Principles of Stable Isotope Fractionation during Anaerobic Respiration

Dissertation

zur Erlangung des Grades eines Doktors der Naturwissenschaften

der Geowissenschaftlichen Fakultät
der Eberhard-Karls-Universität Tübingen

vorgelegt von
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2008

Tag der mündlichen Prüfung: 06.06.2008

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“*Double, double toil and trouble; Fire burn, and cauldron bubble*…”

(MACBETH, ACT IV, SCENE I)

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Ich erkläre hiermit, dass

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ACKNOWLEDGEMENTS

It would not have been possible to carry out this PHD thesis without several people, which I am gratefully indebted to:

First and foremost, I would like to give my special thanks to my supervisor Dr. Florian Einsiedl. Not only, because of his scientific contributions, his guidance and support. He also opened my eyes to new perspectives during innumerous discussions and never lost the faith in me and my work. I would also like to thank him for choosing me for this PHD project and offering me the unique chance to open the mind of a geologist to the microbial world.

I am also especially indebted to Prof. Dr. Rainer U. Meckenstock for his review, advice and helping me to new ideas during instructive discussions. Furthermore, I am very grateful to Prof. Dr. Dr. h. c. Muharrem Satir for reviewing this thesis.

Further, I would like to thank Willibald Stichler and Harald Lowag for conducting the stable isotope analysis without getting desperate, neither on the IRMS, my samples nor on me. A special thanks also goes to Christine Hammerl and Michael Stöckl for their reliable assistance in the lab. Many people also helped me with instructive comments and arguments, where I am very grateful to Prof. Dr. Stefan Peiffer, Dr. Martin Elsner, Dr. Chistian Griebler, Christine Stumpp, Marc Schwientek and Robert Bauer for their interest in my work.

I would furthermore like to thank Christine Stumpp, together with Umakanth Kunapuli and Rita Kleemann for the great time, support and friendship during the good and bad times in Room 123. I also had the luck to have other fantastic colleagues, where I want particularly thank Franz Bergmann, Heike Brielmann, Marc Schwientek, Bettina Anneser, Eva Fritz, Robert Bauer and Christian Winderl for cheering up my time and for bringing me back to the life beyond my culture bottles. I would also like to thank the rest of the IGOE staff for the nice working atmosphere and all the help and comfort during my time there.

I am also indebted to Eva Lattermann for her friendship and for believing in me and I want you to know that I am very proud of you.

It is wonderful to have a family like mine and I want to thank my parents, Elisabeth and Joseph Mangalo at first for their love and support and I am so grateful that whatever future may bring, I know, they stand behind me. Thanks also to my sister Tuti and her husband Anthony O`Connor, my sister Sarah and my brother Toni Mangalo for being there for me and for all the invaluable good times, where I could recharge my batteries.

Last but not least, there is one man left, whom I want to thank for his love, patience and for not leaving me in times of insanity and this Oscar goes to…Harald Graser.

ABSTRACT

The measurements of stable isotope ratios in sulfate and nitrate have proven to be extremely useful for identification and quantification of microbial processes in the environment. Bacterial sulfate reduction as well as denitrification is accompanied by a fractionation of the stable isotopes of the respective electron acceptor leading to an enrichment of the heavier isotope in the remaining fraction. The main objective of this study was to contribute to the fundamental understanding of anaerobic reduction processes and the related isotope effects in the residual sulfate and nitrate.

Therefore, experiments on sulfate reduction and denitrification were performed with pure cultures. The dual isotope approach (O, S/N) was used to elucidate the influences on the oxygen isotope fractionation, as well as to explain the high variability of sulfur and nitrogen isotope enrichment during the respective respiration processes. Hence, the findings of this study on a microscale help to understand and to quantify these processes on a macroscale.

The present thesis includes three chapters and the intention, the used approach and the results of each chapter is briefly summarized below:

