how such an oxygen exchange between water and nitrate most likely occurs can be derived from the knowledge of the pathways and enzymes of denitrification as well as nitrification. Either one of the most common nitrate reductases associated with bacterial nitrate reduction, the enzymes NaR, pNar and Nap could be able to oxidize nitrite in a reversal of nitrate reduction. More likely, however, is a model that includes the inherently reversible nitrite oxidoreductase NXR as a key player in catalyzing an isotope exchange between water and nitrate. A hypothesis drawn from this is that NXR is able to catalyze oxygen exchange between water and nitrate under anoxic conditions, while Nar, pNar and Nap lack that ability. If this process occurs in natural environments in significant intensity, it could explain the variability of Λ observed in nitrate isotope studies and it would have implications on the source determination of nitrate by means of nitrate 2D fingerprinting as well.

1.4 Current methods for measuring nitrate stable isotopes in brief

1.4.1 "Acetone method"

The most recent method of measuring stable isotopes in dissolved nitrate uses a mixture of solvents to extract nitrate from water samples [70]. First, the samples are concentrated by freeze drying them. Then nitrate is extracted from the sample with a solution of acetone/hexane/water. In contrast to nitrate, inorganic carbonates, sulfates and phosphates are not dissolved in this mixture and they can be removed by centrifugation. Subsequently, barium iodide is added to precipitate barium nitrate salt, which then is extracted, dried and measured on a GC-IRMS system. The sensitivity of this method is 20 µmol NO_3^- in the sample volume and its accuracy is claimed to be 0.1‰ for $\delta^{15}N$ and 0.2-0.4‰ for $\delta^{18}O$. It is a cheap and simple procedure. The downside of this method possibly is that not all oxygen or nitrogen bearing substances besides nitrate are removed, especially when high concentrations of organic carbon sources are present in the sample. These could be retained in the prepared sample as a source of interference. A modification of the method also removes dissolved organic matter from the sample [71] but is described only for measuring $\delta^{15}N$.

1.4.2 "Denitrifier method"

Presently, the most commonly applied method utilizes nitrate reducing bacteria to convert nitrate into N_2O, which is then measured by purging the headspace of the reaction vial, trapping the N_2O gas in a capillary submerged in liquid nitrogen and subsequently analyzing this N_2O gas in a GC-IRMS system [30; 97; 119]. Nitrite is removed prior to the preparation by reduction to NO gas with ascorbic acid and flushing the sample with an inert gas [61]. This method requires only small amounts of nitrate. Samples as small as 1 ml with nitrate concentrations as low as 1 µM can be measured with a precision of 0.2‰ in respect to $\delta^{15}N$. Oxygen isotope analysis requires a larger sample of 10 ml at the same nitrate concentrations and provides a precision of 0.3‰ for $\delta^{18}O$ on average. However, a recent error assessment describes the errors as larger due to the correction methods applied and found errors to be up to 2.1‰ for $\delta^{15}N$ and 2.3‰ for $\delta^{18}O$ [145]. The method requires a microbiological laboratory and a continuous pure culture of selected microorganisms. Furthermore, 5 out of 6 oxygen atoms are lost with possible fractionation effects involved and the remaining oxygen atom is subject to a certain degree of isotope exchange with water during the

reduction process. These effects have to be corrected by simultaneously running appropriate standards, by using precisely equal reaction conditions and by using mathematical corrections. When using this method on samples containing distinctly different isotopic compositions of water, these corrections might produce larger errors. Also, $\delta^{18}O\text{-}H_2O$ needs to be measured in this case. The method also is not suitable for samples containing substances that are toxic to the denitrifying bacteria or that inhibit denitrification.

1.4.3 "Cadmium/azide method"

Similarly to the previous method, nitrate in the samples is converted to N_2O and measured by flushing the headspace, trapping the N_2O and analyzing it in an GC-IRMS [96]. However the conversion is done abiotically by chemical means. Nitrate in the sample is reduced to nitrite using spongy (or powdered [112]) cadmium. Subsequently, nitrite is reduced to N_2O by using sodium azide in an acetic acid buffer and then analyzed. Nitrite isotopic composition is determined by omitting the first step. Nitrate isotopic composition then can be calculated from the mixed nitrate/nitrite measurement and the measurement on nitrite alone. Samples of 50 ml with nitrate concentrations of 0.5 µM were measured with a precision of 0.2‰ for $\delta^{15}N$ and 0.5‰ for $\delta^{18}O$. Similar effects as in the previously described biological method occur: 5 out of 6 oxygen atoms are lost and isotope fractionation and exchange are observed and have to be corrected by using standards for calibration and quality control as well as with mathematical methods when water isotopic composition is not equal in all samples. However, due to its purely chemical nature, this method is less sensitive to biopathological compounds in the solution, biological contamination or biologically catalyzed isotope exchange processes that could interfere with the analysis.

1.4.4 "Anion exchange resin method"

A well established method for measuring nitrate isotopes is by means of extracting nitrate with an anion exchange resin from the sample [123]. The anion exchange resin traps major anions including nitrate when flushed with the sample solution. The anions are subsequently eluted from the resin by hydrochloric acid, which then is neutralized with silver oxide to form a silver chloride precipitate. Phosphate is also removed by reaction with the silver oxide to form silver phosphate precipitate. Nitrate remains in solution as silver nitrate. Sulfate is removed by additional steps, involving the addition of barium chloride to precipitate barium sulfate. Barium ions are then removed using a cation exchange resin and

another treatment with silver oxide to neutralize the solution. After removing all precipitates and freeze drying the solution, pure silver nitrate salt is gained, which can be thermally converted to gas or be combusted and subsequently measured in a GC-IRMS. Nitrite has to be removed prior to the first step to avoid interference with nitrate isotopic measurements. This method requires large sample sizes of 10 mg nitrate in the volume sample. Analytical precision is described to be 0.05‰ for $\delta^{15}N$ and 0.5‰ for $\delta^{18}O$.

In chapter 2, a modification of this method is described that was applied in all experiments of this study, as strongly ^{18}O-labeled water was used, making the applicability of the methods using conversion to N_2O (1.4.2 and 1.4.3) questionable. Also, toluene was used as a potentially toxic carbon source, making the biological conversion method (1.4.2) unreliable. The method using nitrate extraction with a solvent (1.4.1) was not available at the time the experiments of this study began.

1.5 Objectives and methodology of this study

The two main goals of this study were to investigate an influence of carbon source on the enrichment factors $\varepsilon^{15}N$ and $\varepsilon^{18}O$ of nitrate during denitrification and to investigate the hypothesis of a microbially catalyzed oxygen isotope exchange between water and nitrate under denitrifying conditions. Both effects are important hypothetical processes that can have a large impact on the use of stable isotopes of nitrogen and oxygen in investigating sources of nitrate and the process of denitrification in the environment. To characterize isotope enrichment factors of nitrate reduction and to determine influences on them can be part of the formation of a model of isotope fractionation during denitrification. Such a model could be useful in the quantification of denitrification using enrichment factors of nitrate stable isotopes. A conditional oxygen isotope exchange between water and nitrate may explain the long standing mystery of a variable slope of $\Delta\delta^{18}O$ vs. $\Delta\delta^{15}N$ described in literature, especially the discrepancy between values derived from laboratory and field experiments. It also would imply that $\delta^{18}O$ of nitrate may not be as reliable as previously thought when it comes to the determination of nitrate sources by 2D fingerprinting. In that case, studies on nitrate sources depending on dual isotope analysis of nitrate alone may misinterpret nitrate sources and for example underestimate the amount of nitrate input from industrial fertilizers or precipitation.

This study was planned to have two parts: The first part focuses on pure cultures of the denitrifiers *Thauera aromatica* and *"Aromatoleum aromaticum"* (strain EbN1) carrying the Nar enzyme grown with nitrate as electron acceptor and different carbon sources as electron donor. Samples from batch experiments with different carbon sources (acetate, toluene and benzoate) were analyzed for nitrate concentrations and the isotopic composition of nitrate. Isotope enrichment factors could be calculated from this data, indicating if there is a significant dependence of $\varepsilon^{15}N$ or $\varepsilon^{18}O$ on the carbon source. Additionally, higher resolution growth curves allowed the determination of nitrate reduction rates. Acetate was chosen as model substance for easily degradable carbon sources while toluene and benzoate represented more complex hydrocarbon electron donors. Adding strongly ^{18}O-labeled water and nitrite to some of these batch cultures was targeted at forcing a change in the isotopic composition of nitrate in case even a minute reverse reaction of nitrate reduction occurs. We expected to see a difference in $\varepsilon^{15}N$ and $\varepsilon^{18}O$

depending on the carbon source and a lack of influence of $\delta^{18}O\text{-}H_2O$ on $\delta^{18}O\text{-}NO_3^-$ indicating that nitrate reduction by the Nar enzyme is irreversible.

Reacting to the results of these first experiments described in chapter 2, the investigation turned towards nitrite oxidizing bacteria with the NXR enzyme to search for the proposed oxygen exchange between water and nitrate. The potential ability of *Nitrobacter vulgaris* to catalyze the exchange of oxygen isotopes between water and nitrate in aquatic environments under anoxic conditions was tested in pure batch cultures by adding ^{18}O-labeled water and measuring the effect of $\delta^{18}O\text{-}H_2O$ on the $\delta^{18}O\text{-}NO_3^-$ over time. A strong exchange of oxygen atoms between water and nitrate and thus the formation of a linear dependence of $\delta^{18}O\text{-}NO_3^-$ on $\delta^{18}O\text{-}H_2O$ was expected. Finally, the applicability of the results on oxygen isotope exchange between water and nitrate in pure cultures to the field was tested in batch incubations of three different natural sediments. Nitrate and ^{18}O-labeled water was added to these anoxic incubations and the consumption and isotopic composition of nitrate as well as the formation of nitrite was monitored. The same three sediment types were incubated under oxic conditions with nitrite to observe their nitrite oxidizing capacity as an indicator for the presence of nitrite oxidizing bacteria. Comparing the results for $\delta^{18}O\text{-}NO_3^-$ of incubations with different $\delta^{18}O\text{-}H_2O$ allowed the calculation of the amount of oxygen exchange between water and nitrate for each sediment type over the course of the experiment.

2 Isotope enrichment factors of denitrifying bacteria carrying the Nar enzyme are affected by different carbon sources

2.1 Introduction

This chapter explores the extent of ^{15}N and ^{18}O isotope fractionation in nitrate reduction when *Thauera aromatica* and "*Aromatoleum aromaticum"* (strain EbN1) were exposed to different carbon sources and water of different isotopic composition. Both strains of denitrifying bacteria use the Nar enzyme to reduce nitrate for respiration. Two parameters were tested simultaneously. First, as hypothesized in section 1.3.3.1, different carbon sources may change the behavior and properties of bacterial cells in a way that affects the observed enrichment factors $\varepsilon^{18}O$ and $\varepsilon^{15}N$ of residual nitrate in the growth medium. Second, the assumption from section 1.3.3.2 that the membrane bound cytoplasmic nitrate reductase enzyme Nar is irreversible and does not catalyze an oxygen isotope exchange between water and nitrate is tested. If nitrate reduction catalyzed by this enzyme would be reversible, the oxygen isotopic composition of water could have an influence on the oxygen isotopic composition of the dissolved nitrate.

2.2 Experimental section

2.2.1 General isotope methodology

Isotopic compositions are all reported in parts per thousands (per mil) using the conventional delta (δ) notation. The simplified "Rayleigh equation" for closed systems was used to assess the stable isotope enrichment factors ($\varepsilon^{15}N$ and $\varepsilon^{18}O$) according to section 1.1. The isotope ratios $^{15}N/^{14}N$ and $^{18}O/^{16}O$ measured in our experiments were within the natural range and R was smaller than 0.01. So we used the approximation described by Mariotti et al. [91], assuming that nitrate concentration is close to the concentrations of light isotopes in nitrate alone. Enrichment factors were calculated from groups of collected data points of all experiments with identical carbon source and microbial strain respectively.

Enrichment factors of oxygen and nitrogen in nitrate in section 2.3.1 were obtained by linear regression from collected plots of biological replicate experiments with identical carbon source and microbial strain ("s*train EbN1*": acetate n=3, toluene n=2; *Thauera aromatica*: acetate n=7, benzoate n=3, toluene n=3). Experiments performed with both "normal" and ^{18}O-enriched water were included in this analysis, since we did not observe an influence of ^{18}O-enriched water on the isotopic composition of residual nitrate (see section 2.3.2). Experiments which had nitrite added during the running experiment are not included in these calculations as nitrite contained minute traces of isotopically labeled nitrate.

2.2.2 Chemicals and preparation of labeled substances

Nitrate for growth medium and silver oxide for sample preparation was purchased from Merck (Darmstadt, Germany); ascorbic acid was obtained from Roth (Karlsruhe, Germany). Water with an ^{18}O content of ~10% was bought from Hyox Rotem GmbH, Leipzig.

A solution of ^{18}O-enriched nitrite with a $\delta^{18}O$ of ~5200‰ (~1.23% ^{18}O) in nitrite was prepared by isotope equilibration of a solution of unlabeled nitrite in ^{18}O-enriched water. 2.3 g of nitrite was dissolved in 45 ml of water amended with 5 ml of anoxic ^{18}O-enriched water and incubated for 2 weeks at 60 °C. The final ion concentrations were determined by chromatography to be 619 mM for NO_2^- and 1 mM for NO_3^- - a purity of >99.8%. The value of $\delta^{18}O$ in nitrite was determined by freeze-drying a small aliquot and subsequent pyrolysis-IRMS measurement. Due to the lack of international standard substances in this isotopic range, the value is to be taken as an approximation.

Nitrate enriched in ^{18}O was prepared by diluting nitric acid with ^{18}O-enriched water and then neutralizing the solution with KOH similar to previously published procedures [15]. The resulting KNO_3 solution was then freeze-dried. The resulting salt was purified by re-dissolving it in boiling deionized water and cooling the solution to 4 °C to precipitate nitrate salts. Subsequent filtration gave pure KNO_3 crystals that were dried at 60 °C. IRMS measurements showed the salt to have a $\delta^{18}O$ of 64.3‰. The salt was used to prepare a sterile 1 M KNO_3 solution, which was measured repeatedly over the duration of its use to ensure stability in $\delta^{18}O$ of nitrate and exclude the occurrence of inorganic oxygen exchange with water. No significant isotopic change in $\delta^{18}O$ was detected within that duration. This solution was used to verify the analytical methods for nitrate extraction and isotope analysis which were used in our experiments.

2.2.3 Organisms and growth in batch cultures

The denitrifying strain *Thauera aromatica* was bought from the German culture collection DSMZ (DSM-6984), "*Aromatoleum aromaticum* strain EbN1" was obtained from F.Widdel. Both strains were grown in a carbonate-buffered fresh water medium [139] with salt concentrations changed to: 100 mg/l NaCl, 40 mg/l $MgCl_2 \times 6H_2O$, 20 mg/l KH_2PO_4, 25 mg/l NH_4Cl, 50 mg/l KCl, 15 mg/l, $CaCl_2 \times 2H_2O$. Trace elements and vitamins were added in quantities described in the referenced literature [139]. Ascorbic acid at a concentration of 700 mg/l was added as a reducing agent. Carbonate buffer concentrations were 3 g/l $NaHCO_3$ to reach a pH of 7.2-7.4. The medium was dispensed in 50 ml aliquots into 120 ml serum bottles via a new method depicted in the appendix: The preparation flask ("Widdel flask") was connected to a three way valve. The other two connections of the valve were attached to a vertically mounted autoclaved glass syringe and the dispensing bell respectively. The glass syringe was covered with an inversely mounted sterile beaker which was adjusted in position to act as a stopper and as protection of the piston against contamination. The glass syringe was filled with medium by overpressure in the Widdel flask and upon turning the valve emptied by gravitational flow into the bottle under the dispenser bell. This way we ensured a quick and easy dispension of precisely equal medium quantities in all bottles and thereby equal growth conditions and concentrations of supplements. The headspace was flushed with a mixture of 80% N_2 and 20% CO_2. A full volume of one such bottle (50 ml) was required for each combined isotope analysis of oxygen and nitrogen in nitrate. Thus, each experiment consisted of several identical bottles with equal growth conditions, which were

sacrificed at different time points. The medium contained acetate (2 mM), benzoate (0.8 mM), or toluene (3 µl/50 ml) as carbon source and sole electron donor and nitrate at a concentration of 10 mM as the sole electron acceptor. Initial carbon source concentrations were set by electron balance calculations to ensure the presence of enough residual nitrate for isotope measurements at the end of the experiment.

Acetate: $CH_3COO^- + 4\ NO_3^- \rightarrow 2\ CO_2 + 4\ NO_2^- + H_2O + HO^-$

Benzoate: $C_6H_5COO^- + 15\ NO_3^- \rightarrow 7\ CO_2 + 15\ NO_2^- + 2\ H_2O + HO^-$

Toluene: $C_7H_8 + 18\ NO_3^- \rightarrow 7\ CO_2 + 18\ NO_2^- + 4\ H_2O$

We assumed no further reduction of nitrite would take place. The validation of this can be seen in Fig. 2.2. In experiments set up for testing the hypothesis of nitrite reoxidation, the bottles were amended with 2 ml of ^{18}O-enriched water to reach a $\delta^{18}O$ in H_2O of ~1700‰ (~0.54% ^{18}O). In another set of experiments, ^{18}O-enriched nitrite with a $\delta^{18}O$ of ~5200‰ (~1.23% ^{18}O) was added during the exponential growth phase to raise the concentration of nitrite by 1 mM. The addition of labeled nitrite was conducted during the growth phase so that microbial activity was immediately high enough to promote possible reoxidation avoiding a lag time during which equilibration of the oxygen atoms with water could take place.

All batch cultures were inoculated with 5 ml (10% v/v) of a preculture grown for at least 3 transfers with the same carbon source and placed at 30 °C in a dark incubator during the experiments. Abiotic controls were performed simultaneously.

2.2.4 Determination of growth parameters

Thauera aromatica was grown in duplicates for each of the three substrates as described above. Each of the 6 bottles was sampled in 2 h intervals for 24h. A sample of 2 ml was taken each time, allowing for analysis of anion concentrations and cell count, but not for isotope analysis. Samples were divided in two 900 µl aliquots – one was preserved for anion analysis by addition of 100 µl NaOH. The other was preserved for cell counting by fixing the cells with glutardialdehyde in a final concentration 2.5% (v/v) and stored at 4 ^{o}C until further use. The anion analysis was performed in a Dionex DX 100 Ion Chromatograph (Dionex GmbH, Idstein, Germany) with an “Ionpac AS14, Analytical 4x250mm” chromatography column. Prior of measuring the cells, the samples were diluted 1:100 and stained with SYBR green I

(Molecular Probes, Invitrogen, Karlsruhe, Germany) at a ratio of 1:10,000 for 15 minutes in the dark. Flow cytometry on the stained cells was performed according to Anneser et al [6]. In brief, the total cell counts were quantified in a flow cytometer (LSR II, Becton Dickinson, Heidelberg, Germany) equipped with a 488 nm and 633 nm laser, using TruCount beads (TruCount Tubes, Becton Dickinson) as internal standard. Instrument settings were as follows: forward scatter (FSC) 350 mV, side scatter (SSC) 300–370 mV, B530 (bandpass filter 350 nm) 500–580 mV. All parameters were collected as logarithmic signals. For minimization of background noise, the threshold was adjusted to 200 mV each for FSC and SSC. Analysis was performed at a low flow rate of ~10 µL/min and with a cutoff of 200 beads using the BD FACSDiva software package (Becton Dickinson). Cell densities in the original samples were calculated using the given dilutions and bead counts for the batch of Trucount tubes utilized.

2.2.5 Sampling procedure for isotope enrichment experiments

Microbial growth was stopped by addition of 50 µl chloroform to each bottle. A small aliquot was taken with a syringe and filtered (0.22 µm PES syringe filter, Millipore, Cork, Ireland) for measurement of the anion concentrations. The bottle was then freed of accumulated nitrite using a modification of the method described in Granger et al. [61]: The 50 ml samples were continuously bubbled with helium, acidified to a pH ~ 3.5 with 10 ml of an anoxic 1 M ascorbic acid stock solution to reduce nitrite to nitric oxide gas (NO). Thereafter, NO gas was stripped out with helium over night. The following day, the bottles were opened and sulfate as well as phosphate was precipitated with 1 ml of a 1 M BaCl solution. The samples were then filtered with a 0.22 µm syringe filter directly onto anion exchange resin columns (BIO-RAD, AG1-X8, and mesh 200-400). The nitrate was retained in the columns for later processing. Since ascorbic acid from the previous step binds to the anion exchange resin, albeit with a lower affinity than nitrate, an additional step was introduced to remove the ascorbate. It was found that an extraction of ascorbic acid from the columns without changing the nitrate isotope ratios was possible by flushing the column with 200 ml of 10 mM HCl. By this procedure, ascorbic acid ions will be protonized, lose their affinity to the resin and are flushed from the column, while the ionic strength of Cl^- in the weak solution is not high enough to elute nitrate from the resin. The columns where then stored at 4 °C until further processing.

For IRMS measurements, the columns were processed similar to the method described by Silva et al. [123]. Nitrate was eluted from the columns with 3x5 ml of 10% HCl into glass beakers. The resulting acidic nitrate solution was then neutralized with ~7g Ag_2O to a pH >5. Accumulating AgCl and residual Ag_2O was removed by vacuum filtration (0.45 µm Cellulose acetate membrane filters, OE67, Whatman, Dassel, Germany). The light sensitive $AgNO_3$ solution was directly filtered into dark centrifuge tubes (Greiner bio-one), frozen at -80 °C and freeze-dried, then stored at room temperature in darkness.

2.2.6 Isotope analysis

Approximately 500 µg of each samples were weighed into tin capsules (^{15}N) or silver capsules (^{18}O) for each isotope measurement (HEKAtech GmbH). Approximately 200 µg of pure graphite were added to the capsules for $\delta^{18}O$ measurement. Each sample was measured at least twice.

Nitrate-nitrogen in the samples was converted to N_2 in an elemental analyzer (EURO EA, Euro Vector Instruments) by combustion of the sample substance $AgNO_3$ to N_xO_y and subsequent reduction to N_2 in a continuous He carrier flow. Combustion gases were separated on a gas chromatography column, and directly transferred into an IRMS (MAT 253, ThermoFisher) for measurement of N_2. Similarly, nitrate-oxygen was converted to CO by pyrolysis (HAT, HEKAtech) with graphite at 1450 °C, separated on a gas chromatography column from other byproducts of pyrolysis, and CO was directly transferred into the IRMS for isotope measurement. The $\delta^{15}N$ values are reported with respect to standard air (AIR), the $\delta^{18}O$ values refer to Vienna Standard Mean Ocean Water (V-SMOW) as a standard. International nitrate salt standards from the IAEA (“N2” and “NO3”) and USGS (“#32”, “#34”, “#35”) were used to calibrate the results. The analytical uncertainty of the IRMS measurements was ±0.5‰ for $\delta^{15}N$ and ±1.0‰ for $\delta^{18}O$.

2.2.7 Validity of the nitrate extraction method

The validity of this procedure and the combination of the two established methods [61; 123] with the new intermittent step was tested with sterile, nitrate free medium amended with nitrate of known isotopic composition. For sample sizes of 50 ml with a NO_3^- concentration between 4 mM and 10 mM, the $\delta^{18}O$ values of these control samples were deviating by -0.21±1.01‰ (for initial values of $\delta^{18}O$=63.79±0.45‰) to -0.58±0.58‰ (for initial values of $\delta^{18}O$=20.79±0.13‰ or 23.54±0.23‰) from the original values. Lower nitrate

concentrations resulted in larger deviations, so experimental samples below 4 mM (=12.4 mg NO_3^- absolute in 50 ml) were discarded.

2.3 Results and discussion

2.3.1 Influence of carbon sources on isotope fractionation

In order to investigate the effect of organic substrates on the stable isotope enrichment factors $\varepsilon^{18}O$ und $\varepsilon^{15}N$ of nitrate during microbial nitrate reduction, we incubated two different organisms with three different carbon sources: acetate, benzoate and toluene (benzoate only with *Thauera aromatica)*.

Cultures with the more complex compounds toluene and benzoate as carbon source produced less negative enrichment factors than cultures grown on acetate (Fig. 2.1, Table 2.1). Based on these results we can estimate a modest shift in isotope fractionation effect of up to ~8‰ for $\delta^{18}O$ as well as $\delta^{15}N$ when comparing acetate with toluene as a carbon source. These results contrast with a similar experiment with *Azoarcus sp.* grown on toluene and succinate as carbon sources, where no significant effect on stable isotope fractionation was observed [79].

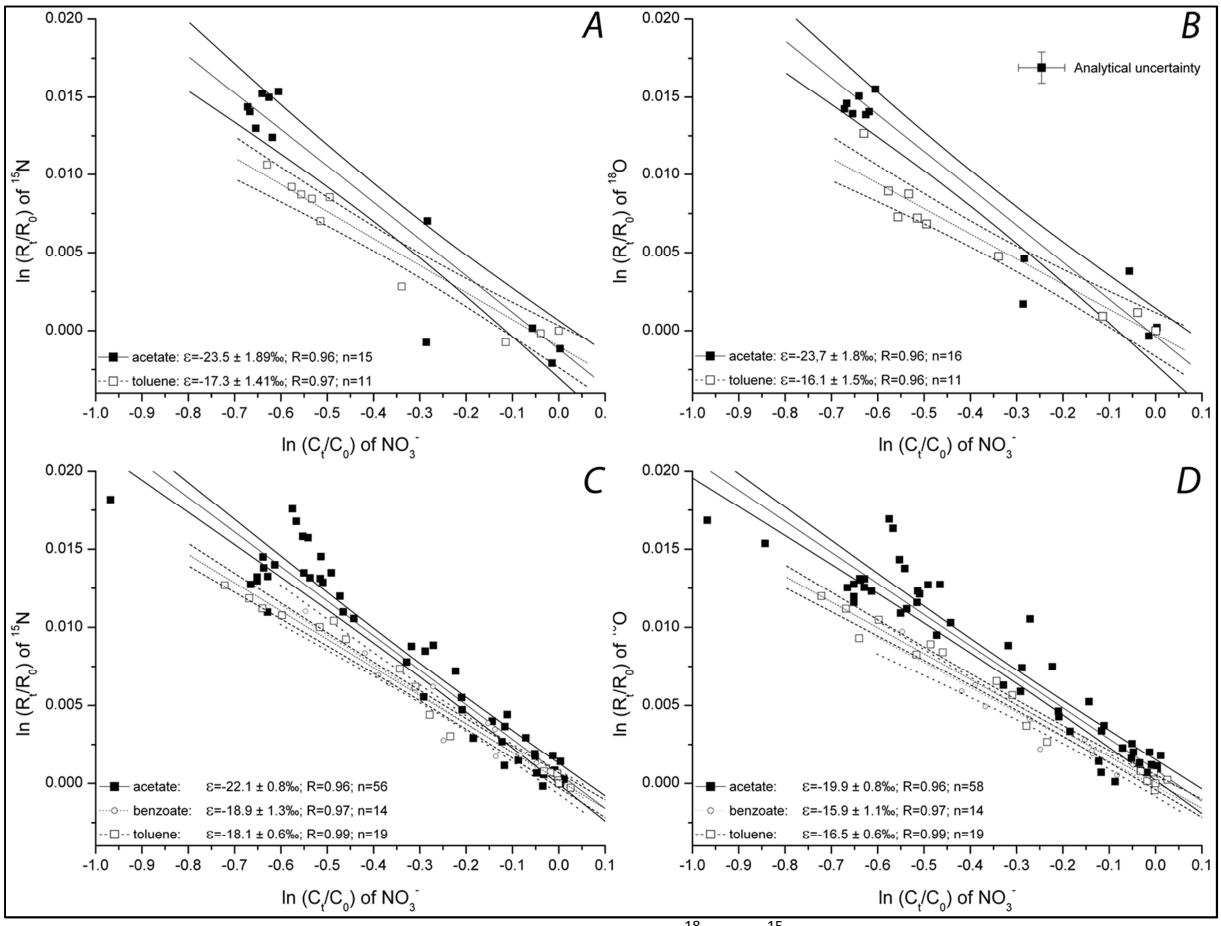

Fig. 2.1 : Calculation of nitrate stable isotope enrichment factors for ^{18}O and ^{15}N during growth of *„strain EbN1"* (A,B) and *Thauera aromatica* (C,D) on the carbon sources acetate, benzoate and toluene. Linear regressions are given with 95% confidence intervals.

Table 2.1: Stable isotope enrichment factors ± standard errors observed in growth experiments with *„strain EbN1"* and *Thauera aromatica*. Experiments with addition of NO_2^- are not included.

Organism	Substrate	^{18}O			^{15}N		
		ε [‰]	R^2	n	ε [‰]	R^2	n
T. aromatica	acetate	-19.9±0.8	0.922	58	-22.1±0.8	0.922	56
T. aromatica	benzoate	-15.9±1.1	0.941	14	-18.9±1.3	0.941	14
T. aromatica	toluene	-16.5±0.6	0.980	19	-18.1±0.6	0.980	19
"strain EbN1"	acetate	-23.7±1.8	0.922	16	-23.5±1.9	0.922	15
"strain EbN1"	toluene	-16.1±1.5	0.922	11	-17.3±1.4	0.941	11

To determine if the carbon source had an influence on the nitrate reduction rate, we conducted additional growth experiments with *Thauera aromatica* under identical conditions to determine growth rates and nitrate reduction rates. The samples were analyzed for concentrations of nitrate and nitrite as well as cell numbers (Fig. 2.2).

From that, cell-specific and total nitrate reduction rates were calculated. The cell densities were quite similar in all cases and increased from a minimum of 2.4×10^6 cells/ml to a maximum of 3.4×10^8 cells/ml. The cell-specific nitrate reduction rate was up to 4.6 pg NO_3^-/cell/h and it was declining throughout the experiment (Fig. 2.3). The cell-specific nitrate reduction rate (csNRR) was similar for all carbon sources within the analytical uncertainty of this method. From this observation, we conclude that the nitrate reduction rate doesn't control the stable isotope fractionation during denitrification in our laboratory experiments.

Different carbon sources are processed by the organisms in different catabolic pathways. However, the electron transfer chain in anaerobic respiration takes the same route at the Nar enzyme. Thus, the influence of the substrate on nitrate reduction via the electron transfer chain is restricted to the reaction kinetics. As we did not observe a dependence of the nitrate fractionation on the growth kinetics, a direct influence of the carbon source on either the gene expression of the Nar enzyme, the transport across the outer cell membrane (by diffusion) or the inner cell membrane (by nitrate transporters) is likely.

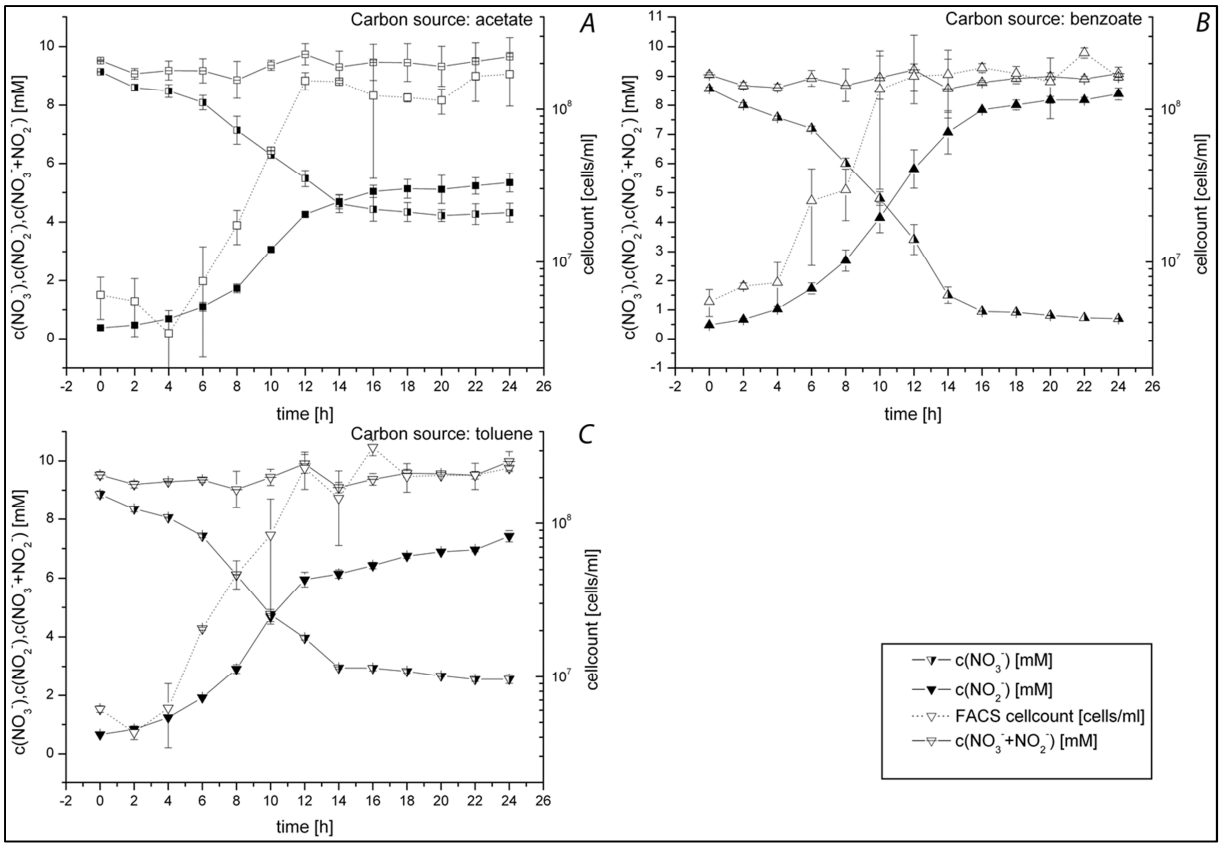

Fig. 2.2: Growth experiments of *Thauera aromatica* with the carbon sources acetate (A), benzoate (B) and toluene (C). The growth curves for each carbon source were performed in biological duplicates; their standard deviation is represented by error bars.

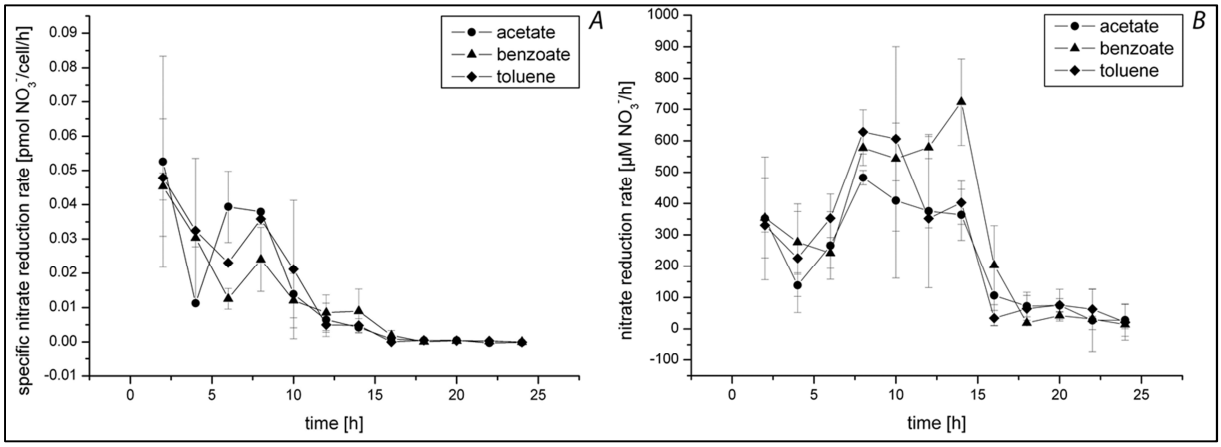

Fig. 2.3: Cell specific nitrate reduction rates (A) and overall nitrate reduction rates (B) during growth of *Thauera aromatica* on three carbon sources. The growth curves for each carbon source were performed in biological duplicates; their standard deviation is represented by error bars.

Theory predicts that the enrichment factors may be influenced by the relative rates of individual steps involved in denitrification, and that they depend on which steps are rate-determining[45]. Here, three steps are involved that can have an influence on extracellular nitrate. First, nitrate is transported from the growth medium across the outer cell membrane by diffusion. Then, nitrate is actively transported into the cytoplasm by transporter enzymes NarK1 and NarK2. Nitrate can also pass through the inner membrane by diffusion; specifically it can leak back out into the periplasm. Once inside the cytoplasm, it is reduced to nitrite via the Nar enzyme in an irreversible third step. Substrate isotope ratios are only affected by the steps leading up to and including the first irreversible one. Subsequent steps therefore cannot influence the observed isotopic composition of extracellular residual nitrate.

This process can be described mathematically by expanding the model given in chapter 1 (Fig 1.1, Eq. 1.7) to a three step process (Fig. 2.4). The apparent kinetic isotope effect AKIE (and thereby ε) is then determined by Eq. 2.1 [45].

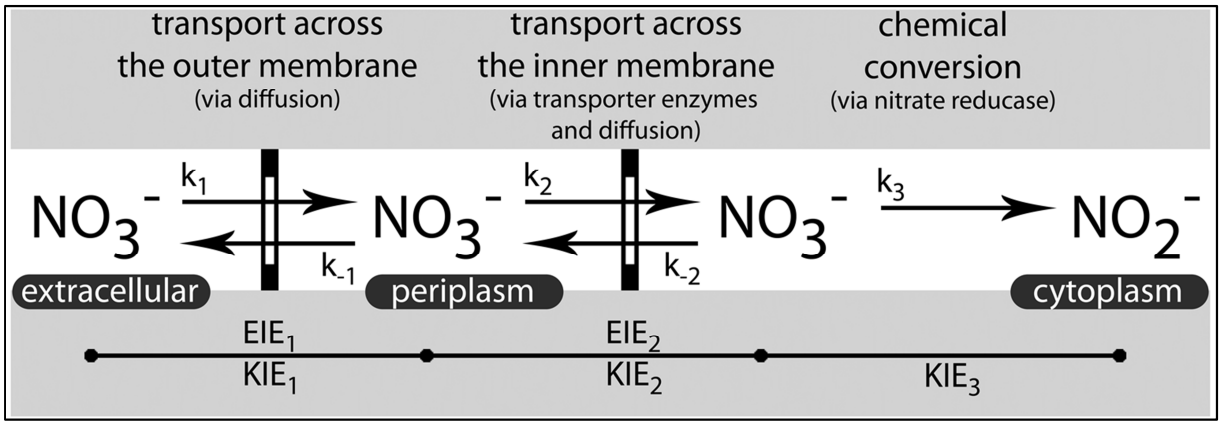

Fig. 2.4: Transport of nitrate into a cell described as a three step process in extension of Fig. 1.1

$$AKIE = \frac{\left(KIE_1 \times \frac{k_2}{k_{-1}} \times \frac{k_3}{k_{-2}}\right) + \left(EIE_1 KIE_2 \times \frac{k_3}{k_{-2}}\right) + \left(EIE_1 EIE_2 KIE_3\right)}{1 + \frac{k_3}{k_{-2}} + \frac{k_2}{k_{-1}} \times \frac{k_3}{k_{-2}}} \qquad \text{Eq. 2.1}$$

Here, EIE and KIE are the equilibrium and kinetic isotope effects of each step and k delimit the forward/backward reaction kinetics as shown in Fig. 2.4.

The kinetics of the first step depends on the permeability of the cell wall and thus can be changed for example by stressors [135] which would increase or decrease k_1 and k_{-1}. In case of a transport limitation, this term becomes rate-determining and k_1 as well as k_{-1} would

decrease, thus increasing the influence of the first of the three terms in Eq. 2.1. As diffusive transport between two similar environments is likely to have neglible isotope effects [19; 62; 87; 91; 92], and EIE_1 and KIE_1 are thus considered to be close to 1, the overall observed isotope effect in this case would get closer to unity.

The second step would be influenced mostly by changes in the activity of the transporter enzymes but also by the permeability of the inner cell membrane to diffusive transport. In the case of an increased transport of nitrate into the cytoplasm, for example by an increased activity of the transporter enzymes, k_2 would increase, also strengthening the influence of the first term in the equation to the same effect as mentioned above. In the case that the outflow of nitrate from the cytoplasm into the periplasm is restricted – by an increased activity of the nitrate transporters or a reduced leakage by diffusion, k_{-2} would decrease. The first two terms in Eq. 2.1 would then both gain more influence. Under these circumstances, the first term approaches unity again as with transport limitation at the outer cell membrane, but as EIE_2 and KIE_2 can also considered being close to 1 [62; 99], the same holds true for the second term. For this situation, the overall observed isotope effect would once again get closer to unity. Only if the nitrate reduction kinetics described by k_3 is lower than the transport kinetics, the kinetic isotope effect KIE_3 of the third step can be observed outside the cell. As this step involves breaking a N-O bond, KIE_3 is considered to be different from 1. Usually it is assumed that this is the rate-determining step [62; 75], that k_3 is small and that KIE_3 can be commonly observed in the residual dissolved nitrate during denitrification leading to strongly negative values for $\varepsilon^{15}N$ and $\varepsilon^{18}O$.

Less negative isotope enrichment factors as seen in our experiments with benzoate and toluene compared to acetate as a carbon source could be the result of shifting kinetics according to the model described above. It was reported that *„strain EbN1“* reacts to toluene by changing the phospholipid composition of the outer cell membrane [148]. The toluene concentrations and bacterial strain in the cited study were nearly identical to those we applied in our experiments. Benzoate is also reported to induce changes in phospholipid composition, however, these changes were not as pronounced as those for toluene [126]. Presumably, these changes are induced by the cell in order to prevent maceration of the cell wall. By stabilizing the cell wall in this way, the cells also decrease the cell membrane fluidity which in turn is thought to inhibit the transport of inorganic anionic substrates such as

nitrate [105]. The factors k_1, k_{-1} and possibly k_{-2} in the isotope kinetics model could be decreased by this behavior of the cells, bringing AKIE closer to unity. A result would be a less negative ε as was observed in our experiments.

Furthermore, it was described that toluene and ethylbenzene in the growth medium lead to an over-expression of enzymes of the denitrification pathway as well as the formation of polyhydroxyalkanoate granules to store carbon [135]. This also has potential to influence the isotope fractionation during denitrification by increasing the availability of nitrate reducing enzymes per intracellular nitrate molecule or by increasing the electron flow to the nitrate reducing enzyme respectively. In both cases, the nitrate reducing step (k_3) would become less rate-determining. In case the enzymes transporting nitrate into the cytoplasm also become more active, k_2 would increase. In both cases, AKIE would once more get closer to 1 and ε would again become less negative, which is in agreement with our results.

2.3.2 Absence of a reoxidation of nitrite

We also wanted to test if the variation of $\Delta\delta^{18}O/\Delta\delta^{15}N$ reported in the literature is due to a putative reoxidation of intermediary nitrite to nitrate, incorporating oxygen from water into nitrate. This was suggested in modeling reports [23] for microbial denitrification after similar effects have become evident for microbial sulfate reduction [43; 90]. Growth experiments with *Thauera aromatica* carried out in ^{18}O-enriched water ($\delta^{18}O$~1700‰) showed no significant differences in oxygen stable isotope fractionation of nitrate compared to experiments carried out in regular water ($\delta^{18}O$~-10‰) (Fig.2.5).

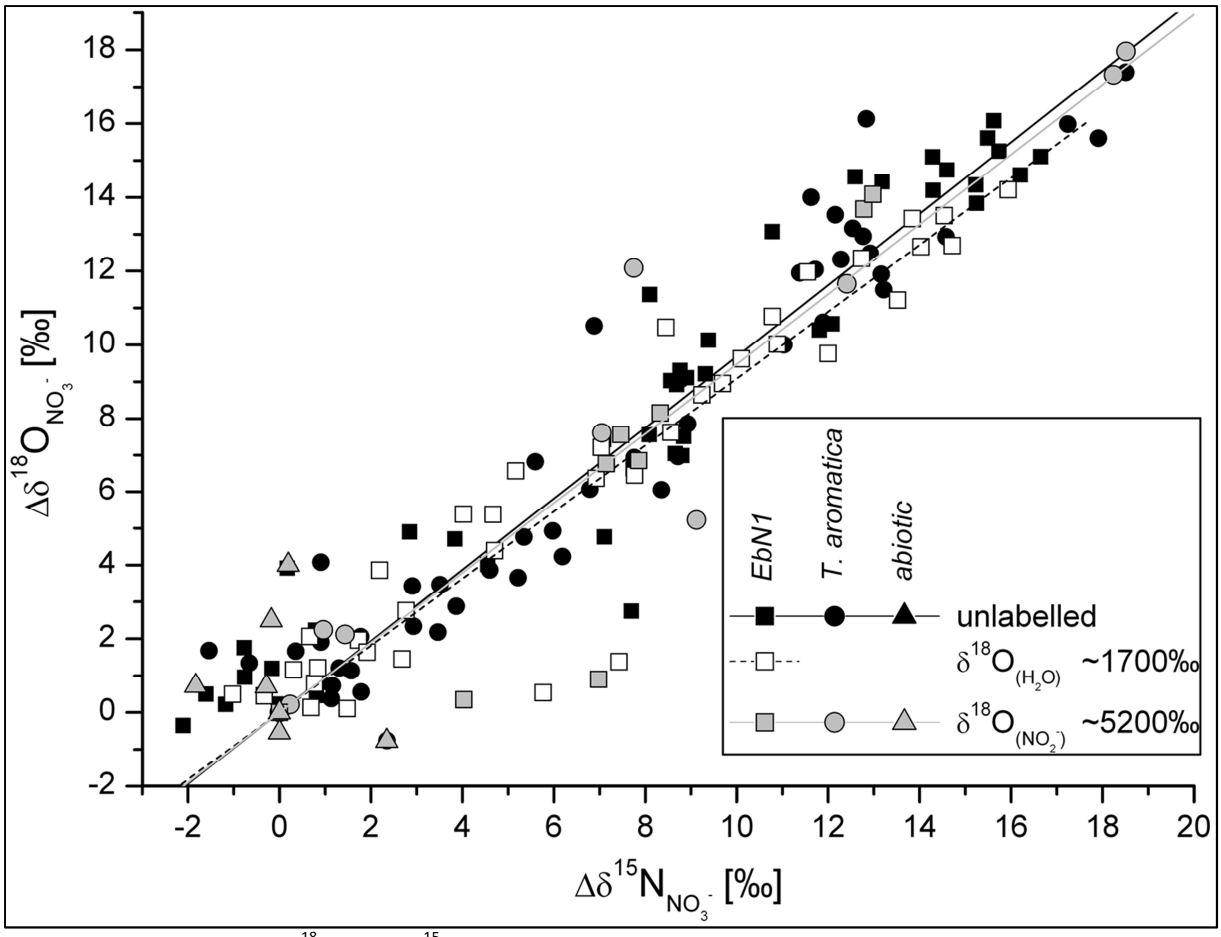

Fig. 2.5: Relative increase in $\delta^{18}O$ versus $\delta^{15}N$ in nitrate during growth of *"strain EbN1"* (squares) and *Thauera aromatica* (circles) in regular water (closed symbols), ^{18}O-labeled water (open symbols) and with addition of labeled nitrite (open symbols with vertical line).

Another test for a putative back reaction to nitrate was the addition of strongly labeled nitrite ($\delta^{18}O$~5200‰) to the experiments. The use of such a strongly labeled substance would enable us to detect even trace amounts of reoxidation. However, amendment of ^{18}O-enriched nitrite during the growth phase of batch cultures of *Thauera aromatica* and *„strain EbN1"* did not affect the oxygen isotope value of the residual nitrate during the continued growth. No significant differences were observed in the respective slopes of the dual isotope plots (Fig. 2.5). The 95% confidence intervals overlapped (data not shown). The slopes inferred from the linear regressions were Λ=0.97±0.02 (unlabeled, R=0.97, n=110), Λ=0.91±0.03 (labeled water, R=0.96, n=46), Λ=0.95±0.05 (labeled NO_2, R=0.94, n=33). This is close to a previously published study [62] describing slopes of 0.86 to 1.02 for various denitrifying microorganisms in growth experiments without labeled water or nitrite. It is also in agreement with previous models that suggest a ratio of ~1 being intrinsic to the reduction of nitrate at the Nar enzyme, however we found no evidence of the influence of transport limitation on element specific isotope fractionation suggested in this model [62]. Since both organisms use closely related variants of the nitrate reductase enzyme Nar for nitrate

reduction [108], it can be concluded that under our growth conditions, nitrate reduction by this enzyme is not reversible.

These results are in agreement with experiments reported recently [79] where no isotope exchange could be found in *Pseudomonas pseudoalcaligenes* and *Azoarcus sp*. when grown in water of different isotopic composition. The reported extent of oxygen exchange between ^{18}O of water and nitrite differs considerably not only depending on the form of Nir (heme- or copper-type) utilized by the denitrifiers but also on the species involved [146; 147]. Different bacterial species with the same Nir type exchanged between <10% and >70% of nitrite oxygen with water [81; 147]. Therefore we recommend that several species of denitrifiers be studied to support the hypothesis that reoxidation of N-intermediates doesn't control the $\delta^{18}O$ in the remaining nitrate under strictly anoxic conditions. We also were concerned that reoxidation processes in a water with $\delta^{18}O$ of -31.2% to 4.5% as used previously to test for reoxidation by Knöller et al. [79], may not be detectable within the analytical uncertainty of $\delta^{18}O$ measurements. The use of strongly labeled substances in his chapter excludes even minor influences of reoxidation of nitrite to nitrate.

The calculated ratio of $\Delta\delta^{18}O/\Delta\delta^{15}N$ in our study was high but comparable to other studies on pure batch cultures of denitrifiers which also show values of Λ close to 1. However, the values were distinctly different from studies on terrestrial freshwater sites where $\Delta\delta^{18}O/\Delta\delta^{15}N$ ratios of about 0.5 were observed. This discrepancy is explored further in the next chapter.

2.3.3 Implications for field studies

Our isotope analyses provide some evidence that the presence of different carbon sources during denitrification may alter the extent of isotopic fractionation in the residual nitrate even though it does not change nitrate reduction rates. Especially denitrification in hydrocarbon contaminant plumes containing toluene can be expected to show less pronounced isotope fractionation in both nitrogen and oxygen isotopes of the residual nitrate as compared to pristine aquifers. For the quantitative description of biodegradation of nitrate in the environment it has to be considered that the stable nitrogen enrichment factors are not very robust to environmental conditions. This may lead to a misjudgment of the amount of denitrification (and thus potential biodegradation of pollutants) at field-sites

that are characterized by different geochemical conditions based on nitrate isotope analysis alone.

Laboratory studies under artificial conditions can provide evidence for fundamental process understanding, but the enrichment factors calculated by growing pure cultures under optimal conditions likely do not reflect enrichment factors expected in field-sites. Lower nitrate concentrations in aquifers compared to our laboratory conditions particularly may cause less negative isotope enrichment factors as transport of nitrate to the cells becomes limiting and the cells will use up all nitrate reaching them. As transport processes have only minute enrichment factors and a complete consumption of a substance will not allow for any isotope fractionation either, overall observed enrichment factors in a nitrate-limited system are expected to be less negative. Consequently, using laboratory-derived enrichment factors that were obtained with high nitrate concentrations to quantitatively describe denitrification occurring in field sites would thus underestimate the extent of actual denitrification rather than overestimate them.

In addition there is some evidence that pH changes between 7.6 and 8.1 and changes in salinity have no influence on the extent of stable isotope fractionation of nitrate during microbial denitrification [62]. However, other factors such as temperature, microbial community composition of nitrate reducing organisms and e^--donor/e^--acceptor limiting conditions could be influential and should be researched to further elucidate environmental parameters that may affect the variability of nitrate stable isotope enrichment during denitrification.

3 Mixed populations of nitrifying and denitrifying bacteria catalyze oxygen isotope exchange between water and dissolved nitrate under anoxic conditions

3.1 Introduction

One of the conclusions drawn in the last chapter was that carbon sources do not affect the ratio of $\Delta\delta^{18}O$ vs. $\Delta\delta^{15}N$. Similar results for laboratory cultures in reaction to variations in pH or salinity were previously reported in literature [62]. Stable isotope studies on sulfate during microbial sulfate reduction in ^{18}O-enriched water have provided an explanation for different ratios of $\Delta\delta^{18}O$ vs. $\Delta\delta^{34}S$ which may be analogues to ratios of $\Delta\delta^{18}O$ vs. $\Delta\delta^{15}N$ during denitrification. In these experiments, a reactive intermediate exchanged oxygen isotopes with water and subsequently was reoxidized to sulfate in the presence of the sulfate reducing enzyme [24; 43; 47; 89; 90; 141]. So far, only denitrifying bacteria with the Nar enzyme were studied in that respect. To show this, the experiments in chapter 2 were conducted and additional results from literature support the conclusion that the Nar enzyme does not express a reoxidation of nitrite and does not catalyze an oxygen isotope exchange between water and nitrate during denitrification [62; 79]. In this chapter, observations of nitrite oxidizing bacteria (NOB) under denitrifying conditions are reported, investigating the hypothesis of such an isotope exchange by the NXR enzyme. Experiments with strongly isotopically labeled substances in conjunction with pure batch cultures of *Nitrobacter vulgaris* were used to investigate the fundamental processes. Then, sediment incubations were used to test the applicability of the observed results to the field.