CHAPTER 1: STABLE ISOTOPE FRACTIONATION DURING BACTERIAL SULFATE REDUCTION IS CONTROLLED BY REOXIDATION OF INTERMEDIATES

Basic principles in stable isotope fractionation of sulfur and a suggested oxygen isotope exchange between residual sulfate and water during bacterial sulfate reduction were investigated by a systematic study. Batch experiments with four sulfate-reducing strains (*Desulfovibrio desulfuricans, Desulfobacca acetoxidans*, *Desulfonatronovibrio hydrogenovorans*, and strain *TRM1*) were performed. These microorganisms metabolize different carbon sources (lactate, acetate, formate, and toluene) and show broad variations in their sulfur isotope enrichment factors. The experiments were conducted with ^{18}O-enriched ($\delta^{18}O_{water}$ = + 700 ‰) and -depleted water ($\delta^{18}O_{water}$ = - 40 ‰), respectively and the stable isotope shifts of oxygen in the residual sulfate were followed. For *Desulfovibrio desulfuricans* and *Desulfonatronovibrio hydrogenovorans,* which are both characterized by low sulfur isotope fractionation ($\varepsilon_S \geq$ - 13.2 ‰), δ^{18}O values in the remaining sulfate increased by only 50 ‰ during growth when ^{18}O-enriched water was used for the growth medium. In contrast, with *Desulfobacca acetoxidans* and strain *TRM1* ($\varepsilon_S \leq$ - 22.7 ‰) the residual sulfate showed an increase of the δ^{18}O close to the values of the enriched water of + 700 ‰. In the experiments with ^{18}O-depleted water, the oxygen isotope values in the residual sulfate remained fairly constant for strains *Desulfovibrio desulfuricans, Desulfobacca acetoxidans* and *Desulfonatronovibrio hydrogenovorans*. However, strain *TRM1*, which exhibits the most negative sulfur isotope enrichment factor ($\varepsilon_S \leq$ - 38.7 ‰) showed slightly decreasing δ^{18}O values.

The results give strong evidence that the oxygen atoms of sulfate are exchanged with the one from ambient water during sulfate reduction. However, this neither takes place in the sulfate itself nor during formation of APS (adenosine-5`-phosphosulfate), but rather in intermediates of the sulfate reduction pathway. These may be partially reoxidized to form sulfate. This reoxidation in turn leads to an incorporation of oxygen from water into the "recycled" sulfate changing the overall ^{18}O isotopic composition of the remaining sulfate fraction. The study shows that such incorporation of ^{18}O is correlated with the stable isotope enrichment factor for sulfur measured during sulfate reduction. The reoxidation of intermediates of the sulfate reduction pathway does also strongly influence the sulfur stable isotope enrichment factor and is probably dependent on the metabolic conversion of the substrate and therefore indirectly influences the stable isotope fractionation factor in a rate dependent manner.

CHAPTER 2: EFFECTS OF NITRITE ON STABLE ISOTOPE FRACTIONATION DURING BACTERIAL SULFATE REDUCTION

This work supports the findings of the previous chapter. In batch experiments with ^{18}O-enriched water the effect of different nitrite concentrations on sulfur isotope fractionation by *Desulfovibrio desulfuricans* was investigated. With increasing nitrite concentrations, the sulfur isotope enrichment factors also became more negative from - 12 to - 25 ‰.

Furthermore, the $\delta^{18}O$ values in the remaining sulfate increased from approximately 50 to 120 ‰ when ^{18}O-enriched water was supplied. Since ^{18}O exchange with ambient water does not take place in sulfate itself, but rather in intermediates of the sulfate reduction pathway (e.g. SO_3^{2-}), it is suggested that nitrite affects the steady-state concentration and the extent of reoxidation of the metabolic intermediate sulfite to sulfate during sulfate reduction. Given that nitrite is known to inhibit the production of the enzyme dissimilatory sulfite reductase, the results suggest that the activity of this enzyme regulates the kinetic isotope fractionation of sulfur and oxygen during bacterial sulfate reduction. These novel results also imply that isotope fractionation during bacterial sulfate reduction strongly depends on the cell internal enzymatic regulation rather than on the physico-chemical features of the individual enzymes.

CHAPTER 3: STABLE ISOTOPE FRACTIONATION DURING DENITRIFICATION BY STRAIN *THAUERA AROMATICA*

For the better understanding of biogeochemical regulators we studied stable isotope fractionation during denitrification in a series of batch experiments with strain *Thauera aromatica.* The experiments were conducted under anoxic conditions with two different carbon sources, acetate and toluene, and oxygen isotope labeled water ($\delta^{18}O_{water}$~ + 700 ‰) to observe putative oxygen isotope exchange reactions of nitrate with water. The experiments with acetate as sole carbon source produced isotope enrichment factors for nitrogen (ε_N) are around - 14 ‰, whereas the isotope enrichment factors for nitrogen (ε_N) in the residual nitrate when toluene was the electron donor are around - 20 ‰ . Furthermore, the $\delta^{18}O$ values of nitrate were fairly constant during the latter experiment, although oxygen isotope labeled water was added to the growth medium. The results presented here suggest that the type of the carbon source indirectly influences the nitrogen isotope fractionation during denitrification by *Thauera aromatica*. Furthermore, the experiments with labeled water give no indication that the oxygen isotopes of residual nitrate are influenced by oxygen isotope exchange with water and a subsequent reoxidation of metabolic intermediates such as nitrite.