3.2 Material and methods

3.2.1 General isotope methodology

Isotope measurements were carried out using the conventional delta (δ) notion. The simplified "Rayleigh equation" for closed systems was used to calculate the stable isotope enrichment factors $\varepsilon^{15}N$ and $\varepsilon^{18}O$ (section 1.1). The isotope ratios $^{15}N/^{14}N$ and $^{18}O/^{16}O$ measured in our experiments were within the natural range and R was smaller than 0.01. So we used the approximation described by Mariotti et al. [91], assuming that nitrate concentration is close to the concentrations of light isotopes in nitrate alone.

3.2.2 Experimental procedures

3.2.2.1 Batch experiment with the pure culture *Nitrobacter vulgaris*

Nitrobacter vulgaris (DSM 10236) was purchased from the "Deutsche Sammlung von Mikroorganismen und Zellkulturen" (DSMZ) and grown aerobically at 30°C in a 10% mixotrophic medium (DSMZ Medium 756b) with nitrite as electron source. After nitrite depletion, two liters of the culture was distributed equally to six airtight sterile bottles for each of the two series of experiments. Thereafter, ^{18}O-labeled water (Hyox Rotem GmbH, Leipzig) was injected to adjust the $\delta^{18}O\text{-}H_2O$ in each bottle. A range of $\delta^{18}O\text{-}H_2O$ between -11‰ and 396‰ was covered by the parallel incubations. The bottles were then flushed with N_2 for 30 minutes to remove oxygen. Noninvasive type PSt3 oxygen sensors (PreSens, Germany) were installed in the bottles of the second series of experiments. All batch experiments were incubated in the dark at 30°C. Two bottles with a high $\delta^{18}O\text{-}H_2O$ value served as control experiments and were autoclaved to assess the potential abiotic oxygen exchange between nitrate and water.

Table 3.1: Overview of the $\delta^{18}O\text{-}H_2O$ and $c(O_2)$ of the experiments. Each setup was conducted in duplicates. $\delta^{18}O\text{-}H_2O$ is given as the average ± the deviation of the min./max. value. $c(O_2)$ is given as average ± the standard deviation over the duplicates and all sampling points. For $c(O_2)$, the smallest and largest values measured are also given.

experiment	setup	$\delta^{18}O\text{-}H_2O$ average and min/max	$c(O_2)$ [ppm]		
			average±stdev.	min.	max.
lake sediments	denitrification	-9.45±0.2	0.08±0.01	0.06	0.11
	denitrification	1364.26±205.02	0.08±0.01	0.06	0.10
	denitrification/control	1473.36±130.94	0.1±0.02	0.06	0.12
	denitrification/control	3.72±1.23	0.14±0.07	0.06	0.23
	nitrification	n.a.	9.8±0.56	7.97	10.46
	nitrification/control	n.a.	10.32±0.29	9.13	10.57
stream sediments	denitrification	-8.4±0.33	0.01±0.01	0.00	0.05
	denitrification	615.16±114.79	0.02±0.02	0.00	0.07
	denitrification/control	-5.8±0.52	0.03±0.03	0.00	0.09
	denitrification/control	902.59±112.35	0.06±0.05	0.00	0.16
	nitrification	n.a.	9.11±1.78	4.22	10.46
	nitrification/control	n.a.	10.98±0.29	10.46	11.60
tidal flat sediments	denitrification	0.32±3.41	0±0.01	-0.01	0.01
	denitrification	1235.27±120.39	0±0.01	-0.02	0.01
	denitrification/control	1312.08±22.5	0.05±0.12	-0.08	0.30
	nitrification	n.a.	9.43±0.81	7.25	10.28
	nitrification/control	n.a.	9.55±1.57	6.00	11.84

3.2.2.2 Sediment incubation experiments

3.2.2.2.1 Denitrification experiments

Sediment and water was sampled from three distinctly different anoxic denitrifying environments: Tidal flats from Dangast near Oldenburg, Germany [18] (fine, black sediment from the upper ~3 cm), a stream sediment from Risby, Denmark that is affected by the infiltration of a contaminated shallow aquifer [102] (coarse sediment from the upper 50 cm) and the seasonally anoxic bottom of lake Fohnsee that is located close to Munich, Germany (the upper ~20 cm of soft sediment from the lake bottom at a depth of 15 m). All sediments were homogenized by shaking in a sterilized vessel and distributed to autoclaved 500 ml Schott bottles. Each bottle contained 200-250 ml of sediment and was filled with water from the respective site. Half of the incubations were labeled with ^{18}O-enriched water to increase $\delta^{18}O\text{-}H_2O$ (Table 3.1). Half the incubations were autoclaved to serve as abiotic controls. Two of the controls were supplemented with a solution of ^{18}O-enriched nitrite ($\delta^{18}O\text{-}NO_2^-$ of ~5200‰ prepared as described in the last chapter to a concentration of ~0.8 mM

(Fig. 3.2 A+D). These experiments served as a control to exclude both, abiotic nitrite oxidation and the potential influence of nitrite isotopic composition on the measurements of nitrate with our analytical method. Afterwards, all bottles were bubbled with nitrogen gas for ~30 minutes to remove oxygen introduced during the field sampling and the filling procedure. All incubations were conducted in duplicates. Bottles were pre-incubated at 12°C for ~48 hours to ensure stable conditions. Then a sterile filtered (0.22 µm PES syringe filter) mixture of nutrients and nitrate was added to allow a wide range of microorganisms to become active. The final concentrations of the additives in the bottles were: yeast extract (50 mg/l), peptone (50 mg/l), Na-pyruvate (10 mg/l), Na-acetate (40 mg/l). The nitrate concentration was set to ~30 mM. Afterwards, the bottles were bubbled with N_2 for an additional ~30 minutes. To ensure that the experiments were performed under strictly anoxic conditions all the bottles were equipped with oxygen sensors.

In summary, we used a total of 8 bottles for each field site: 2 active incubations with site water, 2 active incubations with ^{18}O-labeled water, 2 sterile controls with site water and 2 sterile controls with ^{18}O-labeled water, all of them amended with nutrients and nitrate. Due to a lack of material, we performed the controls for the tidal flat sediments with ^{18}O-labeled water only. The bottles were incubated at 12°C on a shaker at 120 rpm to prevent formation of chemical gradients within the sediments.

3.2.2.2.2 Nitrification experiments

To characterize the nitrification potential, additional sediment samples were incubated in 4 bottles for each location with corresponding site water under oxic conditions. Incubation conditions were identical to the ones of the denitrification experiments, except no flushing with N_2 occurred and no nitrate was added. Instead, nitrite from a 1 M stock solution was added (~10 mM) as an electron source after a 48 hour preincubation which served to stabilize oxic conditions. The bottles were closed with BugStopper™ caps (Whatman) to allow infusion of oxygen while retaining the original microbial community. Half the bottles were autoclaved and served as controls.

3.2.2.3 Sampling procedure

For sampling, the glass bottles were taken from the incubator and the oxygen content was measured with a Fibox 3 detector (PreSens, Germany) with an analytical error of 0.03 ppm. Sediment incubations were then stored in a cooler box for 30 minutes to allow suspended

sediment to settle down. Pure cultures were immediately sampled. Samples were taken from the liquid phase with a syringe flushed with N_2. Sample volume for nitrification experiments was 2 ml, for all other experiments it was 30 ml. The sample volume removed from the bottles was replaced with N_2. A 900 µl aliquot of each sample was filtered (0.22 µm PES syringe filter) and mixed with 100 µl of a 10% NaOH solution for preservation. Samples from bottles stemming from the tidal flat sediments were additionally treated with 0.3 g Ag_2O to precipitate chloride as AgCl. Chloride would otherwise interfere with nitrite concentration measurements [68]. The particles of silver compounds were then removed by centrifugation for 10 minutes at 14000 rpm. This aliquot was subsequently analyzed for nitrate and nitrite concentrations with ion chromatrography in a Dionex DX-500 with an "Ionpac AS14, Analytical 4x250mm" chromatography column (Dionex GmbH, Idstein, Germany). The analytical uncertainty of this method was ±3%.

3.2.2.4 Isotope analysis of nitrate and water

To measure the isotopic composition of nitrate for the denitrification experiments (sections 3.2.2.2.1 and 3.2.2.1), the remaining sample was injected unfiltered into airtight 100 ml serum bottles filled with helium and 50 µl of chloroform. The serum bottles were then flushed with helium for 30 minutes to remove traces of oxygen that could have been introduced by sampling. Subsequently they were treated as described in chapter 2 with the exception that saltwater samples stemming from the tidal flat sediments were treated with 1 g of Ag_2O and shaken after sulfate removal to also remove chloride. Its presence would interfere with the reactivity of the anion exchange resin. Also, all samples were centrifuged at 4000 rpm for 20 minutes after sulfate/chloride removal to ease filtration. In case of small final sample amounts, we re-dissolved the produced $AgNO_3$ salt in 1.5 ml deionized water, transferred them to 1.5 ml brown centrifuge tubes (Eppendorf) and freeze-dried them in a vacuum concentrator (Bachofer) attached to the freeze dryer. Afterwards all samples were stored at room temperature.

The $\delta^{18}O$-H_2O was measured using liquid injection into a pyrolysis oven (HAT, HEKAtech) at 1450 °C and subsequent separation of H_2 and CO on a gas chromatography column. The stable isotope ratios of CO were then analyzed by a connected Isotope Ratio Mass Spectrometer (IRMS) in continuous flow mode (Thermo Finnigan Electron MAT 253). The analytical uncertainty of the IRMS measurements was ±1.0‰ for $\delta^{18}O$ of water at

environmental values for $\delta^{18}O\text{-}H_2O$. For very high $\delta^{18}O\text{-}H_2O$, analytical uncertainty was somewhat higher due to dilution effects.

The isotopic composition of nitrate was determined as described in chapter 2, using the same IRMS in conjunction with a pyrolysis oven for ^{18}O and an elemental analyzer for ^{15}N. Analytical uncertainties were ±1.0‰, for $\delta^{18}O$ and ±0.5‰ for $\delta^{15}N$. International nitrate salt standards from the IAEA ("N2" and "NO3") and USGS ("#32", "#34", "#35") were used for calibration.

To test the validity of our methodology in respect to $\delta^{18}O\text{-}NO_3^-$ measurements, we added 10 mM NO_3^- of two known isotopic compositions ($\delta^{18}O$=27.33±0.80‰ and 64.95±0.01‰) to nitrate-free incubations of the three types of sediment. The sediments were homogenized and two samples were taken from each of the bottles. One sample was taken immediately after shaking and contained suspended sediment, the other one was taken after the suspended sediment had settled. We found that the samples taken without sediment showed a maximum deviation of 1.2‰ (lake sed.), 0.8‰ (stream sed.) and 6.3‰ (tidal flat sed.) from the internal standards. However, the samples with a high amount of suspended sediment showed a reduction in the $\delta^{18}O\text{-}NO_3^-$ of up to 25.9‰ compared to the internal standards. As a consequence we only took water samples when we could clearly separate suspended sediment from the liquid phase.

3.3 Results

3.3.1 Oxygen isotope exchange between water and nitrate by a pure culture of *Nitrobacter vulgaris*

After the transfer of *Nitrobacter vulgaris* to anoxic conditions, 0.25 mM – 1.51 mM of nitrite was produced by nitrate reduction over the course of 88 days. After 33 days, nitrite concentrations reached a minimum of 0.13 mM and the $\delta^{18}O\text{-}NO_3^-$ values increased thereafter in correspondence to $\delta^{18}O\text{-}H_2O$ (Fig. 3.1). The batch experiments with the most strongly labeled water ($\delta^{18}O\text{-}H_2O$ > 298‰) showed an increase in $\delta^{18}O\text{-}NO_3^-$ of up to ~88‰. Less than 1‰ change of the $\delta^{18}O\text{-}NO_3^-$ was observed in the sterile control. The oxygen content in the bottles equipped with oxygen sensors was below 0.1 ppm for active cultures and below 0.3 ppm for the sterile controls (Table 3.1), allowing us to exclude oxygen intrusion.

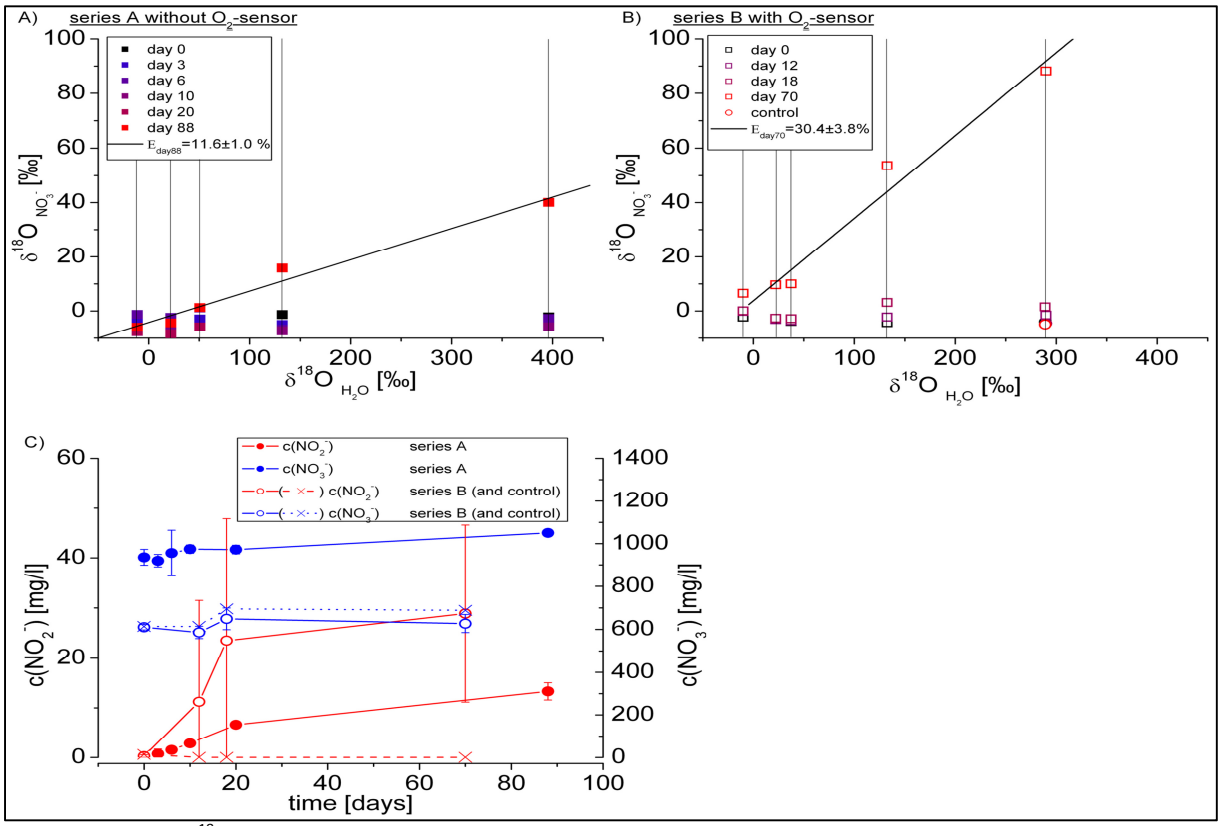

Fig. 3.1: A,B: The $\delta^{18}O\text{-}NO_3^-$ of two series with five parallel anoxic incubations of *Nitrobacter vulgaris* (vertical lines) in relation to the $\delta^{18}O\text{-}H_2O$ of each incubation over the course of 88 days. Colors represent time with the red color being the latest time point, which shows the most pronounced influence of $\delta^{18}O\text{-}H_2O$ on $\delta^{18}O\text{-}NO_3^-$. This time point was also used to calculate the maximum isotope exchange *E* by the slope of a linear regression. C: Nitrate and nitrite concentrations for both series. Error bars show the standard deviation of all five bottles in one series at one time point.

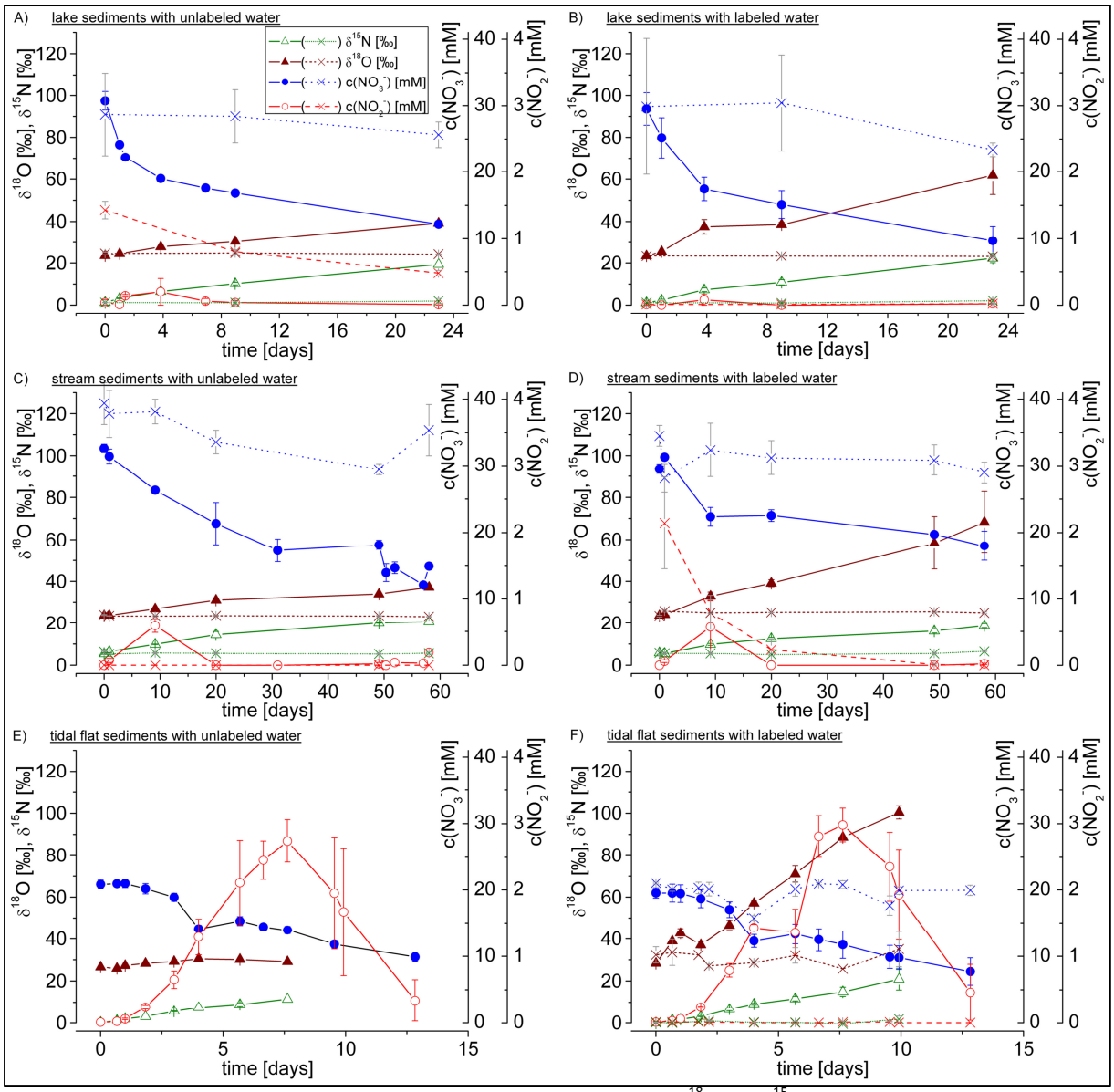

Fig. 3.2: Concentrations of nitrate and nitrite as well as isotopic parameters $\delta^{18}O$ and $\delta^{15}N$ of nitrate during denitrification in anoxic incubation experiments with three types of sediment. A,B : lacustrine sediment; C,D : stream sediment; E,F: tidal flat sediments. "A,C,E" are conducted with site water while "B,D,F" are conducted with ^{18}O-labeled site water according to Table 1. Microbially active incubations have full symbols and lines, control experiments are shown as broken lines with crosses. Error bars represent the duplicates (max./min. values).

The percentage of oxygen exchange was calculated from a plot with $\delta^{18}O\text{-}NO_3^-$ on the Y-axis and $\delta^{18}O\text{-}H_2O$ of the five parallel incubations on the X-axis. We assumed equal initial conditions in all 5 incubations. Over the course of the experiment, $\delta^{18}O\text{-}NO_3^-$ would slightly increase in all of them equally due to microbial nitrate reduction. Assuming that all incubations of one series show equal reaction dynamics and extent of oxygen exchange between nitrate and water, the $\delta^{18}O\text{-}NO_3^-$ of each incubation would shift towards the $\delta^{18}O\text{-}H_2O$ of the respective incubation by the same percentage. This process results in a sloped line when connecting the equitemporal data points of the parallel incubations in the $\delta^{18}O\text{-}NO_3^-$ vs. $\delta^{18}O\text{-}H_2O$ plot. We used a linear regression to determine the slope of this line,

which represents the percentage of the microbial mediated oxygen exchange *E* between water and nitrate. The maximum calculated exchange *E* was 30.4±3.8% (Fig. 3.1). There was no consistent overall trend towards increase in $\delta^{15}N\text{-}NO_3^-$ observable that could be attributed to enrichment due to nitrate reduction. At the end of the experiment, the average value of $\delta^{15}N\text{-}NO_3^-$ (-21.1±0.9‰) was close to the isotopic value measured at the beginning of the experiment (-20.8±1.2‰).

3.3.2 Isotopic shift in dissolved nitrate by oxygen exchange with water during bacterial nitrate reduction in sediment incubations

All incubations showed a rapid nitrate reduction within a few days by the natural microbial community (Fig. 3.2). Denitrification resulted in a temporary accumulation of nitrite and an increase in $\delta^{15}N\text{-}NO_3^-$ of the remaining nitrate. In the lacustrine and stream sediments, the accumulation of nitrite was short lived and nitrite concentrations stayed below 0.6 mM. In the tidal flat sediments, the accumulation was longer lasting and nitrite concentrations in some of the incubations reached values of approximately 3.0 mM. In some experiments, the autoclaved controls initially showed decreasing nitrate and/or nitrite concentrations. This was linked to no or only comparatively minor changes in $\delta^{15}N\text{-}NO_3^-$ (max. difference of 2.4‰ in $\delta^{15}N\text{-}NO_3$ between start- and endpoint). Therefore we exclude significant abiotic nitrate reduction or nitrite oxidation processes during the experiments. We assume that the nitrate concentration most likely is changed by abiotic processes such as sorption to clay minerals [129]. Maximum O_2 concentrations were 0.1 ppm in the active incubations and 0.3 ppm in the sterile controls demonstrating anoxic conditions during the experiment (Table 3.1).

Active incubations were analyzed for nitrate isotope fractionation using Rayleigh plots (Fig. 3.3). Incubations with site water expressed enrichment factors of $\varepsilon^{15}N$=-21.2±2.0‰ to -19.5±3.0‰. Those with ^{18}O-labeled site water showed enrichment factors of $\varepsilon^{15}N$=-25.7±2.5‰ to -18.1±2.0‰ and are in the same range as observed for the experiments with unlabeled site water. The isotope values for $\delta^{18}O\text{-}NO_3^-$ plot as a straight line in most of the Rayleigh plots, allowing the calculation of an apparent enrichment factor of $\varepsilon'^{18}O$=-17.9±0.4‰ to -7.2±2.0‰ in the incubations with site water and of $\varepsilon'^{18}O$=-83.0±11.2‰ to -32.5±4.0‰ in the incubations with ^{18}O-labeled site water.

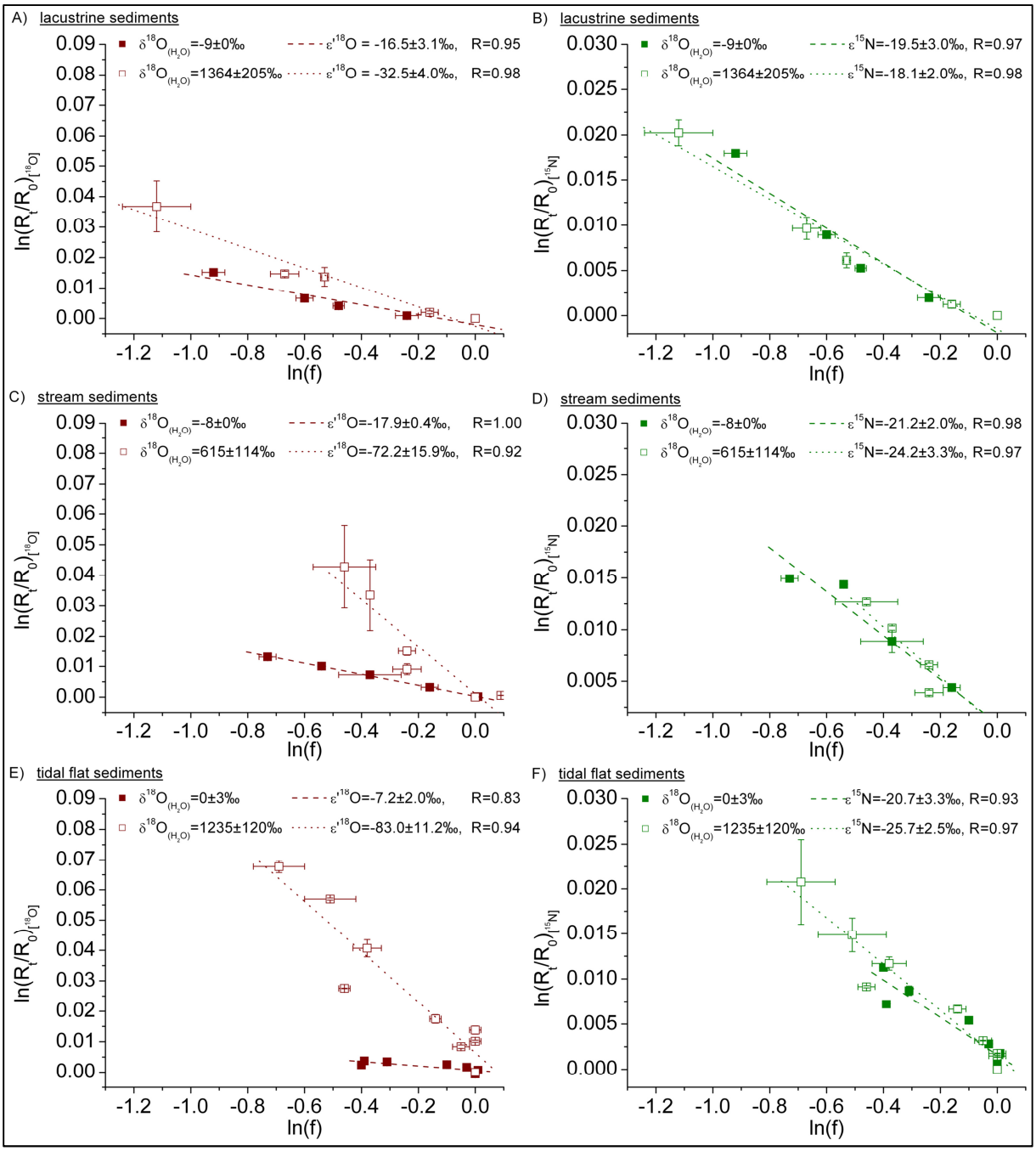

Fig. 3.3: (Apparent) enrichment factors for the sediment incubation experiments are calculated by linear regression in Rayleigh-plots. A,B: lacustrine sediment; C,D: stream sediment; E,F: tidal flat sediment. "A,C,E" show the apparent $\varepsilon'^{18}O$ for oxygen while "B,D,F" show the $\varepsilon^{15}N$ for nitrogen in the residual dissolved nitrate. Open symbols represent the experiments with ^{18}O-labeled water (Table 1). Error bars represent the min./max. values of duplicates.

The controls showed no isotopic shift in $\delta^{18}O\text{-}NO_3^-$ with a maximum difference in $\delta^{18}O\text{-}NO_3^-$ between start- and endpoint of 2.7‰. As some nitrate may be lost due to abiotic processes without influencing the isotopic composition of dissolved nitrate significantly, these calculations may underestimate true values for $\varepsilon^{15}N$ and $\varepsilon'^{18}O$.

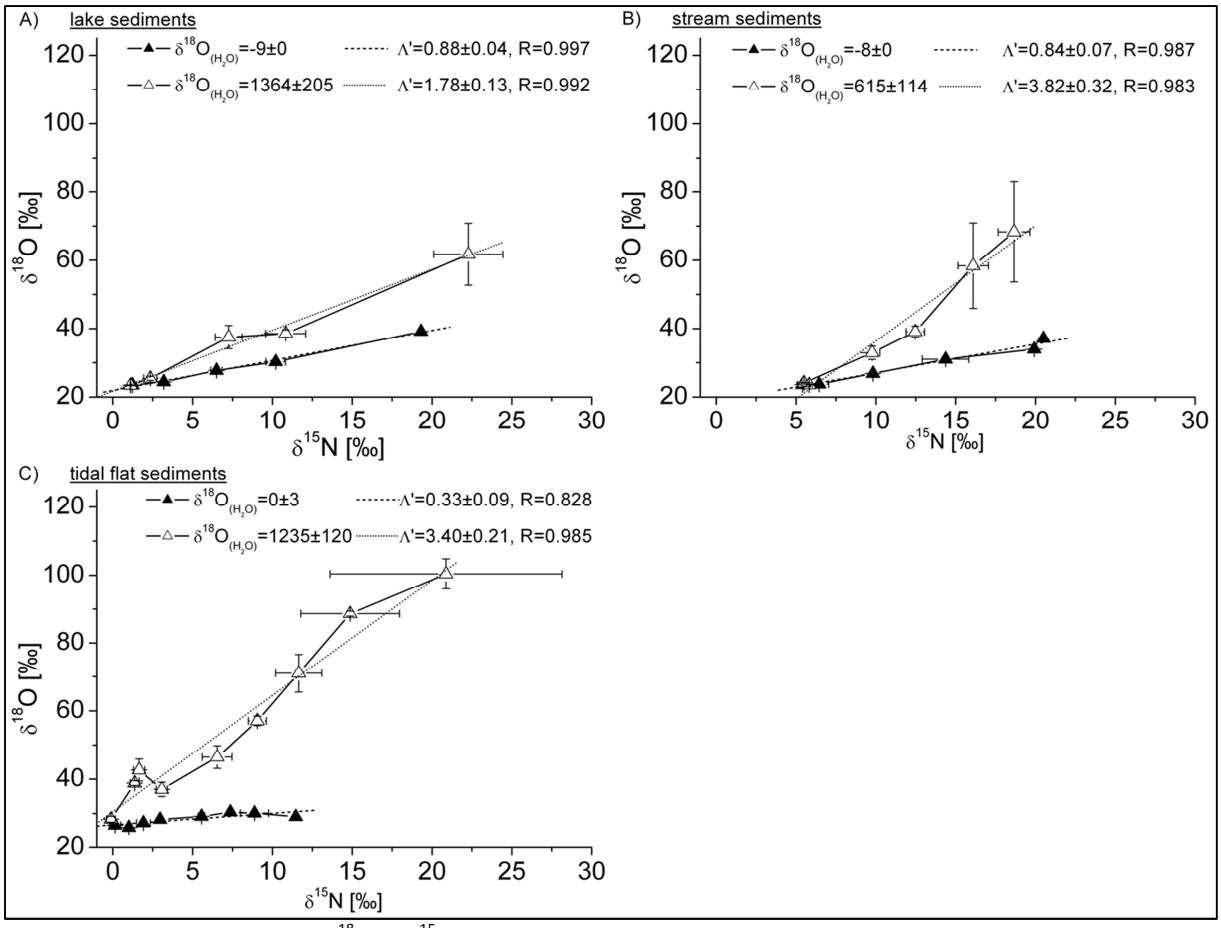

Fig. 3.4: Relative enrichment of $\delta^{18}O$ vs $\delta^{15}N$ in residual dissolved nitrate in the active sediment incubation experiments shown in a dual isotope plot. A: lacustrine sediment; B: stream sediment; C: tidal flat sediment. Open symbols show the experiments with ^{18}O-labeled water. Error bars represent the min./max. values of duplicates. Λ' was calculated by linear regression of the averaged isotope values.

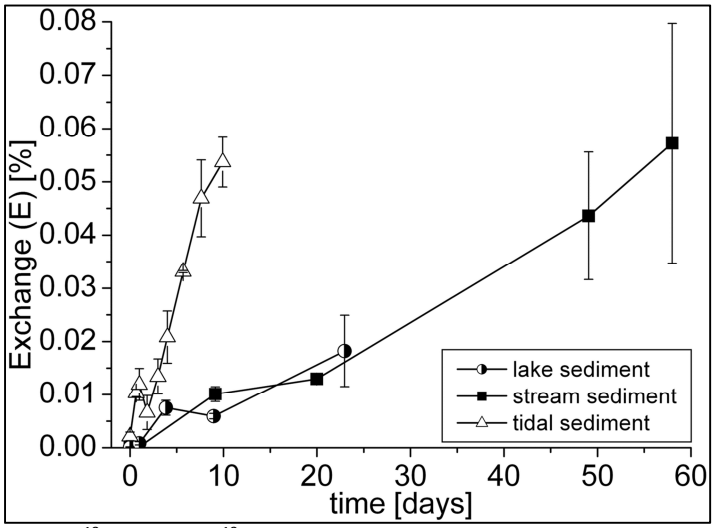

Fig. 3.5: Calculated exchange E of ^{18}O-NO_3^- with ^{18}O-H_2O over the course of the sediment incubation experiments. Each data point for E is calculated from four parallel incubations plotted in a $\delta^{18}O$-NO_3^- vs $\delta^{18}O$-H_2O plot by calculation of the slope of a linear regression. This is analogous to Fig. 1. Error bars represent standard errors of the linear regressions.

Plotting the experimental data with site water in a dual isotope plot (Fig. 3.4), an apparent Λ' of 0.88±0.04 is observed for the lacustrine sediments, Λ' was 0.84±0.07 for stream sediments, and the tidal flat sediments showed a Λ' of 0.33±0.09. For the experiments with ^{18}O-labeled water, the respective slopes were Λ'=1.78±0.13 for lacustrine incubations, Λ'=3.82±0.32 for stream sediment incubations and Λ'=3.40±0.21 for the tidal flat sediment incubations.

We used the four microbially active incubations for each sediment type to calculate the development of the percentage of oxygen exchange *E* with time (Fig. 3.5). The highest value for *E* was 5.7±2.3%, but the experiments did not run long enough for them to reach a plateau. Thus further exchange after the endpoint is likely. The lowest percentage of exchange was observed for the lacustrine sediments, which reached only *E*=1.8±0.7%. The fastest exchange was observable with the tidal flat sediments, which reached *E*=5.4±0.5% after only 8 days.

3.3.3 Nitrite oxidation potential in sediment incubations

Nitrite oxidation to nitrate was observed in all active incubations that were performed under oxic conditions (Fig. 3.6, Table 3.1). The minimum O_2 concentration was 4.22 ppm in the active incubations and 6.00 ppm in the sterile controls. Sterile controls showed no pronounced nitrite oxidation to nitrate. However, as observed for the denitrification experiments, some nitrite was lost during the first 5 days due to abiotic processes. Nitrate concentrations did not change in the sterile controls and was close to zero. The microbial communities in all active sediment incubations were able to completely oxidize all available nitrite within 20 days. The lacustrine sediments however showed a much longer lag-phase than the stream and tidal flat sediments, before microbial nitrite oxidation commenced. The nitrite oxidation rate was variable and thus quantification was not possible.

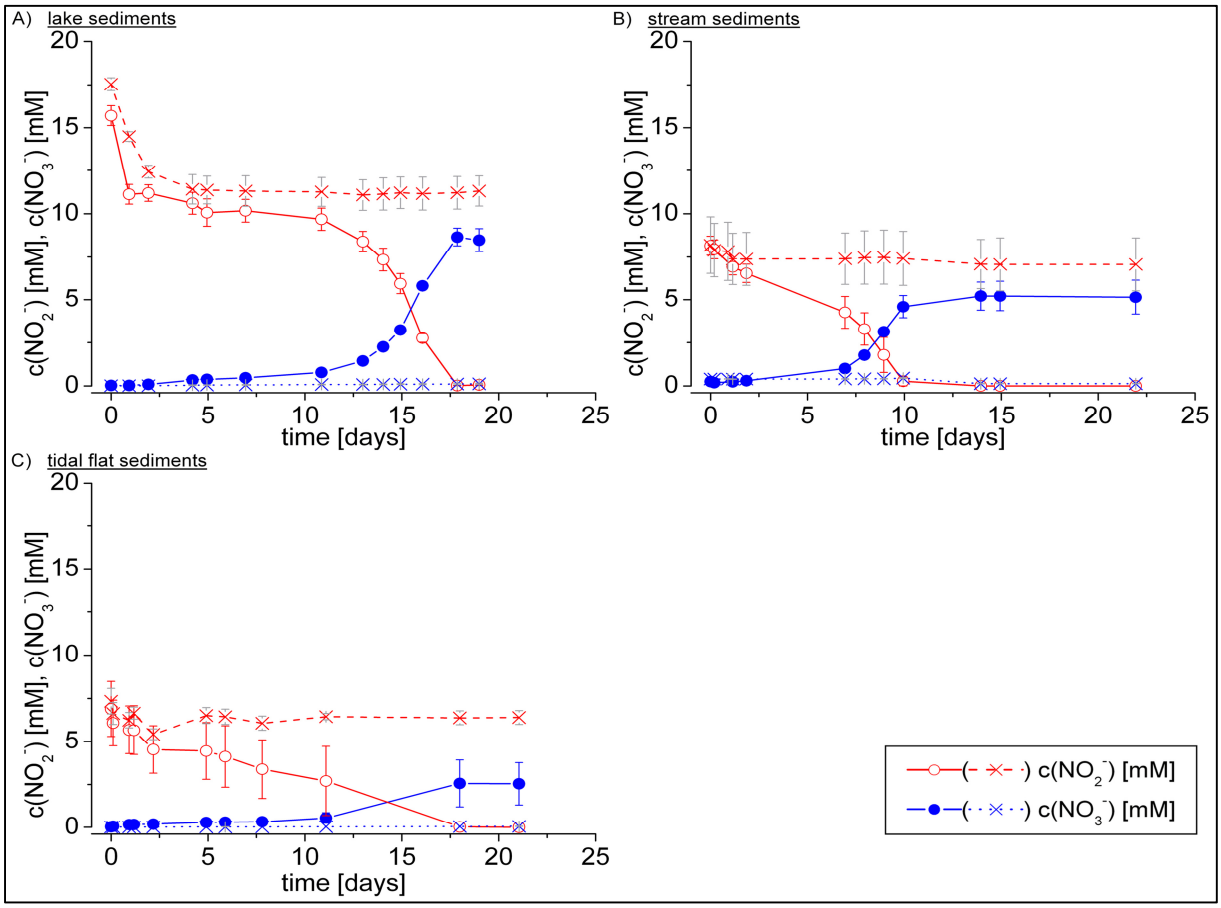

Fig. 3.6: Concentrations of nitrate and nitrite during the nitrite oxidation experiments with sediment incubations under oxic conditions. A: lacustrine sediment; B: stream sediment; C: tidal flat sediment. Microbially active incubations are marked with full symbols and lines, abiotic controls are shown as broken lines with crosses. Error bars represent the min./max. values of duplicates.

3.4 Discussion

Our results with pure cultures of *Nitrobacter vulgaris* performing nitrate reduction under strictly anoxic conditions showed clear evidence of oxygen exchange between nitrate and water. This was only happening, once enough nitrite was produced by *Nitrobacter vulgaris* after 20-33 days. This is in coherence with our proposed pathway of oxygen exchange via intermediary formed nitrite and subsequent reoxidation to nitrate. Our findings are supported by the results reported by Friedman et al. [52]. However the kinetics of the oxygen exchange differed between *Nitrobacter vulgaris* used in our experiments and *Nitrobacter agilis* in the study performed by Friedman et al. [52]. While in our experiments, it took several weeks for the observed oxygen exchange between nitrate and water to become measureable, *Nitrobacter agilis* seems to be able to perform this exchange on the scale of a few hours. However, Friedman added 28 mM of nitrite to a strongly concentrated cell culture, while our experiments started with nitrite concentrations below 0.01 mM. Nitrite available in our experiments was limited to nitrite production of *Nitrobacter vulgaris* during the experiment. Only limited amounts of nitrate were consumed during our experiments, the $\delta^{15}N\text{-}NO_3^-$ did thus not change significantly, however $\delta^{15}N\text{-}NO_3^-$ was also not altered in any way by the addition of ^{18}O-labeled water.

The incubations of natural sediments aimed at elucidating the relevance of NOB-mediated oxygen exchange between water and nitrate in the environment. During these incubations under denitrifying conditions, we observed an isotopic enrichment in ^{15}N of nitrate with $\varepsilon^{15}N$ in the range of previously reported values for denitrification. Looking at $\delta^{18}O\text{-}NO_3^-$ however, we observed a strong microbially induced influence of the $\delta^{18}O\text{-}H_2O$ on the $\delta^{18}O\text{-}NO_3^-$ of residual nitrate under nitrate reducing conditions. No such isotope exchange was observed in the sterilized controls despite strong ^{18}O-labelling of the water and in case of two duplicate incubations also the addition of strongly ^{18}O-labeled nitrite. Subjecting the sediments to nitrite oxidizing conditions showed that all of them were able to oxidize nitrite to nitrate indicating the presence of nitrite oxidizing bacteria in the microbial community. The long lag phase in the lacustrine sediments points towards a low initial presence of active NOB. This correlates with the low oxygen exchange between water and nitrate in the incubations of lacustrine sediment.

The experiments with *Nitrobacter vulgaris* as a model organism for nitrite oxidizing bacteria suggest that the presence of nitrite oxidizing bacteria and nitrite in anoxic aquatic environments are most likely the controlling parameters for isotope exchange reactions between nitrate and water. A possible pathway could be a microbially mediated oxygen isotope exchange between nitrite and water [30; 81; 146] and subsequent reversal of the nitrate reduction step of denitrification by the NXR enzyme. This further adds another oxygen atom from water to nitrite to produce nitrate [3; 13; 66; 84]. This could happen as an equilibrium between a forward (nitrate reducing) and backward (nitrite oxidizing) reaction by the NXR enzyme. High nitrite concentrations might thus correlate with increased backwards reaction rates and thus increased influence of $\delta^{18}O\text{-}H_2O$ on $\delta^{18}O\text{-}NO_3^-$. This was observed in our incubations of the tidal flat sediments, which showed the fastest oxygen exchange correlating with the highest nitrite concentrations (Fig. 3.3E and 3.2 E+F).

An oxygen exchange reaction between water and dissolved nitrate under denitrifying conditions as described here has wide implications. Depending on the dynamics of this exchange the oxygen isotopic value of nitrate may be altered severely. Additional important parameters for observed changes in the oxygen isotopic value of nitrate under these conditions are the original isotopic composition of water and dissolved nitrate, the nitrate reduction rate, the extent of accumulation of intermediary nitrite, and the microbial community composition at a field-site.

As a conclusion, we postulate that the $\delta^{18}O$ of the remaining nitrate can be modified during denitrification and consequently that there is no typical linear slope in dual isotope plots for the identification of denitrification processes in aquatic environments. The slope would depend on the extent of oxygen exchange between nitrate and water as well as the value of $\delta^{18}O\text{-}H_2O$. In some cases, there is an apparent linearity in the relationship between $\Delta\delta^{18}O\text{-}NO_3^-$ and $\Delta\delta^{15}N\text{-}NO_3^-$ misleading to the calculation of a Λ'. This mimics a Λ resulting from denitrification alone. We suggest that a Λ' close to 1 (which is the value found in most of the pure culture experiments), and low concentrations of intermediary nitrite, are indicators of only a minor amount of oxygen exchange between water and nitrate during denitrification. Dual isotope analysis of nitrate in these cases is more likely to give a result that is in line with classical assumptions.

Furthermore, when attempting to calculate enrichment factors $\varepsilon'^{18}O$ from Rayleigh plots, the results do not represent the $\varepsilon^{18}O$ of denitrification alone, but are also influenced by the extent of oxygen exchange with water and the $\delta^{18}O\text{-}H_2O$. When plotting the $\delta^{18}O\text{-}NO_3^-$ data together with nitrate concentrations in a Rayleigh plot, one would not expect to see a linear dependence. Oxygen exchange between nitrate and water would change the $\delta^{18}O\text{-}NO_3^-$ value independently of the decrease in nitrate concentration by denitrification and the dynamics of the nitrate reduction process does not have to match the dynamics of the oxygen exchange process. This seems to be the case for the labeled stream sediment and the unlabeled tidal flat sediment in our incubations (Fig. 3.3 C&E). In both cases, the linear regression has a low coefficient of determination ($R<0.93$). However, in the remaining incubations we found that the $\delta^{18}O\text{-}NO_3^-$ influenced by oxygen exchange with ambient water was mimicking a regular fractionation due to nitrate reduction. This demonstrates that oxygen isotopic exchange between nitrate and water can create a situation in which one can calculate with apparent confidence an $\varepsilon'^{18}O$ that is thought to be the $\varepsilon^{18}O$ of nitrate reduction when in fact it is determined by the oxygen isotope exchange between nitrate and water.

Due to the mounting evidence of oxygen exchange between dissolved nitrate and water in soils [82] as well as in anoxic aquatic environments as demonstrated in our experiments, also the use of $\delta^{18}O\text{-}NO_3^-$ as part of source determination of nitrate in the environment with dual isotope plots may also be unreliable. Given enough time for the oxygen exchange reaction to occur and an ample oxygen exchange capability of the particular environment, the $\delta^{18}O\text{-}NO_3^-$ might eventually get close to the ambient value of $\delta^{18}O\text{-}H_2O$. This would especially mask the high $\delta^{18}O$ isotopic signature of nitrate derived from inorganic fertilizer or nitrate stemming from precipitation. $\delta^{15}N\text{-}NO_3^-$ however is not affected and can still be considered an accurate tool for the analysis of sources and fate of nitrate in the environment.

In our experiments, we observed a maximum oxygen exchange between water and dissolved nitrate of 5.7±2.3%. Under natural conditions this would result in changing the $\delta^{18}O\text{-}NO_3^-$ by only a few ‰. We did, however, not see a limitation to potential oxygen exchange. In our experiments, we applied very high concentrations of nitrate and using concentrations closer to naturally occurring lower nitrate concentrations would probably result in a higher extent of oxygen exchange with water over shorter periods of time. The residence time of water

carrying nitrate also is far longer under natural conditions, making exchange reactions even more likely. Nitrite oxidizing bacteria may be present in many anoxic sediments for various reasons. They could be washed out by precipitation from overlying soils or the redox conditions may be fluctuating allowing nitrate reducers and nitrite oxidizers to thrive in the same spot at different times. There are also nitrite oxidizer species that are able to live under anoxic conditions by nitrate reduction [14; 28; 50]. Nitrite oxidizing bacteria have been found in anoxic marine sediments [134] and anoxic sewage sludges [27; 85] as well.

4 Conclusions and outlook

4.1 The influence of different carbon sources on the stable isotope fractionation of nitrate during microbial demitrification

The results of this study can a partial explanation for the previously observed variability of the isotope enrichment factors $\varepsilon^{15}N$ and $\varepsilon^{18}O$ in the two isotopes of residual dissolved nitrate during denitrification. In chapter 2, the presence of different carbon sources as electron donors was found to be connected to different enrichment factors of nitrate during nitrate reduction. A similar effect was recently also described for bacterial sulfate reduction (BSR) [125]. However, in that case, the carbon source influenced the sulfate reduction rate and thus only indirectly the enrichment factors of sulfate stable isotopes. The experiments in the present study showed such an effect for denitrification without significant change in the nitrate reduction rate. The explanatory model proposed for the influence of carbon sources on nitrate isotope enrichment factors in chapter 2 is thus a new approach compared to those that have yet been suggested for BSR and microbial nitrate reduction. Nitrate concentrations and nitrate reduction rates as well as pH, salinity, temperature and the bacterial strain were kept equal in the parallel experiments and only the carbon source was changed. The changes in enrichment factors for nitrate during nitrate reduction are thus likely connected to the physiology of the cell which is changed by the presence of different carbon sources. A wider variety of carbon sources might show a wider variety of effects on the cells provoking different nitrate isotope enrichment factors as well. It is also thinkable that other environmental influences could have effects on the cells similar to the ones caused by different carbon sources, provoking similar changes in the cell physiology and thus the nitrate isotope enrichment factors. The explanatory hypothesis for the observed effect describes nitrate reduction by denitrifiers with the Nar enzyme as a three step process, two of which are transport processes. Both of these steps are considered to have only small isotope effects on nitrate. If any of these two steps becomes more influential on the overall isotope effect, it would decrease and the observed enrichment factors would get less negative. This is what was observed for the enrichment factors of nitrate during denitrification with complex hydrocarbons as a carbon source in comparison to acetate. In conjunction to that, literature data supports a change in the cell physiology consistent with the hypothesis in chapter 2 by providing evidence of changed outer cell membrane

compositions and modified gene expressions of nitrate reducing bacteria as a reaction to different carbon sources. To elucidate if this hypothesis is correct and which of the two steps is contributing to masking the effects of the third step, batch experiments with pure cultures and a determination of phospholipid composition, gene expression of the enzymes Nar, NarK1 and NarK2 (and possibly Nap, pNar and Nas) paralleled by nitrate isotope analysis could be devised.

Previous studies described another possible influence on the variability of enrichment factors for isotopes in nitrate during denitrification: Nitrate reduction rates are thought to play a role in determining these enrichment factors [87; 91; 92]. This is consistent with the explanations given in this study, as an increase or decrease in nitrate reduction rate could either by itself influence the apparent kinetic isotope effect by changing k_3, or the different nitrate reduction rates may actually be an expression of a limitation in transport of nitrate into the cell. In both cases, Eq. 2.1 can provide insights into the mechanism of changed isotope effects for nitrate during bacterial nitrate reduction.

4.2 Microbially catalyzed oxygen isotope exchange under denitrifying conditions

A major finding of this study is the absence of oxygen isotope exchange between water and nitrate in regular denitrifiers carrying the Nar enzyme and the presence of said exchange in nitrite oxidizing bacteria carrying the NXR enzyme as well as in natural microbial denitrifying communities. As nitrifying bacteria seem to be present in many anoxic environments, especially if these are not permanently anoxic, the microbial communities in most environments can have the potential to catalyze an oxygen exchange of nitrate with ambient water to a certain degree. This is likely the explanation for the variability of the observed slopes Λ for denitrification in field studies and the observed difference in said slope between studies on mixed natural bacterial communities and pure cultures in the laboratory (Fig. 1.5, Table 1.2). Obviously, the relative activity of NXR in relation to Nar in a nitrate reducing environment determines the extent of the oxygen isotope exchange between the residual nitrate and ambient water. Also, long residence times of nitrate in such conditions would increase the likelihood of an isotopic exchange, especially if there is a significant amount of nitrite present. All of these factors can lead to an isotopic exchange between water and nitrate to a varying degree and result in various slopes for Λ'. The extreme would then be Λ'=0 for environments in which nitrate quickly adopts the oxygen isotopic value of water in an equilibrium. If there is no oxygen isotope exchange, a Λ'~1 would likely be the result, as this was observed for nitrate reduction by the Nar enzyme as well as by eukaryotes in pure cultures. A Λ' between 0 and 1 could be the result of a mixing of isotope enrichment due to nitrate reduction and an oxygen isotope exchange of nitrate with water of a low $\delta^{18}O$. A high $\delta^{18}O$-H_2O could on the other hand mimic an isotope enrichment caused by nitrate reduction even though little nitrate reduction is actually happening. A conclusion from these results of this study would be: The presence of dissolved nitrite, a Λ'≠1 as well as a detection of nitrite oxidizing bacteria as part of a microbial community are all factors speaking for the presence of an oxygen exchange between water and nitrate in the environment studied. In these cases, special caution is advised when making assessments of nitrate sources in the water and the amount of denitrification happening. Caution is similarly advised when observing nitrate isotopes in soils. Kool et al. [82] demonstrated that an oxygen isotope exchange between water and nitrate also occurs in soils under oxic conditions, so the same conclusions drawn in this study also apply to nitrate in soils.

There is a possibility to create a mathematical model for the isotope exchange processes comparable to the one created for bacterial sulfate reduction by Brunner et al. [24]. However, there are several enzymatic key players involved in the nitrate reducing step and all but one step of the denitrification pathway are occurring in the periplasm of bacterial cells, not in the cytoplasm. All intermediates of denitrification produced in the cell can thus exchange with the extracellular space by diffusion. This increases the degrees of freedom of such a box-model to an extent that makes it currently impracticable.

To consider if the oxygen isotope exchange between water and nitrate can be an explanation for the observed slopes of Λ in literature, notably the difference between marine (Λ~1) and terrestrial (Λ~0.5) environments, one can look at the common oxygen isotope values of water and nitrate in these environments [22]. In marine environments, $\delta^{18}O\text{-}H_2O$ is close to zero, while terrestrial $\delta^{18}O\text{-}H_2O$ is usually more negative, especially with increasing distance to the ocean. Conversely, initial values for $\delta^{18}O\text{-}NO_3^-$ in terrestrial environments are often higher due to the impact of fertilizer application and nitrate from precipitation compared to marine environments which show values of $\delta^{18}O\text{-}NO_3^-$ close to the $\delta^{18}O\text{-}H_2O$ of seawater (~0‰) [30; 86]. An oxygen isotope exchange between water and nitrite would therefore cause a steeper slope of Λ' for marine environments as the increase in $\delta^{18}O\text{-}NO_3^-$ is not compensated as much by the relatively high $\delta^{18}O\text{-}H_2O$ of marine water compared to terrestrial environments in which the increase of an already high $\delta^{18}O\text{-}NO_3^-$ would be partially compensated by an oxygen isotope exchange with terrestrial water of a low $\delta^{18}O\text{-}H_2O$ (Fig. 4.1).