These findings are important for a quantitative assessment of *in situ* denitrification rates since the experiments demonstrate that generally high fractionations during denitrification can be found in environments where complex substrates such as aromatic compounds and more refractory substrates are oxidized.

ZUSAMMENFASSUNG

Die Ermittlung von stabilen Isotopenverhältnissen im Sulfat und Nitrat haben sich als äusserst hilfreiches Mittel zur Identifikation und Quantifizierung von mikrobiellen Prozessen erwiesen. Sowohl die bakterielle Sulfatreduktion als auch die Denitrifikation werden von einer Fraktionierung der stabilen Isotope des jeweiligen Elektronenakzeptors begleitet, was eine Anreicherung der schwereren Isotope in der residualen Fraktion zur Folge hat.
Gemeinsames Ziel dieser Arbeit ist, zu dem fundamentalen Verständnis anaerober Reduktionsprozesse und deren zugehörigen Isotopeneffekten im residualen Sulfat und Nitrat beizutragen.
Aus diesem Grund wurden Experimente zur Sulfatreduktion und Denitrifikation mit Reinkulturen durchgeführt. Der Ansatz, beide Isotopen (O, S/N) zu analysieren wurde verwendet, um eine Beeinflussung der Isotopenfraktionierungen im Sauerstoff zu klären, sowie die hohe Variabilität der Isotopenanreicherungen während der jeweiligen Respirationsprozesse zu deuten. Daher helfen die Erkenntnisse dieser Arbeit auf der Mikroskala diese Prozesse auf einer Makroskala besser verstehen und quantifizieren zu können. Die vorliegende Doktorarbeit ist in drei Chaptere gegliedert, die Zielsetzung, der angewandte Arbeitsansatz und die Ergebnisse jeder einzelner Arbeit werden im Folgenden kurz zusammengefasst.

KAPITEL 1: KONTROLLE DER STABILEN ISOTOPENFRAKTIONIERUNG WÄHREND DER BAKTERIELLEN SULFATREDUKTION DURCH DIE REOXIDATION VON INTERMEDIATEN

In einer systematischen Studie wurden grundlegende Prinzipien der stabilen Isotopenfraktionierung und ein möglicher Sauerstoffisotopenaustausch zwischen residualem Sulfat und umgebendem Wasser untersucht. Batch-Experimente wurden mit vier sulfatreduzierenden Kulturen (*Desulfovibrio desulfuricans, Desulfobacca acetoxidans, Desulfonatronovibrio hydrogenovorans* und Stamm *TRM1*) durchgeführt. Diese Mikroorganismen metabolisieren jeweils verschiedene Kohlenstoffquellen (Lactat, Acetat, Formiat und Toluol) und zeigen ein breites Spektrum an Anreicherungsfaktoren der Schwefelisotope.
Die Experimente wurden jeweils in ^{18}O-angereichertem ($\delta^{18}O_{Wasser}$ = + 700 ‰) und -abgereichertem Wasser ($\delta^{18}O_{Wasser}$ = - 40 ‰) durchgeführt und die stabilen Isotopenverschiebungen im Sauerstoff beobachtet.
Bei *Desulfovibrio desulfuricans* und *Desulfonatronovibrio hydrogenovorans*, beides Stämme, die durch niedrige Schwefelisotopenfraktionierungen charakterisiert sind ($\varepsilon_S \geq$ - 13.2 ‰), stiegen während des Wachstums im ^{18}O-angereicherten Medium die δ^{18}O Werte im restlichen Sulfat nur um etwa 50 ‰.
Im Gegensatz dazu zeigte das residuale Sulfat bei *Desulfobacca acetoxidans* und dem Stamm *TRM1* ($\varepsilon_S \leq$ - 22.7 ‰) einen ^{18}O Anstieg nahe dem δ-Wert des angereicherten Wassers von

+ 700 ‰. In den Experimenten mit ^{18}O-abgereichertem Wasser blieben die Sauerstoffisotopenwerte im residualen Sulfat für die Stämme *Desulfovibrio desulfuricans, Desulfobacca acetoxidans* und *Desulfonatronovibrio hydrogenovorans* verhältnismässig konstant. Nur der Stamm *TRM1*, der sich durch den negativsten Anreicherungsfaktor in den Schwefelisotopen auszeichnet ($\varepsilon_S \leq$ - 38.7 ‰), zeigte leicht abnehmende $\delta^{18}O$ Werte.

Die Ergebnisse weisen verstärkt darauf hin, dass die Sauerstoffatome des Sulfats mit denen des umgebenden Wassers während der Sulfatreduktion ausgetauscht werden. Jedoch findet dies weder im Sulfat selbst noch während der Bildung von APS (Adenosin-5`-Phosphosulfat) statt, sondern in den Intermediaten des Sulfatreduktionspfades.

Es wird angenommen, dass Letzere teilweise zu Sulfat reoxidiert werden. Diese Reoxidation führt wiederum zu einem Einbau von Sauerstoff aus dem Wassermolekül in das „recyclete" Sulfat und verändert dadurch die gesamte ^{18}O Isotopenzusammensetzung der übrigen Sulfatfraktion.

Die Studie zeigt, dass diese ^{18}O Inkorporation während der Sulfatreduktion mit dem Isotopenanreicherungsfaktor für Schwefel korreliert. Diese Reoxidation der Zwischenprodukte der Sulfatreduktion hat auch starken Einfluss auf die Schwefelisotopenfraktionierung und ist wahrscheinlich abhängig von den metabolischen Umsetzungen des Substrats. Daher beeinflusst es indirekt den stabilen Isotopen Anreicherungsfaktor auf eine ratenabhängige Weise.

KAPITEL 2: DER EFFEKT VON NITRIT AUF DIE STABILE ISOTOPENFRAKTIONIERUNG WÄHREND DER BAKTERIELLEN SULFATREDUKTION

Diese Arbeit unterstützt die Erkenntnisse des vorangegangenen Teils. In Batch-Experimenten mit ^{18}O-angereichertem Wasser wurde der Effekt von verschieden hohen Nitritkonzentrationen auf die Schwefelisotopenfraktionierung durch *Desulfovibrio desulfuricans* untersucht. Mit zunehmenden Nitritkonzentrationen verschoben sich auch die Anreicherungsfaktoren der Schwefelisotopen in Richtung negativere Werte von - 12 ‰ auf - 25 ‰. Ferner stiegen auch die $\delta^{18}O$ Werte im residualen Sulfat von etwa 50 auf 120 ‰, als das ^{18}O-angereicherte Wasser dem Medium zugegeben wurde. Da ein ^{18}O Austausch mit umgebendem Wasser nicht im Sulfat selbst, sondern eher in den Zwischenprodukten des Sulfatreduktionsweges (z.B. SO_3^{2-}) stattfindet, wird angenommen, dass Nitrit die Fliessgleichgewichtskonzentration sowie das Ausmaß der Reoxidation des Stoffwechselintermediates Sulfit während der Sulfatreduktion beeinflusst.

Von Nitrit ist bekannt, dass es die Produktion des Enzyms zur dissimilatorischen Sulfitreduktion hemmt. In Anbetracht dessen weisen die Ergebnisse darauf hin, dass die Aktivität dieses Enzyms die kinetische Isotopenfraktionierung von Schwefel und Sauerstoff während der Sulfatreduktion reguliert. Diese neuen Ergebnisse deuten auch darauf hin, dass die Isotopenfraktionierung während der Sulfatreduktion mehr von der zellinternen

Enzymreguliation als von den physiko-chemischen Eigenschaften der einzelnen Enzyme abhängt.

KAPITEL 3: STABILE ISOTOPENFRAKTIONIERUNG WÄHREND DER DENITRIFIKATION DURCH DEN STAMM *THAUERA AROMATICA*

Batch-Experimente mit der Reinkultur *Thauera aromatica* wurden durchgeführt, um zum Verständnis der biogeochemischen Regulierungen und Einflussfaktoren der stabilen Isotopenfraktionierung durch die Denitrifikation beizutragen.

Die Versuche fanden unter anaeroben Bedingungen mit zwei verschiedenen Kohlenstoffquellen, Acetat und Toluol, und sauerstoffisotopisch markiertem Wasser ($\delta^{18}O_{Wasser}$ ~ + 700 ‰) statt mit dem Ziel, vermeintliche Sauerstoffisotopen Austauschreaktionen von Nitrat mit dem umgebenden Wasser zu untersuchen. Die Experimente mit Acetat als alleinige Kohlenstoffquelle ergaben berechnete Anreicherungsfaktoren für Stickstoff (ε_N) im residualen Nitrat um - 14 ‰, wohingegen die Anreicherungsfaktoren für Stickstoff (ε_N) mit Toluol als Elektronendonor, sich um - 20 ‰ bewegten.

Ferner blieben die $\delta^{18}O$ Werte bei letzterem Experiment trotz der Zugabe des ^{18}O-angereicherten Wassers konstant. Die hier präsentierten Ergebnisse weisen darauf hin, dass die Kohlenstoffquelle indirekt die stabile Isotopenfraktionierung des Stickstoffs während der Denitrifikation von *Thauera aromatica* beeinflusst, da sie offensichtlich die Reaktionsrate reguliert.