There is also an additional possible contribution to the variability in the slope Λ. There can be a difference in the activity of several nitrate reductase enzymes that have a different behavior in respect to the relative enrichment of ^{15}N and ^{18}O in the residual nitrate. The periplasmic nitrate reductase Nap is hypothesized to provoke a Λ of only 0.6, while the cytoplasmic nitrate reductase Nar is thought to result in a Λ close to 1.0 – if both enzymes are active, a mixture between these two values for Λ could be observed in the residual dissolved nitrate [62]. As Nap is considered to be less active under most circumstances [56; 62], its contribution to nitrate isotope enrichment in nature is probably minute in comparison to the isotope exchange processes described in this study.

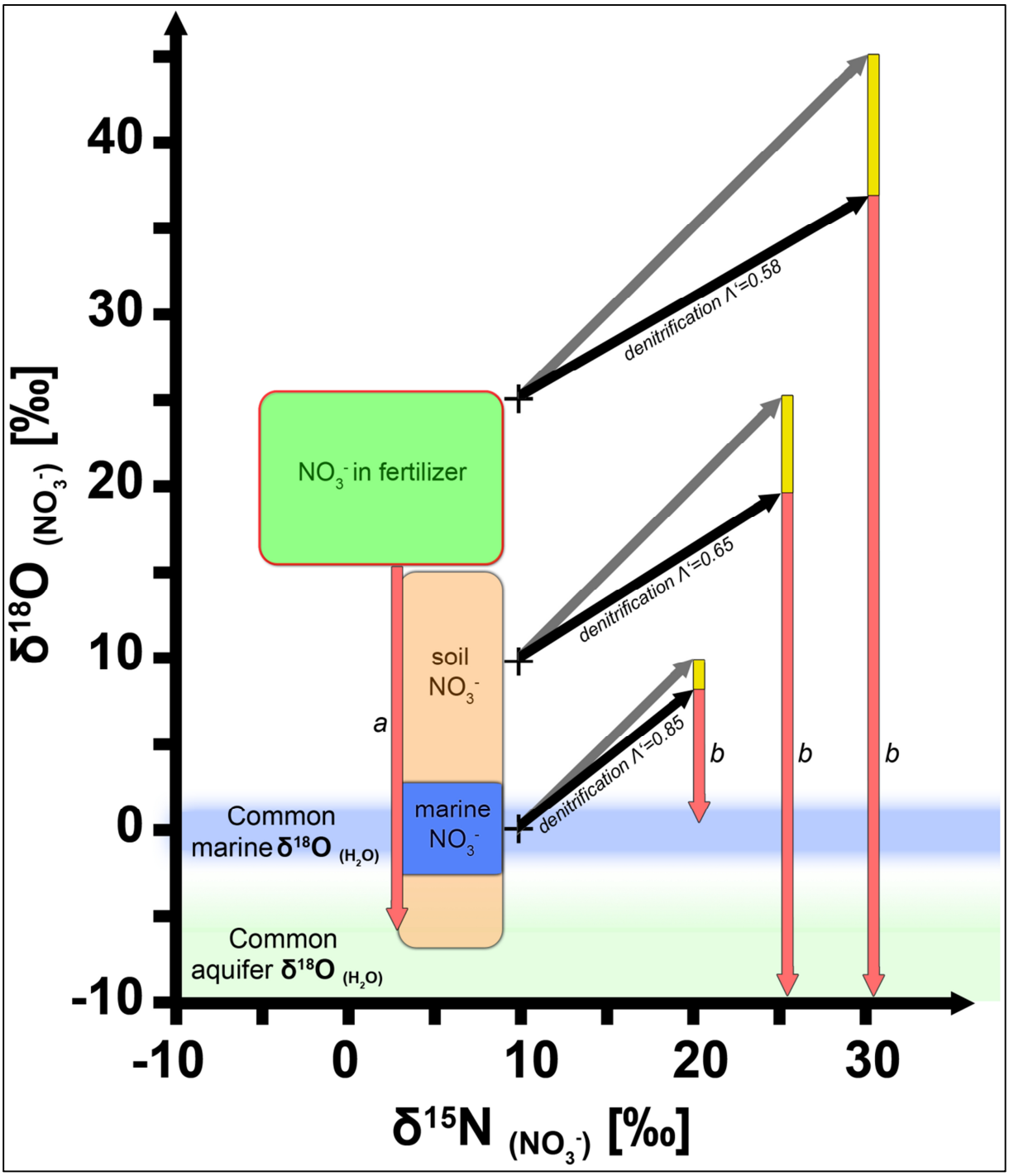

Fig 4.1: Possible effects of the oxygen isotope exchange between water and nitrate on exemplary scenarios in a dual isotope plot. For this plot, the average marine nitrate isotope data was approximated from Casciotti et al. [30]; Lehmann et al. [86]; Sigman et al. [121].

(a) The ^{18}O-signature of nitrate from a source (for example fertilizer) can be lost due to a shift of δ^{18}O in nitrate towards the δ^{18}O of ambient water. Eventually the δ^{18}O of nitrate would be nearly identical to the δ^{18}O of ambient water, in this case δ^{18}O-H_2O=-5‰, and its source could be misjudged.

(b) During denitrification, identical amounts of isotopic exchange *E* can lead to different values for Λ' depending on the extent of the difference in δ^{18}O between water and nitrate. In the examples in this figure, initial δ^{18}O-NO_3^- was 0‰ for marine, 10‰ for soil derived and 25‰ for fertilizer derived nitrate; the ambient δ^{18}O-H_2O was 0‰ for marine and -10‰ for terrestrial water; the grey arrows depict the isotope enrichment caused by denitrification with the hypothetical Λ=1; the red arrows show the difference between δ^{18}O-NO_3^- and δ^{18}O-H_2O; The yellow parts of the red arrows represent an exchange of *E*=15%; the black arrows are the resulting observed trends in both isotopes for denitrification with different values for Λ'.

4.3 Conclusion

This study has provided valuable evidence of two effects on the application of dual isotopes of nitrate that have been mysterious up to now. By providing evidence of an influence of carbon sources on the enrichment factors of nitrate during nitrate reduction and creating a hypothesis on the reasons behind this influence that goes beyond the influence by selected substances, options to further elucidate the enrichment factors during denitrification have been created. The evidence for an oxygen isotope exchange between water and nitrate is a contribution to the isotopic model of denitrification in the environment and provides an explanation of the observed variability in the relative enrichment of $\delta^{18}O$ vs. $\delta^{15}N$ of residual nitrate during denitrification in the literature. It also can serve as an explanation of low $\delta^{18}O$ values of nitrate in waters that were considered to be influenced by fertilizer (Fig. 4.1). As the oxygen isotope exchange of nitrate with water can mask the high $\delta^{18}O$ signature of nitrate fertilizer as well as high $\delta^{18}O$ values of nitrate derived from precipitation, researchers in the future have to consider if an oxygen isotope exchange has occurred in the field sites studied.

5 References

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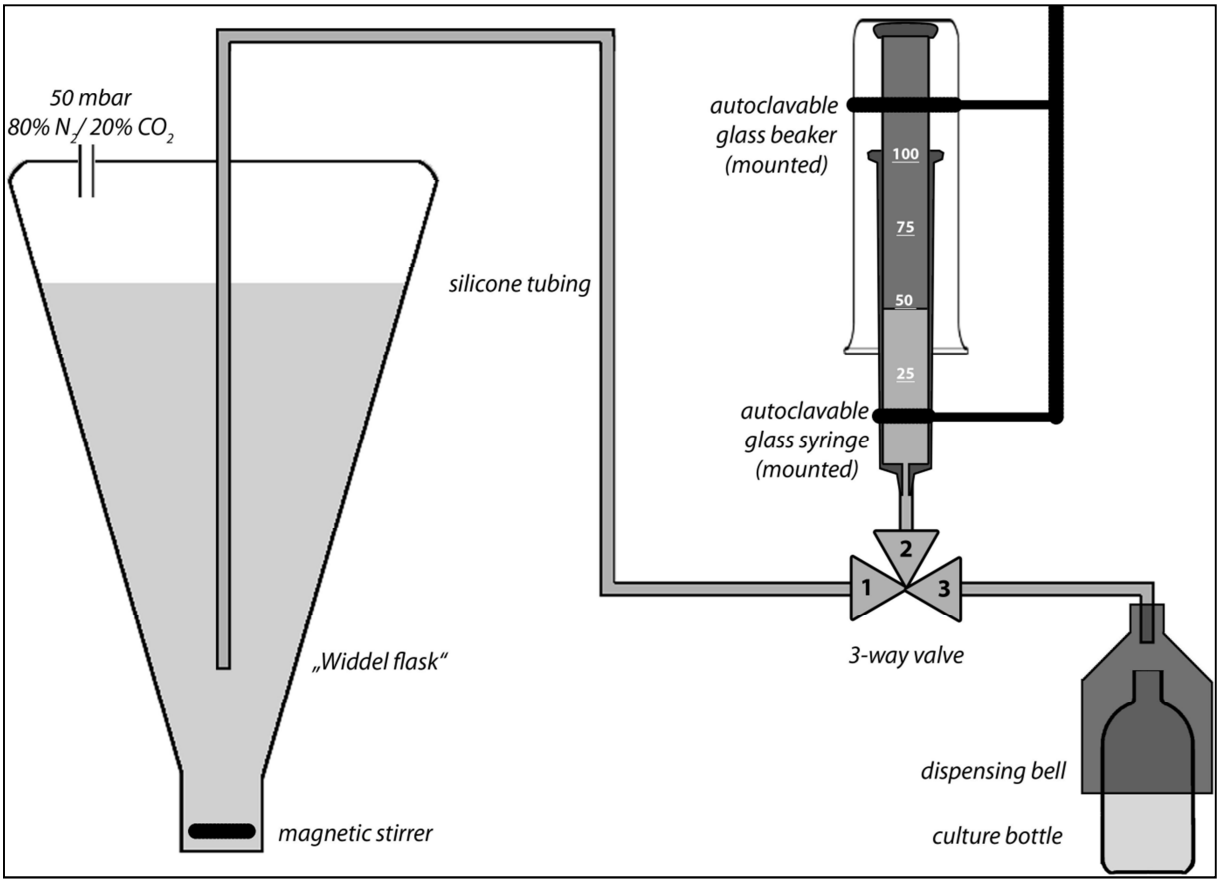
Appendix

Supplementary figure: Schematic of growth medium dispenser (section 2.2.3)



Anoxic growth medium and all parts of the setup are autoclaved under a N_2 atmosphere. Growth medium is then prepared and stirred under 50 mbar pressure of a mixture of 80%N_2/20%CO_2 in a "Widdel flask". In the beginning, the 3-way valve is closed. Take care to remove gas bubbles from the system. Step 1: 3-way valve connects port 1 and 2; Glass syringe fills due to overpressure until it reaches the stop point defined by the glass beaker. Step 2: 3-way valve connects port 2 and 3; Glass syringe empties by gravitational flow into culture bottle. While processing and closing the bottle, step 1 can already be started again. Repeat until all medium is dispensed.

Data tables for chapter 2

Methodology to measure nitrate isotopes									section 2.2.7
sample size=50ml									
original $\delta^{18}O$ and $\delta^{15}N$ of nitrate standards:									
standard name			average $\delta^{18}O$ [‰]		±1σ		average $\delta^{15}N$ [‰]		±1σ
Lab KM			23.54		0.23		5.43		0.01
Lab NM			20.79		0.13		-4.86		0.21
Lab 1			63.79		0.45		5.82		0.02
nitrite tracer			5249.62		51.28		-23.81		0.04
$\delta^{18}O$ and $\delta^{15}N$ of processed samples with nitrate standards:									
$c(NO_3^-)$ [mM]	standard name	target $\delta^{18}O$ [‰]	sample $\delta^{18}O$ [‰]	±1σ	$\Delta\delta^{18}O$ [‰]	target $\delta^{15}N$ [‰]	sample $\delta^{15}N$ [‰]	±1σ	$\Delta\delta^{15}N$ [‰]
9.99	Lab 1	63.79	64.51	0.14	-0.71	5.82	5.97	0.04	-0.14
9.80	Lab 1	63.79	64.54	1.49	-0.75	5.82	6.22	0.06	-0.40
9.35	Lab 1	63.79	63.26	0.11	0.53	5.82	6.16	0.01	-0.33
9.08	Lab 1	63.79	62.21	0.21	1.59	5.82	5.90	0.15	-0.07
8.42	Lab 1	63.79	62.54	0.09	1.25	5.82	6.39	0.01	-0.57
8.08	Lab 1	63.79	63.35	0.17	0.44	5.82	5.87	0.11	-0.05
4.51	Lab 1	63.79	64.67	0.01	-0.87	5.82	5.77	0.01	0.05
11.24	Lab KM	23.54	24.25	1.04	-0.71	5.43	5.19	0.04	0.23
10.93	Lab KM	23.54	23.97	0.54	-0.43	5.43	5.17		0.26
8.88	Lab KM	23.54	23.09	0.52	0.45	-4.86	-4.40	0.12	-0.46
9.80	Lab NM	20.79	21.52	0.44	-0.73	-4.86	-4.66		-0.20
9.66	Lab NM	20.79	21.40	0.30	-0.61	-4.86	-5.10	0.06	0.24
9.17	Lab NM	20.79	20.94	0.55	-0.15	-4.86	-4.63	0.16	-0.23
8.75	Lab NM	20.79	22.31	1.51	-1.52	-4.86	-5.18	0.08	0.32
8.66	Lab NM	20.79	21.75	0.84	-0.96	-4.86	-4.75	0.08	-0.11
average deviation of $\delta^{18}O$ from heavy standards >60‰						+0.21±1.01 [‰]			
average deviation of $\delta^{18}O$ from light standards <25‰						-0.58±0.58 [‰]			
average deviation of $\delta^{15}N$ from heavy standards >0‰						-0.11±0.28 [‰]			
average deviation of $\delta^{15}N$ from light standards <0‰						-0.07±0.30 [‰]			
samples with c(NO3-) <4mM show a larger deviation ($\Delta\delta^{18}O$) and are discarded in the experiments:									
3.99	*Lab KM*	*23.54*	*28.18*	*0.71*	*-4.64*	*5.43*	*5.57*	*0.13*	*-0.15*
3.98	*Lab 1*	*63.79*	*62.48*	*1.48*	*1.31*	*5.82*	*6.40*	*0.01*	*-0.57*
3.97	*Lab KM*	*23.54*	*24.34*	*0.25*	*-0.80*	*5.43*	*5.31*	*0.02*	*0.12*
3.64	*Lab 1*	*63.79*	*64.34*	*0.55*	*-0.55*	*5.82*	*6.79*	*0.03*	*-0.96*
3.59	*Lab 1*	*63.79*	*62.20*	*0.12*	*1.59*	*5.82*	*5.94*	*0.00*	*-0.12*
3.31	*Lab KM*	*23.54*	*24.37*	*0.85*	*-0.83*	*5.43*	*5.29*	*0.01*	*0.14*
2.90	*Lab 1*	*63.79*	*61.30*	*0.96*	*2.50*	*5.82*	*5.86*	*0.08*	*-0.04*
2.66	*Lab 1*	*63.79*	*60.70*	*0.87*	*3.09*	*5.82*	*5.94*	*0.10*	*-0.12*
2.51	*Lab 1*	*63.79*	*63.09*	*0.96*	*0.70*	*5.82*	*6.24*	*0.13*	*-0.41*
2.44	*Lab 1*	*63.79*	*64.50*	*1.86*	*-0.71*	*5.82*	*6.37*	*0.03*	*-0.55*
1.02	*Lab 1*	*63.79*	*57.22*	*0.88*	*6.57*	*5.82*	*6.11*	*0.04*	*-0.28*
0.78	*Lab 1*	*63.79*	*56.09*	*0.37*	*7.70*	*5.82*	*5.79*	*0.26*	*0.03*
0.63	*Lab 1*	*63.79*	*54.59*	*1.68*	*9.20*	*5.82*	*8.73*	*0.30*	*-2.91*

Batch incubations of pure cultures										sections 2.3.1+2.3.2
including the data used for calculating enrichment factors in Fig. 2.1										
Thauera aromatica with acetate and $\delta^{18}O$-H_2O~1700‰										
ID	time [h]	$c(NO_3^-)$ [mM]	$c(NO_2^-)$ [mM]	$\delta^{18}O$ [‰]	±1σ	$\delta^{15}N$ [‰]	±1σ	$\ln(C_t/C_0)$ of NO_3^-	$\ln(R_t/R_0)$ for ^{18}O	$\ln(R_t/R_0)$ for ^{15}N
22	0.3	8.53	0.73	22.18	0.97	-4.78	0.04	0.000000	0.000000	0.000000
22	4.0	7.82	1.47	22.29	0.27	-3.30	0.08	-0.087101	0.000103	0.001484
22	5.0	7.54	1.67	23.63	0.31	-2.10	0.07	-0.122330	0.001416	0.002688
22	6.0	6.92	2.40	26.57	0.38	-0.07	0.12	-0.208505	0.004284	0.004722
22	8.0	6.14	3.30	28.63	0.10	2.99	0.09	-0.328596	0.006290	0.007778
22	10.0	5.32	4.39	31.94	0.03	7.22	0.07	-0.472494	0.009501	0.011987
22	13.8	4.92	4.91	33.39	0.42	8.74	0.05	-0.550538	0.010905	0.013495
22	17.5	4.51	4.99	35.60	1.02	9.06	0.13	-0.637062	0.013039	0.013809
22	19.5	4.62	4.98	34.84	0.41	9.25	0.07	-0.613194	0.012306	0.014002
22	23.5	4.50	5.05	35.68	0.82	9.76	0.03	-0.638530	0.013120	0.014505
24	0.3	7.68	0.85	24.23	0.10	5.19	0.12	0.000000	0.000000	0.000000
24	2.3	7.32	1.10	26.29	0.03	5.85	0.07	-0.047585	0.002011	0.000660
24	3.5	7.61	1.27	25.44	0.67	6.02	0.14	-0.008520	0.001184	0.000834
24	4.5	7.29	1.64	25.86	0.21	7.10	0.14	-0.051788	0.001596	0.001903
24	6.5	6.65	2.31	29.61	1.31	9.21	0.35	-0.143538	0.005238	0.003992
24	8.5	5.75	3.56	31.85	0.39	13.75	0.06	-0.288139	0.007413	0.008483
24	14.5	4.59	4.67	36.92	0.48	19.90	0.30	-0.513489	0.012316	0.014531
24	18.8	4.47	5.02	38.43	0.06	21.12	0.19	-0.541530	0.013773	0.015730
Thauera aromatica with benzoate and $\delta^{18}O$-H_2O~1700‰										
ID	time [h]	$c(NO_3^-)$ [mM]	$c(NO_2^-)$ [mM]	$\delta^{18}O$ [‰]	±1σ	$\delta^{15}N$ [‰]	±1σ	$\ln(C_t/C_0)$ of NO_3^-	$\ln(R_t/R_0)$ for ^{18}O	$\ln(R_t/R_0)$ for ^{15}N
16	0.2	7.97	not measured	21.95	0.69	-4.45	0.03	0.000000	0.000000	0.000000
16	5.8	6.96		23.91	1.42	-2.71	0.03	-0.135848	0.001915	0.001739
16	7.7	6.21		24.20	0.66	-1.68	0.05	-0.249119	0.002200	0.002776
16	10.2	5.40		28.41	0.37	2.48	0.02	-0.389531	0.006306	0.006932
16	10.2	5.52		27.00	1.14	1.96	0.08	-0.367278	0.004930	0.006413
Thauera aromatica with toluene and $\delta^{18}O$-H_2O~1700‰										
ID	time [h]	$c(NO_3^-)$ [mM]	$c(NO_2^-)$ [mM]	$\delta^{18}O$ [‰]	±1σ	$\delta^{15}N$ [‰]	±1σ	$\ln(C_t/C_0)$ of NO_3^-	$\ln(R_t/R_0)$ for ^{18}O	$\ln(R_t/R_0)$ for ^{15}N
13	0.2	8.36	not measured	22.87	0.65	-4.19	0.07	0.000000	0.000000	0.000000
13	5.8	8.21		23.02	0.50	-3.50	0.10	-0.018197	0.000141	0.000694
13	8.1	8.10		23.66	0.95	-3.43	0.13	-0.031866	0.000770	0.000770
13	15.2	5.28		31.51	0.01	5.05	0.05	-0.459474	0.008414	0.009240
13	20.3	4.60		33.65	0.15	6.58	0.10	-0.597647	0.010478	0.010765
13	29.8	4.06		35.21	0.39	8.53	0.13	-0.721591	0.011995	0.012699
14	0.2	8.24		23.24	1.08	-3.97	0.16	0.000000	0.000000	0.000000
14	5.8	8.22		22.79	0.17	-3.64	0.13	-0.001898	-0.000443	0.000332
14	8.1	8.21		23.94	0.32	-3.33	0.01	-0.003203	0.000688	0.000642
14	15.2	5.07		32.41	1.03	6.46	0.07	-0.485907	0.008920	0.010414
14	18.6	4.92		31.74	0.40	6.05		-0.516176	0.008270	0.010012
14	20.3	4.34		32.81	0.22	7.26	0.10	-0.640038	0.009306	0.011207
14	38.8	4.22		34.75	0.17	7.91	0.09	-0.668488	0.011189	0.011854

Thauera aromatica with acetate and $\delta^{18}O$-H_2O~-10‰										
ID	time [h]	$c(NO_3^-)$ [mM]	$c(NO_2^-)$ [mM]	$\delta^{18}O$ [‰]	±1σ	$\delta^{15}N$ [‰]	±1σ	$\ln(C_t/C_0)$ of NO_3^-	$\ln(R_t/R_0)$ for ^{18}O	$\ln(R_t/R_0)$ for ^{15}N
19	2.2	9.07	0.67	22.07	0.77	-4.15	0.01	0.000000	0.000000	0.000000
19	4.0	8.90	0.82	22.78	0.71	instr. failure[1]		-0.019333	0.000690	-
19	4.5	8.96	1.30	24.12	0.79	-2.38	0.19	-0.012663	0.002002	0.001782
19	6.6	8.45	1.73	24.40	0.23	-1.23	0.13	-0.071023	0.002278	0.002933
19	8.0	7.54	1.74	25.50	0.46	-1.25	0.12	-0.184564	0.003343	0.002909
19	9.2	6.60	3.34	31.14	0.98	4.63	0.03	-0.318079	0.008831	0.008783
19	13.0	6.92		32.89	0.86	4.70	0.07	-0.270782	0.010531	0.008851
19	17.5	5.45	4.76	34.55	0.20	8.76	0.01	-0.509465	0.012133	0.012883
19	19.6	5.42	4.87	33.98	0.01	9.00	0.19	-0.514917	0.011582	0.013127
19	22.2	5.30	4.89	33.56	0.50	9.06	0.23	-0.537243	0.011176	0.013179
26	0.0	7.02	0.38	24.81	0.12	9.08	0.54	0.000000	0.000000	0.000000
26	6.5	7.10	0.38	26.64	0.87	9.34	0.22	0.011316	0.001789	0.000260
26	15.8	6.25	1.19	28.27	0.38	12.77	0.27	-0.116192	0.003375	0.003654
26	20.0	5.69	1.71	29.56	0.48	14.65	0.05	-0.209563	0.004624	0.005506
26	22.2	7.04	0.47	25.94	0.69	9.06	0.25	0.003480	0.001107	-0.000020
26	22.2	5.24	1.81	30.86	0.07	14.68	1.40	-0.291387	0.005888	0.005540
26	23.5	4.51	3.03	35.41	0.01	19.79	0.10	-0.442362	0.010289	0.010555
26	26.5	4.41	3.01	37.95	1.39	20.22	0.20	-0.465348	0.012739	0.010982
28	0.0	8.71	0.61	24.32	0.15	9.03	0.45	0.000000	0.000000	0.000000
28	9.0	8.41	1.04	25.65	0.08	8.86	0.45	-0.034216	0.001303	-0.000176
28	21.5	4.54	4.77	37.47	0.53	22.47	0.47	-0.651430	0.012758	0.013225
28	21.5	4.54	4.46	36.63	0.00	22.21	0.12	-0.651430	0.011947	0.012977
28	21.5	4.54	4.60	36.26	0.40	22.21	0.29	-0.651430	0.011591	0.012974
28	25.0	4.64	4.92	37.84	0.01	22.50	0.20	-0.628443	0.013109	0.013262
28	30.0	4.64	4.46	37.26	0.15	20.16	0.05	-0.628758	0.012551	0.010967
28	46.0	4.48	4.73	37.24	0.02	21.98	0.64	-0.665366	0.012537	0.012745
10 3	0.0	10.21	0.20	23.46	0.08	9.03	0.14	0.000000	0.000000	0.000000
10 3	6.2	9.08	0.31	24.18	0.24	10.19	0.22	-0.117263	0.000703	0.001146
10 3	22.5	4.40	6.30	39.31	0.21	instr. failure[1]		-0.843062	0.015368	-
10 3	52.5	3.88	6.46	40.85	0.53	27.53	0.41	-0.967721	0.016843	0.018172
23	0.3	7.39	0.80	24.47	0.43	5.08	0.12	0.000000	0.000000	0.000000
23	2.3	7.16	1.04	25.30	0.31	5.66	0.11	-0.032202	0.000808	0.000573
23	3.5	7.42	1.24	25.42	0.36	6.50	0.31	0.003896	0.000927	0.001408
23	4.5	7.02	1.59	27.09	0.29	6.83	0.03	-0.050579	0.002553	0.001740
23	6.5	6.61	2.14	28.28	1.41	9.53	0.13	-0.110989	0.003709	0.004413
23	8.5	5.91	2.95	32.18	1.54	12.34	0.10	-0.222705	0.007491	0.007193
23	14.5	4.52	4.17	37.59	0.05	18.74	0.03	-0.491287	0.012720	0.013503
23	16.5	4.25	4.60	39.27	0.30	21.10	0.11	-0.552899	0.014337	0.015817
23	18.8	4.19	5.02	41.34	0.43	22.08	0.09	-0.566841	0.016331	0.016775
23	22.8	4.16	5.21	41.96	0.20	22.90	0.08	-0.574962	0.016923	0.017578