Die Versuche mit markiertem Wasser gaben zudem keinerlei Hinweis darauf, dass Sauerstoffisotope des residualen Nitrates durch einen Sauerstoffisotopenaustausch und darauffolgender Reoxidation des Stoffwechselintermediates Nitrit beeinflusst werden.

Diese Erkenntnisse sind für eine quantitative Bestimmung der *in-situ* Denitrifikationsraten bedeutsam. Die Arbeit zeigt, dass hohe Fraktionierungen generell während der Denitrifikation erwartet werden können, wenn komplexere Substrate wie aromatische Verbindungen und andere refraktäre Substrate oxidiert werden

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1. GENERAL INTRODUCTION

1.1 PRINCIPLES OF STABLE ISOTOPE GEOCHEMISTRY

An isotope (Greek *isos* = equal, *topos* = place) is a variety of a chemical element whose atoms have a common number of protons and electrons, but vary in the number of neutrons in the nucleus. In contrast to radioactive isotopes, stable isotopes do not appear to decay to other isotopes over geologic time scales.

Generally, isotopes are written as ^{m}E, where the m denotes the mass number of an element E, which is the sum of protons and neutrons in the nucleus. The principle of measurement in the analysis of stable isotopes is based on their different masses. Most elements possess at least two isotopes where one molecular species occurs dominant, while the others are usually rare. Variations of the abundances of stable isotopes in a compound are based on so called isotope effects, processes that prefer or fractionate one isotope species over the other quantitatively (Seim and Tischendorf, 1990). The isotope fractionation was first described by Urey (1947) who showed that isotope fractionation can be represented as an isotope exchange reaction between any two molecular species that are participating in a physical or chemical reaction. The rate at which an isotopic species passes through a thermodynamic reaction is controlled in part by the energy required to dissociate the molecule (Urey, 1947; Clark, 1999). This activation energy is strongly dependent on the respective mass of the involved isotopes and as a result, the light isotopes of an element require less energy than the heavy ones.

As a consequence one isotope species generally reacts faster during a chemical reaction. If this reaction is in a chemical equilibrium, this isotope species gets enriched in one participating reactant. The so called environmental isotopes of the light elements (i.e. H, C, N, O and S) have relativly large mass differences among their isotope species. Every chemical or physical reaction significantly alters the isotopic signature of a light element bearing compound, which makes them a useful tool for monitoring their geo- and biochemical turnover in nature.

The isotope ratio of an element is generally defined as Equation 1.1:

$$R = \frac{X_h}{X_l} \tag{1.1}$$

where X_h refers to the heavy, and X_l to the lighter isotope. However, for a better international comparison the stable isotope composition of a compound is reported in the δ-value, where the isotope ratio R of a sample is related to an international well known standard and given in permil (‰), as the mass differences are generally very small (see Equation 1.2).

$$\delta R = \left(\frac{R_{sample}}{R_{\text{standard}}} - 1 \right) \times 1000 \quad [‰] \tag{1.2}$$

A compound is considered as enriched relative to the international standard, if it shows a higher δ value in its isotope signature. In contrast, if the compound shows a lower δ value, it denotes a depletion of the heavier isotope and an enrichment of the lighter one relative to the international standard.

Table 1.1 summarizes the stable isotope pairs, their reference standard, and the abundance ratio of the standard according to Kendall and McDonnell (1998). Oxygen isotopes are either referred to the isotopic ratio of the standard mean ocean water (SMOW) or the Pee Dee Belemnite (PDB), a cretaceous fossil from South Carolina. The reference for sulfur is the Canon Diablo troilite (CDT), a sulfur containing meteorite, and atmospheric nitrogen gas is used to compare nitrogen isotope compositions.

Table 1.1: The stable isotope pairs, their reference standard, and the abundance ratio of the standards according to Kendall and McDonnell (1998)

Isotope	Ratio measured	Reference standard	Abundance ratio of standard
^{18}O	$^{18}O/^{16}O$	SMOW or	$2.0052 \cdot 10^{-3}$
		PDB	$2.0672 \cdot 10^{-3}$
^{34}S	$^{34}S/^{32}S$	CDT	$4.5005 \cdot 10^{-2}$
^{15}N	$^{15}N/^{14}N$	Atmospheric N_2	$3.677 \cdot 10^{-3}$

The extend of isotope fractionation in a chemical reaction is quantitatively described by the fractionation factor α (Equation 1.3):

$$\alpha = \frac{R_A}{R_B} \tag{1.3}$$

as the isotope ratio (R_A) of the participating compound A over the isotopic ratio (R_B) of the corresponding compound B.