Thauera aromatica with benzoate and $\delta^{18}O$-H_2O~-10‰										
ID	time [h]	$c(NO_3^-)$ [mM]	$c(NO_2^-)$ [mM]	$\delta^{18}O$ [‰]	±1σ	$\delta^{15}N$ [‰]	±1σ	$\ln(C_t/C_0)$ of NO_3^-	$\ln(R_t/R_0)$ for ^{18}O	$\ln(R_t/R_0)$ for ^{15}N
17	0.2	6.67	0.48	21.00	0.22	-4.94	0.02	0.000000	0.000000	0.000000
17	6.2	5.72	1.66	23.87	0.30	-1.08	0.01	-0.152670	0.002809	0.003874
17	10.3	4.38		30.99	0.50	6.08	0.29	-0.546892	0.009738	0.011021
21	0.3	8.92	0.43	22.15	0.64	-5.24	0.02	0.000000	0.000000	0.000000
21	5.0	8.50	0.94	23.35	0.37	-3.94	0.11	-0.048142	0.001172	0.001306
21	7.0	8.20	1.22	22.70	0.10	-3.46	0.06	-0.083136	0.000535	0.001791
21	8.0	7.77	1.78	24.33	0.10	-1.78	0.08	-0.137653	0.002124	0.003472
21	10.0	6.80	2.62	26.37	0.13	0.95	0.05	-0.271497	0.004123	0.006199
21	11.3	5.87	1.87	28.19	0.01	3.11	0.08	-0.418635	0.005894	0.008357

Thauera aromatica with toluene and $\delta^{18}O$-H_2O~-10‰										
ID	time [h]	$c(NO_3^-)$ [mM]	$c(NO_2^-)$ [mM]	$\delta^{18}O$ [‰]	±1σ	$\delta^{15}N$ [‰]	±1σ	$\ln(C_t/C_0)$ of NO_3^-	$\ln(R_t/R_0)$ for ^{18}O	$\ln(R_t/R_0)$ for ^{15}N
12	5.7	7.81	not measured	22.64	0.28	-2.99	0.10	0.000000	0.000000	0.000000
12	8.0	8.02		22.87	0.30	-3.26	0.05	0.025544	0.000228	-0.000279
12	15.8	6.18		25.38	0.05	0.04	0.04	-0.234164	0.002672	0.003034
12	17.8	5.91		26.43	0.12	1.42	0.15	-0.278641	0.003699	0.004408
12	21.3	5.54		29.36	0.23	4.37	0.10	-0.343371	0.006552	0.007351
12	24.0	5.73		28.44	0.02	3.21	0.05	-0.309360	0.005656	0.006191

strain EBN1 with acetate and $\delta^{18}O$-H_2O~-10‰										
ID	time [h]	$c(NO_3^-)$ [mM]	$c(NO_2^-)$ [mM]	$\delta^{18}O$ [‰]	±1σ	$\delta^{15}N$ [‰]	±1σ	$\ln(C_t/C_0)$ of NO_3^-	$\ln(R_t/R_0)$ for ^{18}O	$\ln(R_t/R_0)$ for ^{15}N
40 2	0.0	11.22	0.52	28.38	3.85	7.71	0.60	0.000000	0.000000	0.000000
40 2	8.0	8.45	2.93	33.13	1.33	14.81	0.85	-0.283412	0.004614	0.007024
40 2	24.0	5.83	6.25	42.79	0.47	20.89	2.14	-0.654088	0.013920	0.012990
40 2	32.0	5.44	6.68	50.79	0.42	instr. failure[1]		-0.724004	0.021558	-
37	0.0	8.29	0.41	27.21	1.21	8.36	0.40	0.000000	0.000000	0.000000
37	5.0	8.31	0.64	27.42	1.10	7.18	0.01	0.002911	0.000205	-0.001169
37	9.5	7.83	1.12	31.13	1.83	8.53	0.19	-0.056665	0.003801	0.000166
37	15.8	4.25	4.57	42.31	0.08	22.64	0.81	-0.667060	0.014586	0.014064
37	19.8	4.52	4.52	43.28	1.03	23.98	0.01	-0.605258	0.015523	0.015369
37	24.0	4.43	4.66	41.55	0.93	23.58	0.53	-0.625756	0.013856	0.014986
38	0.0	8.47	0.38	27.89	0.70	8.86	0.78	0.000000	0.000000	0.000000
38	5.0	8.35	0.62	27.53	0.97	6.75	0.08	-0.014354	-0.000351	-0.002094
38	9.5	6.36	0.80	29.64	0.99	8.08	0.05	-0.285817	0.001699	-0.000765
38	15.8	4.56	4.59	42.44	1.61	21.44	0.03	-0.618319	0.014055	0.012398
38	19.8	4.46	4.74	43.49	0.94	24.34	0.46	-0.640388	0.015066	0.015231
38	24.0	4.33	4.64	42.62	1.40	23.45	0.20	-0.671654	0.014233	0.014362

strain EBN1 with toluene and $\delta^{18}O$-H_2O~-10‰										
ID	time [h]	$c(NO_3^-)$ [mM]	$c(NO_2^-)$ [mM]	$\delta^{18}O$ [‰]	±1σ	$\delta^{15}N$ [‰]	±1σ	$\ln(C_t/C_0)$ of NO_3^-	$\ln(R_t/R_0)$ for ^{18}O	$\ln(R_t/R_0)$ for ^{15}N
39	0.0	8.35	0.37	27.44	1.18	8.64	0.66	0.000000	0.000000	0.000000
39	9.5	7.45	0.93	28.39	0.45	7.88	0.32	-0.114480	0.000920	-0.000756
39	15.8	5.09	3.42	34.48	0.33	17.29	0.19	-0.494751	0.006828	0.008541
39	19.8	4.79	3.45	34.95	1.38	17.47	0.13	-0.556200	0.007278	0.008718
39	25.2	4.90	3.60	36.46	0.41	17.19	0.46	-0.533023	0.008743	0.008442
35	0.0	10.52	0.23	25.84	1.37	6.91	0.85	0.000000	0.000000	0.000000
35	14.5	10.11	0.50	27.03	1.14	6.74	0.00	-0.039039	0.001160	-0.000169
35	24.0	7.49	1.67	30.73	1.60	9.75	0.36	-0.339371	0.004757	0.002819
35	30.0	6.29	3.98	33.26	0.64	14.00	0.21	-0.514466	0.007206	0.007014
35	37.7	5.91	3.71	35.04	0.82	16.22	0.25	-0.576761	0.008933	0.009202
35	46.0	5.60	4.58	38.90	3.35	17.68	0.25	-0.629917	0.012656	0.010639

[1)] due to instrument failure, some samples could not be measured - there was not enough material left for a second analysis

Growth curves of *Thauera aromatica* with different carbon sources — **section 2.3.1**

carbon source: acetate

	duplicate #1			duplicate #2			average of both duplicates										
time [h]	$c(NO_3^-)$ [mM]	$c(NO_2^-)$ [mM]	cells/ml	$c(NO_3^-)$ [mM]	$c(NO_2^-)$ [mM]	cells/ml	$c(NO_3^-)$ [mM]	±1σ	$c(NO_2^-)$ [mM]	±1σ	cells/ml	±1σ	$c(NO_3^-+NO_2^-)$ [mM]	±1σ	csNRR [pmol/cell/h]	±1σ	NRR [µM/h]	±1σ
0	9.17	0.37	4.72E+06	9.14	0.39	7.37E+06	9.15	0.02	0.38	0.01	6.04E+06	1.87E+06	9.53	0.01				
2	8.53	0.43	3.88E+06	8.69	0.52	7.08E+06	8.61	0.12	0.47	0.06	5.48E+06	2.26E+06	9.08	0.18	0.0526463	0.0308376	352.30	195.83
4	8.65	0.62	4.37E+06	8.34	0.76	2.41E+06	8.49	0.22	0.69	0.10	3.39E+06	1.39E+06	9.19	0.12	0.0112242	0.0370944	138.54	35.26
6	8.27	0.99	3.85E+06	7.91	1.21	1.11E+07	8.09	0.25	1.10	0.15	7.45E+06	5.10E+06	9.18	0.10	0.0393349	0.0104281	265.67	107.88
8	7.47	1.62	1.42E+07	6.80	1.84	2.04E+07	7.14	0.48	1.73	0.15	1.73E+07	4.33E+06	8.87	0.32	0.0396095	0.0060889	482.51	23.47
10	6.39	3.03	5.35E+07	6.21	3.10	5.33E+07	6.30	0.12	3.07	0.05	5.34E+07	1.18E+05	9.37	0.08	0.0119844	0.0057147	408.93	247.16
12	5.28	4.32	1.40E+08	5.68	4.21	1.68E+08	5.48	0.28	4.26	0.08	1.54E+08	2.02E+07	9.74	0.20	0.0040620	0.0023443	375.45	244.35
14	4.40	4.86	1.55E+08	4.83	4.52	1.50E+08	4.62	0.30	4.69	0.24	1.52E+08	3.28E+06	9.31	0.06	0.0028240	0.0002162	363.73	81.36
16	4.15	5.19	1.85E+08	4.71	4.89	6.08E+07	4.43	0.40	5.04	0.21	1.23E+08	8.79E+07	9.47	0.19	0.0006440	0.0001384	106.45	95.19
18	4.11	5.36	1.24E+08	4.56	4.90	1.14E+08	4.34	0.32	5.13	0.32	1.19E+08	7.58E+06	9.46	0.00	0.0005012	0.0005187	72.42	33.84
20	4.08	5.45	1.30E+08	4.36	4.77	9.91E+07	4.22	0.20	5.11	0.48	1.14E+08	2.15E+07	9.33	0.28	0.0005185	0.0005879	74.85	21.47
22	4.03	5.43	2.03E+08	4.52	5.03	1.28E+08	4.27	0.35	5.23	0.28	1.65E+08	5.28E+07	9.50	0.06	-0.0002525	0.0005933	27.23	98.97
24	4.09	5.56	2.17E+08	4.55	5.11	1.24E+08	4.32	0.32	5.34	0.32	1.70E+08	6.55E+07	9.66	0.01	-0.0001332	0.0000198	28.34	51.14

carbon source: benzoate

	duplicate #1			duplicate #2			average of both duplicates										
time [h]	$c(NO_3^-)$ [mM]	$c(NO_2^-)$ [mM]	cells/ml	$c(NO_3^-)$ [mM]	$c(NO_2^-)$ [mM]	cells/ml	$c(NO_3^-)$ [mM]	±1σ	$c(NO_2^-)$ [mM]	±1σ	cells/ml	±1σ	$c(NO_3^-+NO_2^-)$ [mM]	±1σ	csNRR [pmol/cell/h]	±1σ	NRR [µM/h]	±1σ
0	8.53	0.47	4.69E+06	8.62	0.50	6.24E+06	8.57	0.06	0.48	0.02	5.47E+06	1.09E+06	9.05	0.08				
2	7.95	0.64	7.15E+06	8.07	0.69	6.67E+06	8.01	0.08	0.67	0.04	6.91E+06	3.40E+05	8.68	0.12	0.0453967	0.0039600	354.35	127.67
4	7.55	0.97	5.46E+06	7.62	1.09	9.16E+06	7.58	0.05	1.03	0.09	7.31E+06	2.61E+06	8.61	0.13	0.0303312	0.0026100	276.64	97.91
6	7.26	1.59	1.40E+07	7.14	1.86	3.60E+07	7.20	0.08	1.73	0.19	2.50E+07	1.55E+07	8.93	0.11	0.0125455	0.0030000	241.90	49.26
8	6.14	2.43	2.18E+07	5.86	2.95	3.72E+07	6.00	0.19	2.69	0.37	2.95E+07	1.09E+07	8.69	0.17	0.0244036	0.0093500	577.12	55.77
10	4.96	3.79	6.08E+07	4.63	4.52	2.10E+08	4.80	0.23	4.15	0.51	1.36E+08	1.06E+08	8.95	0.28	0.0096112	0.0079000	543.07	71.07
12	3.77	5.34	1.42E+08	3.04	6.28	1.89E+08	3.41	0.51	5.81	0.66	1.65E+08	3.37E+07	9.22	0.15	0.0049438	0.0051600	578.78	35.23
14	1.70	6.55	1.17E+08	1.31	7.59	2.23E+08	1.50	0.28	7.07	0.74	1.70E+08	7.45E+07	8.57	0.46	0.0061112	0.0063900	723.18	138.26
16	0.94	7.82	1.96E+08	0.97	7.86	1.79E+08	0.95	0.02	7.84	0.03	1.88E+08	1.23E+07	8.79	0.06	0.0016275	0.0014300	202.96	126.07
18	0.92	7.89	1.59E+08	0.93	8.12	1.86E+08	0.92	0.01	8.01	0.17	1.73E+08	1.94E+07	8.93	0.18	0.0000860	0.0000403	19.34	8.42
20	0.81	8.09	1.06E+08	0.82	8.26	1.98E+08	0.81	0.01	8.18	0.12	1.52E+08	6.54E+07	8.99	0.13	0.0003535	0.0000887	42.47	12.64
22	0.73	8.12	2.48E+08	0.73	8.25	2.23E+08	0.73	0.00	8.18	0.09	2.35E+08	1.78E+07	8.91	0.09	0.0002139	0.0000048	31.23	9.15
24	0.70	8.24	1.63E+08	0.71	8.54	1.64E+08	0.70	0.01	8.39	0.21	1.64E+08	7.05E+05	9.09	0.22	0.0000659	0.0000226	14.18	12.98

carbon source: toluene																		
	duplicate #1			duplicate #2			average of both duplicates											
time [h]	$c(NO_3^-)$ [mM]	$c(NO_2^-)$ [mM]	cells/ml	$c(NO_3^-)$ [mM]	$c(NO_2^-)$ [mM]	cells/ml	$c(NO_3^-)$ [mM]	±1σ	$c(NO_2^-)$ [mM]	±1σ	cells/ml	±1σ	$c(NO_3^-+NO_2^-)$ [mM]	±1σ	csNRR [pmol/cell/h]	±1σ	NRR [µM/h]	±1σ
0	8.78	0.67	5.90E+06	8.94	0.65	6.32E+06	8.86	0.12	0.66	0.01	6.11E+06	2.99E+05	9.52	0.11				
2	8.42	0.86	3.99E+06	8.28	0.83	4.61E+06	8.35	0.10	0.84	0.02	4.30E+06	4.38E+05	9.20	0.12	0.0479515	0.0171000	330.36	21.53
4	8.03	1.24	4.23E+06	8.06	1.25	8.17E+06	8.05	0.02	1.24	0.01	6.20E+06	2.79E+06	9.29	0.03	0.0324776	0.0211300	225.33	173.23
6	7.47	1.91	2.02E+07	7.38	1.93	2.08E+07	7.43	0.06	1.92	0.02	2.05E+07	4.38E+05	9.35	0.05	0.0231363	0.0003065	352.74	77.15
8	6.45	3.02	4.65E+07	5.77	2.80	4.62E+07	6.11	0.48	2.91	0.15	4.64E+07	2.78E+05	9.02	0.63	0.0196859	0.0015500	628.00	70.11
10	4.74	4.50	4.07E+07	4.78	4.86	1.27E+08	4.76	0.03	4.68	0.25	8.38E+07	6.09E+07	9.44	0.28	0.0126915	0.0201600	606.04	294.27
12	4.01	5.77	2.75E+08	3.90	6.12	1.86E+08	3.96	0.07	5.95	0.25	2.31E+08	6.32E+07	9.90	0.18	0.0025498	0.0021300	351.88	29.58
14	2.98	6.04	1.99E+08	2.92	6.24	9.35E+07	2.95	0.04	6.14	0.14	1.46E+08	7.48E+07	9.09	0.10	0.0028414	0.0019800	402.21	68.95
16	3.00	6.36	3.42E+08	2.87	6.51	2.89E+08	2.94	0.09	6.43	0.11	3.15E+08	3.70E+07	9.37	0.02	0.0000448	0.0001065	34.47	24.52
18	2.87	6.69	1.73E+08	2.79	6.80	2.34E+08	2.83	0.06	6.75	0.08	2.04E+08	4.35E+07	9.58	0.02	0.0002057	0.0004548	64.11	52.64
20	2.69	6.90	2.05E+08	2.66	6.87	2.08E+08	2.67	0.03	6.89	0.02	2.06E+08	2.28E+06	9.56	0.04	0.0003884	0.0001258	75.61	50.66
22	2.50	6.92	1.79E+08	2.59	7.00	2.37E+08	2.55	0.07	6.96	0.06	2.08E+08	4.08E+07	9.51	0.13	0.0003202	0.0002613	62.46	64.45
24	2.45	7.28	2.35E+08	2.67	7.55	2.26E+08	2.56	0.15	7.42	0.19	2.31E+08	6.72E+06	9.98	0.34	-0.0000255	0.0001823	21.25	56.41

Data tables for chapter 3

$\delta^{18}O$-methodology; c(NO_3^-~10 mM)				section 3.2.2.4		
original $\delta^{18}O$ of nitrate standards:						
standard name				average $\delta^{18}O$	±1σ	
'Lab 3'				64.95	0.01	
'Lab 4'				27.33	0.80	
						sample $\delta^{18}O$:
sediment type	phase	standard name	target $\delta^{18}O$	sample $\delta^{18}O$	±1σ	$\Delta\delta^{18}O$ [‰]
lake	sediment slurry	'Lab 3'	64.95	64.48	0.19	0.47
lake	sediment slurry	'Lab 3'	64.95	63.19	0.72	1.76
lake	sediment slurry	'Lab 4'	27.33	27.61	0.35	-0.28
lake	sediment slurry	'Lab 4'	27.33	27.75	0.21	-0.42
lake	supernatant	'Lab 3'	64.95	63.76	0.53	1.19
lake	supernatant	'Lab 4'	27.33	27.67	0.16	-0.34
stream	sediment slurry	'Lab 3'	64.95	44.11	2.73	20.84
stream	sediment slurry	'Lab 3'	64.95	39.26	0.73	25.69
stream	sediment slurry	'Lab 4'	27.33	27.49	0.37	-0.16
stream	sediment slurry	'Lab 4'	27.33	18.27	1.87	9.06
stream	supernatant	'Lab 3'	64.95	64.11	0.47	0.83
stream	supernatant	'Lab 4'	27.33	27.62	0.76	-0.29
tidal flat	sediment slurry	'Lab 3'	64.95	54.43	7.67	10.51
tidal flat	sediment slurry	'Lab 3'	64.95	52.45	0.25	12.50
tidal flat	sediment slurry	'Lab 3'	64.95	39.06	0.35	25.89
tidal flat	sediment slurry	'Lab 4'	27.33	27.92	0.08	-0.59
tidal flat	sediment slurry	'Lab 4'	27.33	26.50	0.15	0.83
tidal flat	sediment slurry	'Lab 4'	27.33	25.41	0.13	1.92
tidal flat	sediment slurry	'Lab 4'	27.33	24.57	0.16	2.76
tidal flat	sediment slurry	'Lab 4'	27.33	22.28	0.03	5.05
tidal flat	supernatant	'Lab 3'	64.95	58.62	1.68	6.32
tidal flat	supernatant	'Lab 3'	64.95	58.80	0.83	6.15
tidal flat	supernatant	'Lab 4'	27.33	25.17	0.11	2.16

Incubations of *Nitrobacter vulgaris*				**section 3.3.1**	
series A (without oxygen sensor):					
bottle 1				$\delta^{18}O$ of water:	-11.32±0.17‰
time [days]	$c(NO_3^-)$ [mg/l]	$c(NO_2^-)$ [mg/l]	$\delta^{18}O$ [‰]	$\delta^{15}N$ [‰]	$c(O_2)$[ppm]
0	961.70	0.00	-1.73	-20.56	n.a.
3	933.76	0.00	-4.47	-21.05	n.a.
6	853.21	1.13	-1.24	-19.35	n.a.
10	985.70	2.36	-7.62	-20.68	n.a.
20	988.06	6.29	-7.48	-17.46	n.a.
88	1054.47	12.31	-5.99	-21.95	n.a.
bottle 2				$\delta^{18}O$ of water:	21.91±1.38‰
time [days]	$c(NO_3^-)$ [mg/l]	$c(NO_2^-)$ [mg/l]	$\delta^{18}O$ [‰]	$\delta^{15}N$ [‰]	$c(O_2)$[ppm]
0	929.44	0.00	-5.29	-19.82	n.a.
3	931.26	0.00	-5.40	-21.24	n.a.
6	994.47	1.42	-2.34	-20.85	n.a.
10	986.05	2.43	-7.81	-21.82	n.a.
20	983.18	5.76	-8.48	-19.68	n.a.
88	1061.00	11.50	-4.39	-19.97	n.a.
bottle 3				$\delta^{18}O$ of water:	50.71±0.52‰
time [days]	$c(NO_3^-)$ [mg/l]	$c(NO_2^-)$ [mg/l]	$\delta^{18}O$ [‰]	$\delta^{15}N$ [‰]	$c(O_2)$[ppm]
0	939.62	0.00	-4.37	-21.59	n.a.
3	916.65	0.00	-2.91	-18.58	n.a.
6	990.74	1.41	1.12	-19.81	n.a.
10	sample was lost				n.a.
20	972.44	6.35	-5.85	-20.29	n.a.
88	1057.09	13.07	1.29	-20.94	n.a.
bottle 4				$\delta^{18}O$ of water:	132.23±1.34‰
time [days]	$c(NO_3^-)$ [mg/l]	$c(NO_2^-)$ [mg/l]	$\delta^{18}O$ [‰]	$\delta^{15}N$ [‰]	$c(O_2)$[ppm]
0	955.06	0.00	-1.31	-19.01	n.a.
3	923.64	1.55	-4.97	-20.49	n.a.
6	990.11	1.59	-6.67	-21.66	n.a.
10	980.46	2.85	-7.30	-21.73	n.a.
20	976.80	6.93	instr. fail. [1)]		n.a.
88	1059.36	13.60	15.85	-19.69	n.a.
bottle 5				$\delta^{18}O$ of water:	396.15±1.98‰
time [days]	$c(NO_3^-)$ [mg/l]	$c(NO_2^-)$ [mg/l]	$\delta^{18}O$ [‰]	$\delta^{15}N$ [‰]	$c(O_2)$[ppm]
0	892.48	0.00	-2.14	-21.77	n.a.
3	892.46	2.12	-3.73	-16.51	n.a.
6	950.64	2.07	-2.77	-20.84	n.a.
10	944.15	3.78	-5.82	-21.11	n.a.
20	940.35	7.62	-0.95	-19.63	n.a.
88	1020.16	16.10	40.08	-17.61	n.a.

series B (with oxygen sensor):					
bottle 1				$\delta^{18}O$ of water:	-10.06±0.09‰
time [days]	$c(NO_3^-)$ [mg/l]	$c(NO_2^-)$ [mg/l]	$\delta^{18}O$ [‰]	$\delta^{15}N$ [‰]	$c(O_2)$[ppm]
0	619.32	0.36	-2.21	-20.35	0.02
12	533.07	49.61	-0.11	-17.39	0.04
18	572.01	69.46	0.06	-18.18	0.07
70	550.04	62.09	6.57	instr. fail. [1)]	0.07
bottle 2				$\delta^{18}O$ of water:	22.29±0.23‰
time [days]	$c(NO_3^-)$ [mg/l]	$c(NO_2^-)$ [mg/l]	$\delta^{18}O$ [‰]	$\delta^{15}N$ [‰]	$c(O_2)$[ppm]
0	617.58	0.49	-3.13	-20.66	0.02
12	600.82	1.07	-3.13	instr. fail. [1)]	0.01
18	702.52	14.20	-2.82	-20.66	0.02
70	633.15	19.66	9.67	-21.62	0.02
bottle 3				$\delta^{18}O$ of water:	37.55±0.40‰
time [days]	$c(NO_3^-)$ [mg/l]	$c(NO_2^-)$ [mg/l]	$\delta^{18}O$ [‰]	$\delta^{15}N$ [‰]	$c(O_2)$[ppm]
0	600.22	0.00	-3.84	-20.58	0.02
12	601.93	1.16	-3.54	-22.24	0.02
18	665.38	13.50	-2.66	-22.10	0.02
70	647.27	16.07	10.03	-22.08	0.03
bottle 4				$\delta^{18}O$ of water:	132.65±2.08‰
time [days]	$c(NO_3^-)$ [mg/l]	$c(NO_2^-)$ [mg/l]	$\delta^{18}O$ [‰]	$\delta^{15}N$ [‰]	$c(O_2)$[ppm]
0	592.35	0.00	-4.27	-22.47	0.02
12	595.59	2.45	-2.22	-19.29	0.01
18	618.03	7.00	3.17	-19.76	0.02
70	663.31	23.55	53.64	-21.34	0.02
bottle 5				$\delta^{18}O$ of water:	289.85±4.23‰
time [days]	$c(NO_3^-)$ [mg/l]	$c(NO_2^-)$ [mg/l]	$\delta^{18}O$ [‰]	$\delta^{15}N$ [‰]	$c(O_2)$[ppm]
0	598.21	0.32	-3.73	-23.24	0.03
12	596.67	1.74	-1.51	-18.16	0.01
18	686.72	13.20	1.56	-19.26	0.02
70	640.64	22.90	88.19	-21.46	0.02
bottle 6 (sterile control)				$\delta^{18}O$ of water:	289.05±4.59‰
time [days]	$c(NO_3^-)$ [mg/l]	$c(NO_2^-)$ [mg/l]	$\delta^{18}O$ [‰]	$\delta^{15}N$ [‰]	$c(O_2)$[ppm]
0	613.39	0.60	-3.97	-23.00	0.15
12	613.49	0.00	-4.14	-22.85	0.11
18	695.01	0.00	-4.54	-21.38	0.09
70	688.09	0.00	-4.96	-22.73	0.30

[1)] due to instrument failure, some samples could not be measured - there was not enough material left for a second analysis

Incubations of *Nitrobacter vulgaris*				**section 3.3.1**
series A (without oxygen sensor):				
average anion concentrations of bottle 1-5				
time [days]	$c(NO_3^-)$ [mg/l]	±1σ	$c(NO_2^-)$ [mg/l]	±1σ
0	935.66	37.31	0.00	0.00
3	919.55	29.34	0.73	0.97
6	955.83	107.39	1.52	0.39
10	974.09	19.47	2.86	0.62
20	972.17	20.50	6.59	0.72
88	1050.41	16.11	13.32	1.73
series B (with oxygen sensor):				
average anion concentrations of bottle 1-5				
time [days]	$c(NO_3^-)$ [mg/l]	±1σ	$c(NO_2^-)$ [mg/l]	±1σ
0	609.76	12.54	0.24	0.26
12	585.62	28.83	11.20	20.26
18	648.93	50.43	23.47	24.39
70	626.88	41.84	28.85	17.74

Oxygen content in aerobic incubations of sediment samples														**section 3.3.3**
oxic lake sediment incubations					oxic stream sediment incubations					oxic tidal flat sediment incubations				
c (O_2) [ppm] in bottle number					c (O_2) [ppm] in bottle number					c (O_2) [ppm] in bottle number				
time [days]	9	10	11	12	time [days]	9	10	11	12	time [days]	9	10	11	12
0	10.27	10.36	10.37	9.13	0	7.97	5.51	11.39	11.17	0	8.27	8.50	10.67	7.85
1	10.36	10.46	10.37	10.57	0	4.22	9.20	11.28	11.38	1	8.81	9.42	10.38	7.44
2	8.33	7.97	10.37	10.28	1	8.33	8.80	10.96	10.96	2	9.21	9.91	10.57	7.13
4	9.80	9.99	10.27	10.47	1	6.69	6.88	10.96	10.96	3	9.47	10.09	10.67	7.51
5	9.71	10.08	10.08	10.38	2	9.80	10.08	10.86	11.07	4	7.25	7.53	8.41	6.00
7	9.90	10.08	10.17	10.57	7	10.08	10.27	10.86	10.76	5	9.91	10.09	11.29	8.50
11	9.80	10.17	10.08	10.57	10	10.27	10.17	10.76	10.86	6	10.00	10.09	11.51	8.57
13	9.71	9.99	10.37	10.38	13	10.36	10.08	11.49	11.60	7	9.73	10.00	11.29	8.57
14	9.63	9.81	10.46	10.57	15	10.36	10.27	10.86	10.96	8	10.19	10.28	11.84	8.96
15	9.63	9.81	10.37	10.57	17	10.27	10.46	10.76	10.66	12	10.09	10.09	11.51	8.96
18	9.63	9.90	10.37	10.47	22	10.08	10.36	10.46	10.56	19	10.19	10.09	11.40	9.21
19	9.90	9.99	10.37	10.28										
mean value	9.80		10.32		mean value	9.11		10.98		mean value	9.43		9.55	
±1σ	0.56		0.29		±1σ	1.78		0.29		±1σ	0.81		1.57	
min.	7.97		9.13		min.	4.22		10.46		min.	7.25		6.00	
max.	10.46		10.57		max.	10.46		11.60		max.	10.28		11.84	

Oxygen content in anaerobic incubations of sediment samples								**section 3.3.2**
anoxic lake sediment incubations								
	c (O_2) [ppm] in bottle number							
time [days]	1	2	3	4	5	6	7	8
0	0.06	0.06	0.06	0.06	sensor defunct[1]	0.06	0.06	0.06
4	0.08	0.08	0.09	0.09		0.12	0.08	0.07
9	0.08	0.09	0.08	0.10		0.11	0.16	0.16
23	0.11	0.10	0.10	0.10		0.12	0.23	0.22
mean value	0.08		0.08		0.10		0.14	
standard deviation	0.01		0.01		0.02		0.07	
min.	0.06		0.06		0.06		0.06	
max.	0.11		0.10		0.12		0.23	
anoxic stream sediment incubations								
	c (O_2) [ppm] in bottle number							
time [days]	1	2	3	4	5	6	7	8
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
9	0.01	0.01	0.01	0.02	0.02	0.05	0.10	0.07
20	0.03	0.04	0.03	0.06	0.04	0.08	0.16	0.08
31	0.02	0.03	0.03	0.05	0.04	0.07	0.13	0.07
49	0.02	0.02	0.02	0.05	0.04	0.07	0.11	0.05
58	0.05	0.03	0.04	0.07	0.05	0.09	0.09	0.07
mean value	0.01		0.02		0.03		0.06	
standard deviation	0.01		0.02		0.03		0.05	
min.	0.00		0.00		0.00		0.00	
max.	0.05		0.07		0.09		0.16	
anoxic tidal flat sediment incubations								
	c (O_2) [ppm] in bottle number							
time [days]	1	2	3	4	5	6	7	8
0	0.00	sensor defunct[1]	-0.01	0.00	0.27	sensor defunct[1]	n.a.	
1	-0.01		-0.01	0.00	-0.05			
2	0.00		-0.01	-0.01	0.09			
3	0.00		-0.01	-0.01	0.07			
4	-0.01		-0.01	-0.02	0.30			
5	-0.01		0.00	-0.01	0.04			
6	-0.01		0.00	0.00	0.01			
7	0.00		0.00	0.00	-0.01			
8	0.01		0.00	0.01	-0.02			
9	0.00		0.00	0.00	-0.04			
12	0.01		0.00	0.00	-0.06			
19	-0.01		0.00	0.00	-0.08			
mean value	0.00		0.00		0.05			
standard deviation	0.01		0.01		0.12			
min.	-0.01		-0.02		-0.08			
max.	0.01		0.01		0.30			
1) three of the oxygen sensors were damaged by shaking with coarse sediment								

Anaerobic incubations of lake sediment samples					**section 3.3.2**	
bottle 1: biologically active, no addition of ^{18}O-labeled water (duplicate #1)			$\delta^{18}O$ of water:		-9.24±0.27‰	
time [days]	$c(NO_2^-)$ [mg/l]	$c(NO_3^-)$ [mg/l]	$\delta^{15}N$ [‰]	±1σ	$\delta^{18}O$ [‰]	±1σ
0	0.06	1847.01	1.50	0.25	23.39	1.05
1	0.31	1507.94	3.33	1.12	24.45	1.00
1.5	7.14	1379.62	conc. only[1]			
4	18.08	1165.33	6.85	0.80	27.89	0.08
7	4.76	1105.47	conc. only[1]			
9	2.78	1040.77	9.60		29.82	0.11
23	0.00	771.13	conc. only[1]			
bottle 2: biologically active, no addition of ^{18}O-labeled water (duplicate #2)			$\delta^{18}O$ of water:		-9.65±0.45‰	
time [days]	$c(NO_2^-)$ [mg/l]	$c(NO_3^-)$ [mg/l]	$\delta^{15}N$ [‰]	±1σ	$\delta^{18}O$ [‰]	±1σ
0	3.83	2023.12	0.96	0.03	23.62	0.21
1	0.17	1530.77	3.05	0.56	24.38	0.12
1.5	5.74	1416.56	conc. only[1]			
4	0.11	1225.71	6.15	0.07	27.57	0.73
7	1.16	1109.97	conc. only[1]			
9	0.45	1079.38	10.82		30.71	0.33
23	0.70	769.70	19.32	0.39	39.16	0.21
bottle 3: biologically active, addition of ^{18}O-labeled water (duplicate #1)			$\delta^{18}O$ of water:		1159.24±51.69‰	
time [days]	$c(NO_2^-)$ [mg/l]	$c(NO_3^-)$ [mg/l]	$\delta^{15}N$ [‰]	±1σ	$\delta^{18}O$ [‰]	±1σ
0	0.59	2012.23	1.09	0.06	23.26	0.36
1	0.07	1774.76	1.94	0.57	24.81	0.04
4	7.56	1208.15	8.10		34.11	0.27
9	0.18	1083.22	9.58	0.68	instr. failure[2]	
23	0.74	746.56	20.11	0.36	52.68	0.99
bottle 4: biologically active, addition of ^{18}O-labeled water (duplicate #2)			$\delta^{18}O$ of water:		1569.28±24.21‰	
time [days]	$c(NO_2^-)$ [mg/l]	$c(NO_3^-)$ [mg/l]	$\delta^{15}N$ [‰]	±1σ	$\delta^{18}O$ [‰]	±1σ
0	0.11	1703.61	2.50		23.54	0.12
1	0.37	1393.88	2.76	0.47	26.04	0.03
4	0.14	989.70	6.42	0.32	40.88	0.06
9	0.15	822.12	12.08	1.01	39.61	0.08
23	0.79	470.33	24.45	0.80	70.82	1.03

Anaerobic incubations of lake sediment samples					**section 3.3.2**	
bottle 1: biologically active, no addition of ^{18}O-labeled water (duplicate #1)			$\delta^{18}O$ of water:		-9.24±0.27‰	
time [days]	$c(NO_2^-)$ [mg/l]	$c(NO_3^-)$ [mg/l]	$\delta^{15}N$ [‰]	±1σ	$\delta^{18}O$ [‰]	±1σ
0	0.06	1847.01	1.50	0.25	23.39	1.05
1	0.31	1507.94	3.33	1.12	24.45	1.00
1.5	7.14	1379.62	conc. only[1]			
4	18.08	1165.33	6.85	0.80	27.89	0.08
7	4.76	1105.47	conc. only[1]			
9	2.78	1040.77	9.60		29.82	0.11
23	0.00	771.13	conc. only[1]			
bottle 2: biologically active, no addition of ^{18}O-labeled water (duplicate #2)			$\delta^{18}O$ of water:		-9.65±0.45‰	
time [days]	$c(NO_2^-)$ [mg/l]	$c(NO_3^-)$ [mg/l]	$\delta^{15}N$ [‰]	±1σ	$\delta^{18}O$ [‰]	±1σ
0	3.83	2023.12	0.96	0.03	23.62	0.21
1	0.17	1530.77	3.05	0.56	24.38	0.12
1.5	5.74	1416.56	conc. only[1]			
4	0.11	1225.71	6.15	0.07	27.57	0.73
7	1.16	1109.97	conc. only[1]			
9	0.45	1079.38	10.82		30.71	0.33
23	0.70	769.70	19.32	0.39	39.16	0.21
bottle 3: biologically active, addition of ^{18}O-labeled water (duplicate #1)			$\delta^{18}O$ of water:		1159.24±51.69‰	
time [days]	$c(NO_2^-)$ [mg/l]	$c(NO_3^-)$ [mg/l]	$\delta^{15}N$ [‰]	±1σ	$\delta^{18}O$ [‰]	±1σ
0	0.59	2012.23	1.09	0.06	23.26	0.36
1	0.07	1774.76	1.94	0.57	24.81	0.04

Anaerobic incubations of stream sediment samples					**section 3.3.2**	
bottle 1: biologically active, no addition of ^{18}O-labeled water (duplicate #1)			$\delta^{18}O$ of water:		-8.07±0.12‰	
time [days]	$c(NO_2^-)$ [mg/l]	$c(NO_3^-)$ [mg/l]	$\delta^{15}N$ [‰]	±1σ	$\delta^{18}O$ [‰]	±1σ
0	0.00	1889.77	5.69	0.64	23.78	0.03
1	1.76	1909.94	5.85	0.59	23.32	0.13
9	22.32	1652.53	9.92	0.07	27.23	0.20
20	0.00	1146.30	15.83	0.39	31.63	0.04
31	0.00	980.86	conc. only[1]			
49	0.00	1097.89	19.80	1.29	34.31	0.14
50	0.08	792.28	conc. only[1]			
52	1.85	868.25	conc. only[1]			
57	1.57	759.54	conc. only[1]			
58	8.69	937.29	20.51	5.55	37.12	0.30
bottle 2: biologically active, no addition of ^{18}O-labeled water (duplicate #2)			$\delta^{18}O$ of water:		-8.75±0.43‰	
time [days]	$c(NO_2^-)$ [mg/l]	$c(NO_3^-)$ [mg/l]	$\delta^{15}N$ [‰]	±1σ	$\delta^{18}O$ [‰]	±1σ
0	0.00	2015.21	5.10	2.29	23.41	0.04
1	4.44	2049.23	7.05	0.63	24.19	0.17
9	31.70	1660.73	9.71	0.08	26.50	0.08
20	0.00	1540.60	12.91	0.28	30.45	
31	0.00	1196.17	conc. only[1]			
49	2.09	1186.11	20.05	1.31	33.60	0.19
50	0.37	961.26	conc. only[1]			
52	2.27	977.82	conc. only[1]			
bottle 3: biologically active, addition of ^{18}O-labeled water (duplicate #1)			$\delta^{18}O$ of water:		500.37±5.14‰	
time [days]	$c(NO_2^-)$ [mg/l]	$c(NO_3^-)$ [mg/l]	$\delta^{15}N$ [‰]	±1σ	$\delta^{18}O$ [‰]	±1σ
0	0.00	1814.35	5.10	0.23	23.44	0.24
1	1.93	1943.08	5.39	0.03	24.04	0.10
9	44.67	1495.35	9.28	0.22	30.94	0.13
20	0.00	1364.39	11.89	0.37	37.47	0.40
49	0.42	1232.68	15.15	0.53	45.89	0.11
58	1.07	1272.27	17.67	0.56	53.65	0.16
bottle 4: biologically active, addition of ^{18}O-labeled water (duplicate #2)			$\delta^{18}O$ of water:		729.97±2.94‰	
time [days]	$c(NO_2^-)$ [mg/l]	$c(NO_3^-)$ [mg/l]	$\delta^{15}N$ [‰]	±1σ	$\delta^{18}O$ [‰]	±1σ
0	0.00	1776.73	6.57	0.17	23.69	0.01
1	3.63	2003.71	5.62	0.04	24.22	0.28
9	8.01	1320.68	10.25	0.07	34.84	0.07
20	0.00	1474.00	13.04	0.06	40.69	0.21
49	0.00	1250.21	17.04	0.31	70.87	0.08
58	0.37	994.67	19.64	0.92	82.95	0.51

bottle 5: sterile, no addition of ^{18}O-labeled water (duplicate #1)			$\delta^{18}O$ of water:			-6.34‰
time [days]	$c(NO_2^-)$ [mg/l]	$c(NO_3^-)$ [mg/l]	$\delta^{15}N$ [‰]	±1σ	$\delta^{18}O$ [‰]	±1σ
0	0.00	2281.21	6.63	1.09	23.72	0.41
1	0.00	2611.84	5.22	0.05	23.44	0.11
9	0.00	2519.68	5.55	0.28	23.15	0.08
20	0.00	2004.94	5.39	0.15	23.35	0.06
49	0.00	1905.30	5.46	0.33	23.25	0.10
58	0.00	1985.31	5.16	0.10	23.20	0.23

bottle 6: sterile, no addition of ^{18}O-labeled water (duplicate #2)			$\delta^{18}O$ of water:			-5.28‰
time [days]	$c(NO_2^-)$ [mg/l]	$c(NO_3^-)$ [mg/l]	$\delta^{15}N$ [‰]	±1σ	$\delta^{18}O$ [‰]	±1σ
0	0.00	2683.60	6.06	0.16	24.11	0.33
1	0.00	2158.97	5.34	0.46	23.59	0.33
9	0.00	2287.60	5.94	0.39	23.68	0.09
20	0.00	2227.41	5.73	0.30	23.84	0.30
49	0.00	1801.24	5.10	0.11	23.67	0.17
58	0.00	2470.93	6.09	0.91	23.07	0.01

bottle 7: sterile, addition of ^{18}O-labeled water and ^{18}O-labeled nitrite at day 1 (duplicate #1)			$\delta^{18}O$ of water:		1014.95±10.47‰	
time [days]	$c(NO_2^-)$ [mg/l]	$c(NO_3^-)$ [mg/l]	$\delta^{15}N$ [‰]	±1σ	$\delta^{18}O$ [‰]	±1σ
0	0.00	2273.30	5.13	0.01	23.01	0.15
1	129.81	1905.55	5.66	0.49	26.21	0.17
9	48.18	1784.95	5.35	0.06	25.34	0.03
20	17.96	1803.07	4.45	1.78	25.32	0.02
49	0.87	1800.47	5.36	0.03	25.80	0.26
58	0.00	1724.02	6.63	0.03	25.42	0.06

bottle 8: sterile, addition of ^{18}O-labeled water and ^{18}O-labeled nitrite at day 1 (duplicate #2)			$\delta^{18}O$ of water:		790.24±10.58‰	
time [days]	$c(NO_2^-)$ [mg/l]	$c(NO_3^-)$ [mg/l]	$\delta^{15}N$ [‰]	±1σ	$\delta^{18}O$ [‰]	±1σ
0	0.00	2076.76	6.50	0.90	23.86	0.11
1	66.66	1638.62	5.41	0.64	25.38	0.25
9	24.43	2294.07	5.68	0.19	24.64	0.35
20	3.09	2128.78	5.53	0.13	25.15	0.11
49	0.00	2088.39	5.71	0.80	25.18	0.02
58	0.00	1925.80	6.40		24.57	0.05

[1)] for some timepoints, only the anion concentrations have been sampled, either to conserve sampling material for later timepoints or because not enough sediment-free sampling material was extractable

Anaerobic incubations of tidal flat sediment samples					**section 3.3.2**	
bottle 1: biologically active, no addition of ^{18}O-labeled water (duplicate #1)			$\delta^{18}O$ of water:		3.73±0.1‰	
time [days]	$c(NO_2^-)$ [mg/l]	$c(NO_3^-)$ [mg/l]	$\delta^{15}N$ [‰]	±1σ	$\delta^{18}O$ [‰]	±1σ
0	0.34	1273.94	0.49	0.22	26.05	0.33
0.5	1.19	1287.40	1.08	0.02	25.42	0.23
1	2.92	1281.27	2.35	0.10	27.28	0.11
2	12.59	1221.41	2.96	0.03	28.29	0.10
3	23.75	1150.20	5.34	0.11	28.31	0.06
4	46.99	854.71	7.37	0.02	30.45	0.01
6	67.63	920.49	9.75	0.25	30.50	0.53
7	99.18	880.94	conc. only[1]			
8	111.11	840.39	conc. only[1]			
9	50.73	707.10	conc. only[1]			
10	32.33	672.34	conc. only[1]			
13	1.24	580.35	conc. only[1]			
bottle 2: biologically active, no addition of ^{18}O-labeled water (duplicate #2)			$\delta^{18}O$ of water:		-3.09±0.09‰	
time [days]	$c(NO_2^-)$ [mg/l]	$c(NO_3^-)$ [mg/l]	$\delta^{15}N$ [‰]	±1σ	$\delta^{18}O$ [‰]	±1σ
0	0.42	1351.80	-0.20	0.29	27.12	0.21
0.5	0.97	1348.72	0.95	0.01	26.47	0.80
1	2.20	1360.53	1.49	0.02	27.31	0.07
2	9.91	1319.83	conc. only[1]			
3	35.71	1227.16	5.77	1.15	30.07	0.21
4	71.66	922.86	conc. only[1]			
6	126.87	1005.62	8.00	0.59	29.76	0.18
7	126.17	935.14	conc. only[1]			
8	140.73	913.21	11.47	0.57	29.09	0.35
9	128.25	776.53	conc. only[1]			
10	120.97	752.89	17.28	1.23	32.94	0.14
13	30.12	665.16	conc. only[1]			

bottle 3: biologically active, addition of ^{18}O-labeled water (duplicate #1)			$\delta^{18}O$ of water:		1114.88±1.19‰	
time [days]	$c(NO_2^-)$ [mg/l]	$c(NO_3^-)$ [mg/l]	$\delta^{15}N$ [‰]	±1σ	$\delta^{18}O$ [‰]	±1σ
0	0.00	1181.72	-0.39	0.35	27.50	0.03
0.5	1.11	1144.45	1.57	0.05	39.36	0.54
1	2.79	1141.11	1.90	0.12	40.40	0.21
2	11.38	1091.17	2.82	0.07	35.55	0.03
3	31.45	997.91	7.20	0.06	44.13	0.33
4	63.44	712.98	9.45	0.30	56.10	0.24
6	78.65	745.37	12.68	0.28	74.95	0.17
7	143.68	680.33	conc. only[1]			
8	148.73	610.29	17.06	0.32	89.16	0.01
9	132.26	515.56	conc. only[1]			
10	120.15	505.86	26.02	0.45	103.49	0.18
13	40.38	357.47	conc. only[1]			

bottle 4: biologically active, addition of ^{18}O-labeled water (duplicate #2)			$\delta^{18}O$ of water:		1355.67±2.51‰	
time [days]	$c(NO_2^-)$ [mg/l]	$c(NO_3^-)$ [mg/l]	$\delta^{15}N$ [‰]	±1σ	$\delta^{18}O$ [‰]	±1σ
0	0.46	1278.51	0.17	0.45	29.22	0.13
0.5	1.26	1313.22	1.18	0.45	38.33	0.43
1	2.82	1307.91	1.40	0.07	44.92	0.11
2	10.87	1259.73	3.31	0.29	38.48	0.58
3	40.92	1146.92	5.88		48.80	0.09
4	67.84	832.63	8.66	0.16	58.04	0.13
6	46.43	934.00	10.63	0.05	67.14	0.28
7	114.90	891.17	conc. only[1]			
8	125.31	869.41	12.69	0.02	88.13	0.13
9	84.01	730.85	conc. only[1]			
10	57.62	726.22	15.73	0.17	97.29	2.43
13	1.67	613.89	conc. only[1]			

bottle 5: sterile, addition of ^{18}O-labeled water (duplicate #1)			$\delta^{18}O$ of water:		1334.59±2.88‰	
time [days]	$c(NO_2^-)$ [mg/l]	$c(NO_3^-)$ [mg/l]	$\delta^{15}N$ [‰]	±1σ	$\delta^{18}O$ [‰]	±1σ
0	0.59	1332.42	-0.09	0.42	36.13	0.28
0.5	0.43	1278.46	0.48	0.37	39.66	0.11
1.5	0.62	1334.15	conc. only[1]			
2	0.73	1330.93	0.63	0.10	27.97	0.06
4	0.00	1015.44	0.27	0.04	27.97	0.21
6	0.00	1338.17	0.66	0.47	28.38	0.15
7	0.00	1330.69	conc. only[1]			
8	0.38	1345.51	-0.53	0.04	25.73	0.14
9	0.68	1018.87	conc. only[1]			
10	0.00	1288.98	3.09	0.94	43.31	0.08
13	0.00	1303.85	conc. only[1]			

bottle 6: sterile, addition of ^{18}O-labeled water (duplicate #2)			$\delta^{18}O$ of water:		1289.57±1.32‰	
time [days]	$c(NO_2^-)$ [mg/l]	$c(NO_3^-)$ [mg/l]	$\delta^{15}N$ [‰]	±1σ	$\delta^{18}O$ [‰]	±1σ
0	0.31	1315.59	0.23	0.31	28.50	0.30
0.5	0.44	1247.32	-0.60	0.13	27.30	0.09
2	0.49	1223.98	0.65	0.08	32.31	0.35
3	0.58	1203.01	1.13	0.05	26.24	0.06
4	0.00	966.51	0.15	0.44	29.23	0.07
6	0.00	1199.22	-0.36	0.09	35.69	0.29
7	0.40	1307.55	conc. only[1]			
8	0.83	1272.22	conc. only[1]			
9	0.00	1203.10	conc. only[1]			
10	0.39	1217.68	0.32	0.06	26.77	0.17
13	0.00	1206.87	conc. only[1]			

[1)] for some timepoints, only the anion concentrations have been sampled, either to conserve sampling material for later timepoints or because not enough sediment-free sampling material was extractable

Average values of incubations of lake sediment samples														section 3.3.2
including the data used for calculating enrichment factors in Fig. 3.3														
average of bottle 1+2: biologically active, no addition of ^{18}O-labeled water										$\delta^{18}O$ of water:		-9.45±0.20‰ (min/max)		
time [days]	$c(NO_2^-)$ [mg/l]	±min/ max	$c(NO_3^-)$ [mg/l]	±min/ max	$\delta^{15}N$ [‰]	±min/ max	$\delta^{18}O$ [‰]	±min/ max	$\ln(C_t/C_0)$ of NO_3^-	±min/max	$\ln(R_t/R_0)$ for ^{15}N	±min/max	$\ln(R_t/R_0)$ for ^{18}O	±min/max
0	1.94	1.88	1935.07	88.05	1.23	0.27	23.50	0.12	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000
1	0.24	0.07	1519.35	11.42	3.19	0.14	24.41	0.04	-0.241865	0.037014	0.001955	0.000134	0.000889	0.000078
1.5	6.44	0.70	1398.09	18.47	[1]conc. only									
4	9.09	8.99	1195.52	30.19	6.50	0.35	27.73	0.16	-0.481579	0.019561	0.005250	0.000077	0.004119	0.000043
7	2.96	1.80	1107.72	2.25	[1]conc. only									
9	1.62	1.17	1060.07	19.31	10.21	0.61	30.27	0.45	-0.601839	0.026453	0.008929	0.000335	0.006588	0.000319
23	0.35	0.35	770.41	0.71	19.32		39.16		-0.921036	0.043576	0.017902		0.015180	
average of bottle 3+4: biologically active, addition of ^{18}O-labeled water										$\delta^{18}O$ of water:		1364.26±205.02‰ (min/max)		
time [days]	$c(NO_2^-)$ [mg/l]	±min/ max	$c(NO_3^-)$ [mg/l]	±min/ max	$\delta^{15}N$ [‰]	±min/ max	$\delta^{18}O$ [‰]	±min/ max	$\ln(C_t/C_0)$ of NO_3^-	±min/max	$\ln(R_t/R_0)$ for ^{15}N	±min/max	$\ln(R_t/R_0)$ for ^{18}O	±min/max
0	0.35	0.24	1857.92	154.31	1.80	0.70	23.40	0.14	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000
1	0.22	0.15	1584.32	190.44	2.35	0.41	25.43	0.61	-0.159306	0.033724	0.000551	0.000290	0.001982	0.000463
4	3.85	3.71	1098.92	109.23	7.26	0.84	37.50	3.39	-0.525158	0.014972	0.005437	0.000133	0.013684	0.003121
9	0.16	0.01	952.67	130.55	10.83	1.25	39.61		-0.667979	0.048641	0.008978	0.000532	0.015720	
23	0.76	0.03	608.44	138.12	22.28	2.17	61.75	9.07	-1.116391	0.124785	0.020240	0.001423	0.036795	0.008367

average of bottle 5+6: sterile, addition of ^{18}O-labeled water										$\delta^{18}O$ of water:	1473.36±130.95‰ (min/max)			
time [days]	$c(NO_2^-)$ [mg/l]	±min/ max	$c(NO_3^-)$ [mg/l]	±min/ max	$\delta^{15}N$ [‰]	±min/ max	$\delta^{18}O$ [‰]	±min/ max	$\ln(C_t/C_0)$ of NO_3^-	±min/max	$\ln(R_t/R_0)$ for ^{15}N	±min/max	$\ln(R_t/R_0)$ for ^{18}O	±min/max
0	0.61	0.76	1882.11	644.86	1.99	1.22	23.14	0.31	n.a.					
9	0.62	0.78	1916.36	455.29	0.98	0.68	23.28	0.07						
23	0.80	0.62	1471.89	64.72	2.09	1.08	23.14	0.32						
average of bottle 7+8: sterile, no addition of ^{18}O-labeled water, addition of ^{18}O-labeled NO_2^-										$\delta^{18}O$ of water:	3.73±1.23‰ (min/max)			
time [days]	$c(NO_2^-)$ [mg/l]	±min/ max	$c(NO_3^-)$ [mg/l]	±min/ max	$\delta^{15}N$ [‰]	±min/ max	$\delta^{18}O$ [‰]	±min/ max	$\ln(C_t/C_0)$ of NO_3^-	±min/max	$\ln(R_t/R_0)$ for ^{15}N	±min/max	$\ln(R_t/R_0)$ for ^{18}O	±min/max
0	65.73	6.08	1806.98	393.94	1.19	0.12	24.52	0.55	n.a.					
9	36.85	1.48	1789.03	251.18	1.16	0.65	24.74	0.56						
23	21.96	1.71	1614.08	121.15	1.95	1.19	24.13							

[1)] for some timepoints, only the anion concentrations have been sampled, either to conserve sampling material for later timepoints or because not enough sediment-free sampling material was extractable