Processes, where no isotope fractionation occurs have therefore the fractionation factor $\alpha = 1$. As α has generally a value close to 1 and its variations are very small, so the fractionation is usually converted to the isotope enrichment factor ε (according to Equation 1.4) and given in permil [‰].

$$\varepsilon = (\alpha - 1) \times 1000 \quad [‰] \tag{1.4}$$

Two types of isotope fractionation can be differentiated through either isotope exchange reactions (equilibrium fractionation) or kinetic fractionation.

Equilibrium fractionation occurs in closed, well mixed systems, where a chemical equilibrium has established. The consequence is the existence of equal forward and backward reactions and the isotopes of an element are redistributed among the reactants. Generally, an enrichment of the heavier isotope can be found in the participating chemical species with the highest oxidation state. Most physical and chemical reactions show equilibrium isotope fractionations, which are therefore closely related to the reaction rate constant.

Kinetic fractionation comes about, if forward and backward reactions are not identical or if the product is physically removed from the system. In the latter case, kinetic fractionation gets unidirectional. As mentioned above, depending on their masses, heavy and light isotopes react differently fast. Consequently, if a reaction is in imbalance or occurs unidirectional, the lighter isotopes become enriched in the reaction product, whereas the heavier isotope accumulates in the reactant. Enzymatic reactions in biological systems are often accompanied by kinetic fractionations. The degree of kinetic fractionation is therefore highly dependent on the nature of the reaction and its efficiency.

The Rayleigh-distillation equation (Rayleigh, 1896; Hoefs, 1997) is commonly used to describe kinetic isotope fractionations. The equation depicts an exponential relation of the distribution of stable isotopes between two reservoirs as one reservoir decreases in concentration:

$$R_t / R_0 = (C_t / C_0)^{(\alpha - 1)} \tag{1.5}$$

Where R_t and R_0 denote the stable isotope ratios of the studied compound at times t and zero. C_t and C_0 represent the respective concentration of the residuum, and α is the stable isotope fractionation factor.

1.2 THE BIOGEOCHEMICAL CYCLE OF SULFUR

The fourteenth most abundant element on this planet, sulfur, underlies constant abiotic and biotic transformations in the litho-, hydro-, atmo-, and biosphere. It succumbs this constant turnover as it is an essential element for all living organisms (up to 1% dry mass) (Cypionka, 2005). Sulfur also is a constituent of many organic tissues such as amino acids (cysteine, methionine), vitamins, as it is part and parcel of many proteins and coenzymes. The element occurs in different oxidation states (0, -2, +2, +4 and +6). It is very redox-sensitive and gets constantly converted as it is required for a variety of metabolic activities.

Essential steps of the biogeochemical sulfur cycle are described in the following Chapter and can also be followed in Figure 1.1.

The most oxidized species sulfate (SO_4^{2-}) is a highly abundant anion in natural waters and gets reduced by assimilatory as well as dissimilatory processes in oxic and anoxic environments. The assimilatory sulfate reduction, mainly performed by bacteria, fungi and green plants, is the biosynthesis of organic sulfur-containing compounds by the reduction of sulfate without any energy conservation. Sulfate assimilation does not lead to a direct excretion of reduced sulfur, but biomass. Only the decay of the biomass releases the therein contained reduced sulfur.

Solely prokaryotes are able to reduce sulfate during dissimilatory anaerobe respiration gaining energy and releasing sulfide as an end product. Sulfate acts as a terminal electron acceptor for the oxidation of organic substrate or hydrogen as described further below. Both sulfate reducing processes produce reduced sulfur species, which are the basis of another branch of the biogeochemical sulfur cycle.

Reduced sulfur compounds operate as important electron donors in oxic environments for sulfur oxidizing bacteria and are also used by anaerobic photosynthetic sulfur bacteria. This backward oxidation to sulfate can therefore be conducted in the presence of molecular oxygen or dissolved nitrate, where elemental sulfur can occur as an intermediate product.

Elemental sulfur can be of different origin, whether it is deposited geologically or derives from abiotic or biotic oxidation of reduced sulfur compounds. It can also act on the one hand as electron acceptor as well as on the other hand as electron donor for biological processes.

Therefore, bacterial sulfate reduction and bacterial sulfur oxidation next to the turnover of elemental sulfur constitute the key cycle for sulfur in the environment. Disturbance of the cycling can have severe impacts on the environment. For example a high rate of microbial sulfide oxidation causes acidity, high sulfate concentrations, and mobilization of sulfide metals. An increase of sulfate reduction results in turn in ascending toxic H_2S gas formation.

Bacterial sulfate reduction is one of the most common anaerobic processes for cell synthesis and growth as dissolved sulfate is among other inorganic sulfur species highly abundant in nearly all ecosystems. Sulfate is an energetically poor electron acceptor, because of the more unfavourable redox conditions (Thauer et al., 1977) compared to oxygen or nitrate. However, it is profitable, if a sufficient amount of electron donor is present. Sulfate reducing bacteria have also the ability to degrade a broad variety of organic compounds ranging from simple fatty acids to complex aromatic hydrocarbons and therefore they contribute highly to natural attenuation processes.

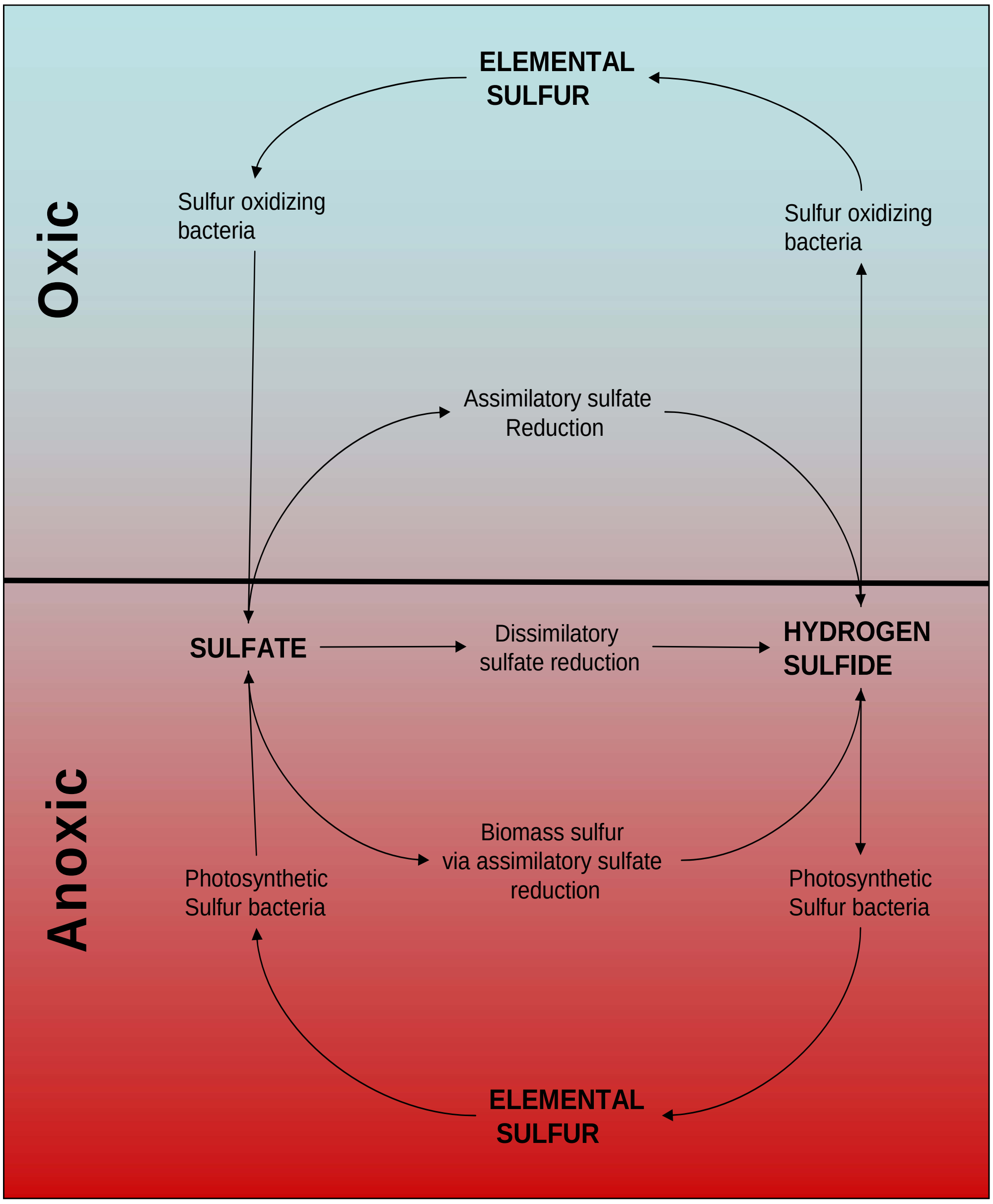

Figure 1.1: The biogeochemical sulfur cycle

1.2.1 Dissimilatory Sulfate Reduction

Two groups of sulfate reducing bacteria can be differentiated (Fritsche and Laplace, 1999). The incomplete oxidizers that degrade organic substrate to acetate and the complete oxidizing sulfate reducing bacteria that oxidize the carbon source completely to CO_2.