Average values of incubations of stream sediment samples														**section 3.3.2**
including the data used for calculating enrichment factors in Fig. 3.3														
average of bottle 1+2: biologically active, no addition of ^{18}O-labeled water										$\delta^{18}O$ of water:		-8.41±0.34‰ (min/max)		
time [days]	$c(NO_2^-)$ [mg/l]	±min/ max	$c(NO_3^-)$ [mg/l]	±min/ max	$\delta^{15}N$ [‰]	±min/ max	$\delta^{18}O$ [‰]	±min/ max	$\ln(C_t/C_0)$ of NO_3^-	±min/max	$\ln(R_t/R_0)$ for ^{15}N	±min/max	$\ln(R_t/R_0)$ for ^{18}O	±min/max
0	0.00	0.00	1952.49	62.72	5.40	0.30	23.59	0.19	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000
1	3.10	1.34	1979.59	69.65	6.45	0.60	23.75	0.44	0.013783	0.002960	0.001046	0.000305	0.000156	0.000244
9	27.01	4.69	1656.63	4.10	9.82	0.10	26.86	0.37	-0.164327	0.029146	0.004386	0.000194	0.003190	0.000175
20	0.00	0.00	1343.45	197.15	14.37	1.46	31.04	0.59	-0.373882	0.105315	0.008883	0.001141	0.007250	0.000389
31	0.00	0.00	1088.52	107.66	[1]conc. only									
49	1.05	1.05	1142.00	44.11	19.93	0.12	33.95	0.36	-0.536348	0.006281	0.014347	0.000173	0.010070	0.000163
50	0.22	0.14	876.77	84.49	[1]conc. only									
52	2.06	0.21	923.04	54.79	[1]conc. only									
57	1.57		759.54		[1]conc. only									
58	8.69		937.29		20.51		37.12		-0.733894	0.031617	0.014920	0.000295	0.013129	0.000183
average of bottle 3+4: biologically active, addition of ^{18}O-labeled water										$\delta^{18}O$ of water:		615.17±114.80‰ (min/max)		
time [days]	$c(NO_2^-)$ [mg/l]	±min/ max	$c(NO_3^-)$ [mg/l]	±min/ max	$\delta^{15}N$ [‰]	±min/ max	$\delta^{18}O$ [‰]	±min/ max	$\ln(C_t/C_0)$ of NO_3^-	±min/max	$\ln(R_t/R_0)$ for ^{15}N	±min/max	$\ln(R_t/R_0)$ for ^{18}O	±min/max
0	0.00	0.00	1795.54	18.81	5.83	0.73	23.57	0.13	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000
1	2.78	0.85	1973.39	30.31	5.50	0.12	24.13	0.09	0.094449	0.004821	-0.000328	0.000613	0.000552	0.000034
9	26.34	18.33	1408.02	87.33	9.76	0.49	32.89	1.95	-0.243142	0.049757	0.003900	0.000246	0.009069	0.001764
20	0.00	0.00	1419.19	54.81	12.47	0.58	39.08	1.61	-0.235247	0.027469	0.006571	0.000159	0.015039	0.001426
49	0.21	0.21	1241.44	8.77	16.10	0.95	58.38	12.49	-0.369100	0.003385	0.010149	0.000205	0.033448	0.011610
58	0.72	0.35	1133.47	138.80	18.66	0.99	68.30	14.65	-0.460112	0.105098	0.012666	0.000239	0.042775	0.013500

average of bottle 5+6: sterile, no addition of ^{18}O-labeled water										δ^{18}O of water:		-5.81±0.53‰ (min/max)		
time [days]	c(NO_2^-) [mg/l]	±min/ max	c(NO_3^-) [mg/l]	±min/ max	δ^{15}N [‰]	±min/ max	δ^{18}O [‰]	±min/ max	ln(C_t/C_0) of NO_3^-	±min/max	ln(R_t/R_0) for ^{15}N	±min/max	ln(R_t/R_0) for ^{18}O	±min/max
0	0.00	0.00	2482.40	201.19	6.35	0.29	23.92	0.20	n.a.					
1	0.00	0.00	2385.40	226.43	5.28	0.06	23.51	0.08						
9	0.00	0.00	2403.64	116.04	5.74	0.20	23.41	0.26						
20	0.00	0.00	2116.17	111.23	5.56	0.17	23.59	0.25						
49	0.00	0.00	1853.27	52.03	5.28	0.18	23.46	0.21						
58	0.00	0.00	2228.12	242.81	5.62	0.46	23.13	0.06						

average of bottle 7+8: sterile, addition of ^{18}O-labeled water and ^{18}O-labeled nitrite										δ^{18}O of water:		902.59±112.36‰ (min/max)		
time [days]	c(NO_2^-) [mg/l]	±min/ max	c(NO_3^-) [mg/l]	±min/ max	δ^{15}N [‰]	±min/ max	δ^{18}O [‰]	±min/ max	ln(C_t/C_0)	±min/max	ln(R_t/R_0) for ^{15}N	±min/max	ln(R_t/R_0) for ^{18}O	±min/max
0	0.00	0.00	2175.03	98.27	5.82	0.69	23.43	0.43	n.a.					
1	98.23	31.57	1772.08	133.47	5.54	0.13	25.80	0.42						
9	36.31	11.88	2039.51	254.56	5.52	0.17	24.99	0.35						
20	10.52	7.44	1965.92	162.86	4.99	0.54	25.23	0.08						
49	0.43	0.43	1944.43	143.96	5.53	0.18	25.49	0.31						
58	0.00	0.00	1824.91	100.89	6.51	0.11	24.99	0.42						

[1)] for some timepoints, only the anion concentrations have been sampled, either to conserve sampling material for later timepoints or because not enough sediment-free sampling material was extractable

Average values of incubations of tidal flat sediment samples														section 3.3.2
including the data used for calculating enrichment factors in Fig. 3.3														
average of bottle 1+2: biologically active, no addition of ^{18}O-labeled water										$\delta^{18}O$ of water:		0.32±3.42‰ (min/max)		
time [days]	$c(NO_2^-)$ [mg/l]	±min/ max	$c(NO_3^-)$ [mg/l]	±min/ max	$\delta^{15}N$ [‰]	±min/ max	$\delta^{18}O$ [‰]	±min/ max	$\ln(C_t/C_0)$ of NO_3^-	±min/max	$\ln(R_t/R_0)$ for ^{15}N	±min/max	$\ln(R_t/R_0)$ for ^{18}O	±min/max
0	0.38	0.04	1312.87	38.93	0.14	0.35	26.59	0.53	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000
0.5	1.08	0.11	1318.06	30.66	1.01	0.07	25.94	0.53	0.003947	0.006226	0.000869	0.000280	-0.000628	0.000009
1	2.56	0.36	1320.90	39.63	1.92	0.43	27.29	0.02	0.006096	0.000340	0.001770	0.000084	0.000689	0.000504
2	11.25	1.34	1270.62	49.21	2.96		28.29		-0.032714	0.008777	0.002812		0.001659	
3	29.73	5.98	1188.68	38.48	5.56	0.21	29.19	0.88	-0.099379	0.002636	0.005398	0.000132	0.002532	0.000336
4	59.33	12.34	888.79	34.08	7.37		30.45		-0.390123	0.008401	0.007197		0.003753	
6	97.25	29.62	963.06	42.57	8.88	0.88	30.13	0.37	-0.309867	0.014028	0.008694	0.000523	0.003442	0.000162
7	112.68	13.50	908.04	27.10	[1]conc. only									
8	125.92	14.81	876.80	36.41	11.47		29.09		-0.403697	0.011465	0.011257		0.002432	
9	89.49	38.76	741.82	34.72	[1]conc. only									
10	76.65	44.32	712.62	40.28	[1]conc. only									
13	15.68	14.44	622.76	42.41	[1]conc. only									
average of bottle 3+4: biologically active, addition of ^{18}O-labeled water										$\delta^{18}O$ of water:		1235.28±120.40‰ (min/max)		
time [days]	$c(NO_2^-)$ [mg/l]	±min/ max	$c(NO_3^-)$ [mg/l]	±min/ max	$\delta^{15}N$ [‰]	±min/ max	$\delta^{18}O$ [‰]	±min/ max	$\ln(C_t/C_0)$ of NO_3^-	±min/max	$\ln(R_t/R_0)$ for ^{15}N	±min/max	$\ln(R_t/R_0)$ for ^{18}O	±min/max
0	0.23	0.23	1230.12	48.40	-0.11	0.28	28.36	0.86	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000
0.5	1.19	0.08	1228.84	84.39	1.37	0.20	38.84	0.52	-0.001062	0.022693	0.001483	0.000084	0.010144	0.000335
1	2.81	0.01	1224.51	83.40	1.65	0.25	42.66	2.26	-0.004595	0.020305	0.001756	0.000032	0.013810	0.001334
2	11.13	0.26	1175.45	84.28	3.07	0.24	37.02	1.47	-0.045474	0.026417	0.003174	0.000038	0.008384	0.000578
3	36.18	4.74	1072.42	74.51	6.54	0.66	46.46	2.34	-0.137231	0.019730	0.006629	0.000375	0.017450	0.001395
4	65.64	2.20	772.81	59.83	9.06	0.40	57.07	0.97	-0.464893	0.021976	0.009127	0.000111	0.027538	0.000084
6	62.54	16.11	839.68	94.32	11.65	1.02	71.04	3.91	-0.381924	0.047124	0.011695	0.000729	0.040670	0.002808
7	129.29	14.39	785.75	105.42	[1]conc. only									
8	137.02	11.71	739.85	129.56	14.87	2.19	88.64	0.51	-0.508535	0.093612	0.014875	0.001873	0.056969	0.000363
9	108.14	24.13	623.21	107.65	[1]conc. only									
10	88.89	31.27	616.04	110.18	20.88	5.14	100.39	3.10	-0.691694	0.091201	0.020770	0.004744	0.067700	0.001982
13	21.03	19.36	485.68	128.21	[1]conc. only									

average of bottle 5+6: sterile, addition of ^{18}O-labeled water											$\delta^{18}O$ of water:	1312.09±22.51‰ (min/max)		
time [days]	$c(NO_2^-)$ [mg/l]	±min/ max	$c(NO_3^-)$ [mg/l]	±min/ max	$\delta^{15}N$ [‰]	±min/ max	$\delta^{18}O$ [‰]	±min/ max	$\ln(C_t/C_0)$ of NO_3^-	±min/max	$\ln(R_t/R_0)$ for ^{15}N	±min/max	$\ln(R_t/R_0)$ for ^{18}O	±min/max
0	0.45	0.14	1324.01	8.42	0.07	0.16	32.31	3.82	n.a.					
0.5	0.44	0.01	1262.89	15.57	-0.06	0.54	33.48	6.18						
1.5	0.56	0.07	1279.07	55.09	0.65		32.31							
2	0.66	0.08	1266.97	63.96	0.88	0.25	27.11	0.86						
4	0.00	0.00	990.98	24.47	0.21	0.06	28.60	0.63						
6	0.00	0.00	1268.69	69.47	0.15	0.51	32.03	3.66						
7	0.20	0.20	1319.12	11.57	[1]conc. only									
8	0.61	0.23	1308.87	36.65	-0.53		25.73							
9	0.34	0.34	1110.99	92.12	[1]conc. only									
10	0.20	0.20	1253.33	35.65	1.70	1.39	35.04	8.27						
13	0.00	0.00	1255.36	48.49	[1]conc. only									

[1)] for some timepoints, only the anion concentrations have been sampled, either to conserve sampling material for later timepoints or because not enough sediment-free sampling material was extractable

Aerobic incubations of lake sediment samples													section 3.3.3
	bottle 9: biol. active		bottle 10: biol. active		average of bottles 9+10								
time [days]	$c(NO_2^-)$ [mg/l]	$c(NO_3^-)$ [mg/l]	$c(NO_2^-)$ [mg/l]	$c(NO_3^-)$ [mg/l]	$c(NO_2^-)$ [mg/l]	±min/ max	$c(NO_3^-)$ [mg/l]	±min/ max	$c(NO_2^-)$ [mM]	±min/ max	$c(NO_3^-)$ [mM]	±min/ max	
0	748.66	2.21	695.32	1.84	721.99	26.67	2.03	0.18	15.70	0.58	0.03	0.00	
1	485.41	1.27	537.98	2.18	511.70	26.29	1.72	0.45	11.12	0.57	0.03	0.01	
2	536.76	4.72	492.47	6.07	514.62	22.14	5.40	0.67	11.19	0.48	0.09	0.01	
4	516.62	20.79	458.44	21.11	487.53	29.09	20.95	0.16	10.60	0.63	0.34	0.00	
5	499.26	23.92	426.39	22.81	462.82	36.43	23.36	0.56	10.06	0.79	0.38	0.01	
7	498.30	29.78	437.21	28.89	467.76	30.54	29.33	0.45	10.17	0.66	0.47	0.01	
11	475.11	49.33	415.79	47.63	445.45	29.66	48.48	0.85	9.68	0.64	0.78	0.01	
13	413.24	89.92	359.07	88.65	386.16	27.08	89.28	0.64	8.39	0.59	1.44	0.01	
14	367.87	136.49	307.34	142.08	337.61	30.26	139.28	2.80	7.34	0.66	2.25	0.05	
15	300.28	198.66	246.28	202.15	273.28	27.00	200.40	1.75	5.94	0.59	3.23	0.03	
16	138.29	373.74	115.68	346.46	126.99	11.30	360.10	13.64	2.76	0.25	5.81	0.22	
18	0.36	567.80	1.42	504.53	0.89	0.53	536.16	31.63	0.02	0.01	8.65	0.51	
19	0.58	565.90	4.62	484.56	2.60	2.02	525.23	40.67	0.06	0.04	8.47	0.66	
	bottle 11: sterile		bottle 12: sterile		average of bottles 11+12								
time [days]	$c(NO_2^-)$ [mg/l]	$c(NO_3^-)$ [mg/l]	$c(NO_2^-)$ [mg/l]	$c(NO_3^-)$ [mg/l]	$c(NO_2^-)$ [mg/l]	±min/ max	$c(NO_3^-)$ [mg/l]	±min/ max	$c(NO_2^-)$ [mM]	±min/ max	$c(NO_3^-)$ [mM]	±min/ max	
0	789.50	0.24	823.84	0.25	806.67	17.17	0.41	0.00	17.54	0.37	0.01	0.00	
1	679.06	0.20	653.24	0.46	666.15	12.91	0.55	0.22	14.48	0.28	0.01	0.00	
2	588.53	0.31	554.67	0.68	571.60	16.93	0.81	0.30	12.43	0.37	0.01	0.00	
4	563.55	0.74	485.74	2.37	524.65	38.91	2.56	1.34	11.41	0.85	0.04	0.02	
5	559.93	1.35	486.10	2.18	523.02	36.91	2.91	0.68	11.37	0.80	0.05	0.01	
7	560.83	1.56	479.73	2.83	520.28	40.55	3.62	1.05	11.31	0.88	0.06	0.02	
11	555.88	1.96	479.92	3.72	517.90	37.98	4.69	1.45	11.26	0.83	0.08	0.02	
13	550.06	2.33	468.95	4.02	509.51	40.56	5.24	1.39	11.08	0.88	0.08	0.02	
14	555.01	2.76	469.51	4.25	512.26	42.75	5.79	1.23	11.14	0.93	0.09	0.02	
15	557.50	3.00	473.96	4.71	515.73	41.77	6.36	1.41	11.21	0.91	0.10	0.02	
16	556.91	2.73	469.50	5.07	513.20	43.70	6.43	1.93	11.16	0.95	0.10	0.03	
18	558.63	2.82	472.36	5.72	515.49	43.14	7.05	2.39	11.21	0.94	0.11	0.04	
19	560.42	3.00	479.95	5.92	520.19	40.24	7.36	2.41	11.31	0.87	0.12	0.04	

Aerobic incubations of stream sediment samples													section 3.3.3
	bottle 9: biol. active		bottle 10: biol. active		average of bottles 9+10								
time [days]	$c(NO_2^-)$ [mg/l]	$c(NO_3^-)$ [mg/l]	$c(NO_2^-)$ [mg/l]	$c(NO_3^-)$ [mg/l]	$c(NO_2^-)$ [mg/l]	±min/ max	$c(NO_3^-)$ [mg/l]	±min/ max	$c(NO_2^-)$ [mM]	±min/ max	$c(NO_3^-)$ [mM]	±min/ max	
0	349.95	20.16	400.31	10.88	375.13	25.18	15.52	4.64	8.15	0.55	0.25	0.07	
0.5	339.95	11.49	390.76	11.58	365.35	25.41	11.54	0.04	7.94	0.55	0.19	0.00	
1	296.59	14.81	340.07	14.87	318.33	21.74	14.84	0.03	6.92	0.47	0.24	0.00	
2	276.60	17.86	324.95	19.10	300.78	24.17	18.48	0.62	6.54	0.53	0.30	0.01	
7	153.06	62.59	238.96	61.55	196.01	42.95	62.07	0.52	4.26	0.93	1.00	0.01	
8	107.76	117.10	194.65	102.78	151.20	43.45	109.94	7.16	3.29	0.94	1.77	0.12	
9	35.95	203.85	128.50	184.28	82.23	46.28	194.06	9.78	1.79	1.01	3.13	0.16	
10	0.00	243.75	24.62	324.92	12.31	12.31	284.34	40.59	0.27	0.27	4.59	0.65	
14	0.00	272.15	0.00	374.40	0.00	0.00	323.28	51.12	0.00	0.00	5.21	0.82	
15	0.00	270.28	0.00	376.90	0.00	0.00	323.59	53.31	0.00	0.00	5.22	0.86	
22	0.00	257.61	0.00	380.88	0.00	0.00	319.25	61.63	0.00	0.00	5.15	0.99	
	bottle 11: sterile		bottle 12: sterile		average of bottles 11+12								
time [days]	$c(NO_2^-)$ [mg/l]	$c(NO_3^-)$ [mg/l]	$c(NO_2^-)$ [mg/l]	$c(NO_3^-)$ [mg/l]	$c(NO_2^-)$ [mg/l]	±min/ max	$c(NO_3^-)$ [mg/l]	±min/ max	$c(NO_2^-)$ [mM]	±min/ max	$c(NO_3^-)$ [mM]	±min/ max	
0	273.80	15.23	182.17	15.17	376.18	75.60	25.07	0.05	8.18	1.64	0.40	0.00	
0.5	263.06	15.10	176.91	14.91	362.97	71.08	24.76	0.16	7.89	1.55	0.40	0.00	
1	264.92	15.15	170.92	14.68	359.56	77.55	24.61	0.38	7.82	1.69	0.40	0.01	
2	247.24	15.04	164.17	14.43	339.41	68.54	24.32	0.50	7.38	1.49	0.39	0.01	
7	248.75	15.25	162.98	15.04	339.67	70.76	24.99	0.17	7.38	1.54	0.40	0.00	
8	247.52	15.42	164.29	14.84	339.75	68.66	24.97	0.48	7.39	1.49	0.40	0.01	
9	251.01	15.62	164.73	15.07	342.99	71.18	25.32	0.45	7.46	1.55	0.41	0.01	
10	252.21	15.88	164.73	15.22	343.97	72.16	25.66	0.55	7.48	1.57	0.41	0.01	
14	250.35	18.06	162.77	15.97	340.82	72.25	28.07	1.72	7.41	1.57	0.45	0.03	
15	237.28	5.21	157.35	4.53	325.57	65.94	8.04	0.56	7.08	1.43	0.13	0.01	
22	239.75	5.51	154.00	4.63	324.84	70.74	8.36	0.73	7.06	1.54	0.13	0.01	

Aerobic incubations of tidal flat sediment samples													section 3.3.3
	bottle 9: biol. active		bottle 10: biol. active		average of bottles 9+10								
time [days]	$c(NO_2^-)$ [mg/l]	$c(NO_3^-)$ [mg/l]	$c(NO_2^-)$ [mg/l]	$c(NO_3^-)$ [mg/l]	$c(NO_2^-)$ [mg/l]	±min/ max	$c(NO_3^-)$ [mg/l]	±min/ max	$c(NO_2^-)$ [mM]	±min/ max	$c(NO_3^-)$ [mM]	±min/ max	
0	243.86	2.13	389.78	2.12	316.82	72.96	2.13	0.01	6.89	1.59	0.03	0.00	
0.5	218.66	2.95	340.43	2.63	279.54	60.88	2.79	0.16	6.08	1.32	0.05	0.00	
1	197.69	11.93	324.70	6.22	261.20	63.50	9.08	2.86	5.68	1.38	0.15	0.05	
1.5	195.33	13.98	325.44	6.23	260.38	65.05	10.10	3.88	5.66	1.41	0.16	0.06	
2	145.44	16.04	272.03	6.28	208.74	63.30	11.16	4.88	4.54	1.38	0.18	0.08	
5	129.01	23.76	280.51	10.42	204.76	75.75	17.09	6.67	4.45	1.65	0.28	0.11	
6	107.27	25.10	272.25	10.95	189.76	82.49	18.02	7.07	4.13	1.79	0.29	0.11	
8	77.37	28.74	234.34	11.46	155.85	78.49	20.10	8.64	3.39	1.71	0.32	0.14	
11	31.61	52.70	217.14	14.08	124.37	92.76	33.39	19.31	2.70	2.02	0.54	0.31	
18	1.11	73.76	1.02	243.71	1.06	0.04	158.74	84.98	0.02	0.00	2.56	1.37	
21	0.00	80.48	0.40	234.81	0.20	0.20	157.64	77.16	0.00	0.00	2.54	1.24	
	bottle 11: sterile		bottle 12: sterile		average of bottles 11+12								
time [days]	$c(NO_2^-)$ [mg/l]	$c(NO_3^-)$ [mg/l]	$c(NO_2^-)$ [mg/l]	$c(NO_3^-)$ [mg/l]	$c(NO_2^-)$ [mg/l]	±min/ max	$c(NO_3^-)$ [mg/l]	±min/ max	$c(NO_2^-)$ [mM]	±min/ max	$c(NO_3^-)$ [mM]	±min/ max	
0	371.49	0.56	303.43	0.55	337.46	34.03	0.56	0.01	7.34	0.74	0.01	0.00	
0.5	334.11	0.50	276.01	0.74	305.06	29.05	0.62	0.12	6.63	0.63	0.01	0.00	
1	307.46	0.73	266.59	0.38	287.03	20.43	0.55	0.17	6.24	0.44	0.01	0.00	
1.5	321.71	0.99	289.96	0.75	305.83	15.88	0.87	0.12	6.65	0.35	0.01	0.00	
2	263.90	0.80	235.27	0.92	249.59	14.31	0.86	0.06	5.43	0.31	0.01	0.00	
5	320.51	1.34	278.35	1.08	299.43	21.08	1.21	0.13	6.51	0.46	0.02	0.00	
6	316.70	1.67	276.07	1.02	296.38	20.31	1.35	0.33	6.44	0.44	0.02	0.01	
8	298.34	1.79	260.18	1.27	279.26	19.08	1.53	0.26	6.07	0.41	0.02	0.00	
11	305.08	2.27	288.06	1.82	296.57	8.51	2.05	0.22	6.45	0.18	0.03	0.00	
18	311.85	3.02	274.66	2.41	293.26	18.60	2.71	0.31	6.38	0.40	0.04	0.00	
21	312.80	3.45	275.71	2.49	294.25	18.54	2.97	0.48	6.40	0.40	0.05	0.01	

Percentage of oxygen exchange between nitrate and water in sediment incubations				**section 3.3.2**	
by linear regression of $\delta^{18}O\text{-}NO_3^-$ vs. $\delta^{18}O\text{-}H_2O$ in 4 parallel incubations for each time					
lake sediment incubations					
	bottle 1 $\delta^{18}O\text{-}H_2O$=-9.25‰	bottle 2 $\delta^{18}O\text{-}H_2O$=-9.65‰	bottle 3 $\delta^{18}O\text{-}H_2O$=1159.24‰	bottle 4 $\delta^{18}O\text{-}H_2O$=1569.28‰	
time [days]	$\delta^{18}O\text{-}NO_3^-$ [‰]	$\delta^{18}O\text{-}NO_3^-$ [‰]	$\delta^{18}O\text{-}NO_3^-$ [‰]	$\delta^{18}O\text{-}NO_3^-$ [‰]	exchange [%]
0	23.39	23.62	23.26	23.54	0.00±0.01
1	24.45	24.38	24.81	26.04	0.08±0.03
4	27.89	27.57	34.11	40.88	0.75±0.14
9	29.82	30.71	not available[1]	39.61	0.59±0.05
23	not available[1]	39.16	52.68	70.82	1.82±0.67
stream sediment incubations					
	bottle 1 $\delta^{18}O\text{-}H_2O$=-8.07‰	bottle 2 $\delta^{18}O\text{-}H_2O$=--8.75‰	bottle 3 $\delta^{18}O\text{-}H_2O$=500.37‰	bottle 4 $\delta^{18}O\text{-}H_2O$=729.97‰	
time [days]	$\delta^{18}O\text{-}NO_3^-$ [‰]	$\delta^{18}O\text{-}NO_3^-$ [‰]	$\delta^{18}O\text{-}NO_3^-$ [‰]	$\delta^{18}O\text{-}NO_3^-$ [‰]	exchange [%]
0	23.78	23.41	23.44	23.69	0.00±0.03
1	23.32	24.19	24.04	24.22	0.01±0.07
9	27.23	26.50	30.94	34.84	1.01±0.14
20	31.63	30.45	37.47	40.69	1.30±0.09
49	34.31	33.60	45.89	70.87	4.36±1.20
58	37.12	not available[1]	53.65	82.95	5.72±2.25
tidal flat sediment incubations					
	bottle 1 $\delta^{18}O\text{-}H_2O$=-3.74‰	bottle 2 $\delta^{18}O\text{-}H_2O$=-3.09‰	bottle 3 $\delta^{18}O\text{-}H_2O$=1114.88‰	bottle 4 $\delta^{18}O\text{-}H_2O$=1355.67‰	
time [days]	$\delta^{18}O\text{-}NO_3^-$ [‰]	$\delta^{18}O\text{-}NO_3^-$ [‰]	$\delta^{18}O\text{-}NO_3^-$ [‰]	$\delta^{18}O\text{-}NO_3^-$ [‰]	exchange [%]
0	26.05	27.50	29.22	29.22	0.21±0.08
0.5	25.42	26.47	38.33	39.36	1.03±0.07
1	27.28	27.31	44.92	40.40	1.19±0.30
2	28.29	not available[1]	38.48	35.55	0.66±0.32
3	28.31	30.07	48.80	44.13	1.34±0.33
4	30.45	not available[1]	58.04	56.10	2.08±0.49
6	30.50	29.76	67.14	74.95	3.31±0.02
8	not available[1]	29.09	88.13	89.16	4.69±0.72
10	not available[1]	32.94	97.29	103.49	5.37±0.47
[1)] some isotope values are not available due to the reasons given in the corresponding previous tables					