Since eight electrons are demanded to reduce S^{+VI} to S^{-II}, a relatively large amount of carbon is needed to gain the required energy for ATP synthesis, which results in the accumulation of hydrogen sulfide, the final product of dissimilatory sulfate reduction.

Sulfate reduction is an eight electron step process and therefore requires an array of catalyzing enzymes. The metabolic regulation and the formation of the various sulfuroxy-intermediates are not fully understood until now. A general description of the sulfate reduction pathway can also be followed in Figure 1.2.

$$SO_{4\ ex}^{2-} \longrightarrow SO_{4\ in}^{2-} \longrightarrow APS \longrightarrow SO_3^{2-} \longrightarrow H_2S_{in} \longrightarrow H_2S_{ex}$$

Figure 1.2: The sulfate reduction pathway

The initial reaction is the uptake of external sulfate, as all enzymatic steps occur in the cytoplasm or in association with the inner side of the cytoplasmic membrane. As the sulfate anion is very stable it needs an activation process, where ATP and sulfate form the high energy compound APS (adenosine-5`-phosphosulfate). This is done by the enzyme ATP sulfurylase. The sulfate residue of the APS molecule is then subsequently reduced to sulfite by the enzyme APS reductase and then finally reduced to sulfide (by the dissimilatory sulfite reductase), which is afterwards excreted from the cell. The reductive pathway from APS to sulfide is a matter of debate since long time. Two theories can be found in literature describing two possible mechanisms (Rabus et al., 2000).

The first mechanism is a direct six electron reduction of sulfite to sulfide, occuring without the formation of intermediates. The second mechanism, the so called “trithionate pathway”, proposes that the reduction of sulfite to sulfide takes place via the formation of trithionate and thiosulfate as intermediates. The final product sulfide is mainly present as HS^- inside the microbial cell, but accompanied by an additional proton, it is transported out of the cell as H_2S by diffusion.

Sulfate reducing bacteria are a quite ubiquitous bacterial group, which can be found in seawater, groundwater, soil and even in the intestinal tract in man and other animals (Brieuc et al., 1996; Morvan et al., 1996; Singleton, 1996; Blaut, 2002). They are adapted to many extreme environments as sulfate-reducers are reported from saline habitats or hydrothermal deep sea vents (Nissenbaum, 1975; Canfield, 1991; Jörgensen et al., 1992; Morvan et al., 1996; Knoblauch et al., 1999; Brandt et al., 2001; Audiffrin et al., 2003).

Bacterial sulfate reduction is also highly contributing to the degradation of subsurface contaminations, where oxygen is already depleted due to aerobic oxidation processes and sulfate acts as an alternative electron acceptor (Coates et al., 1996; Vroblesky et al., 1996; Schroth et al., 2001; Sullivan et al., 2001; Rothermich et al., 2002).

Whenever sulfate reducing bacteria are active in anoxic environments it leads to high levels of accumulating sulfide. The latter can cause beneficial advantages as well as unpleasant disadvantages. So are sulfate reducing bacteria next to their high contribution in degradation of organic contaminants, also of agricultural use. For example, it has been reported, that the production of sulfide in rice fields affects the growth of parasitic organisms, such as nematodes (Rodriguez-Kabana et al., 1965; Jatala, 1986). But furthermore, high concentrations of sulfide can also have fatal consequences on other organisms, due to its high toxicity. Increased sulfide concentrations also cause corrosion of underground iron pipes and can be responsible for the degeneration of concrete and stonework (Peng et al., 1994; Londry and Suflita, 1999; Casey et al., 2005; Greene et al., 2006).

Therefore, it is of high importance to assess dissimilatory sulfate reduction. Microbiologists as well as geochemists have to put special emphasis on understanding the basic mechanisms of bacterial sulfate reduction and its consequences on the environment.

1.2.2 Stable Isotope Fractionation during Bacterial Dissimilatory Sulfate Reduction

Sulfur has four stable isotopes with following abundances ^{32}S = 95.02 %, ^{33}S = 0.75 %, ^{34}S = 4.21 % and ^{36}S = 0.02 % (Hoefs, 1997). Generally, it is possible to compare the ratios of all stable isotopes of sulfur, but the focus is clearly set on the ratio of ^{32}S and ^{34}S due to their higher abundance on earth.

Next to nine radioactive isotopes, oxygen has three stable ones: ^{16}O, ^{17}O and ^{18}O, with the respective abundances of 99.76 %, 0.04 % and 0.20 % (Hoefs, 1997), where the emphasis lies in the isotopic ratio of ^{16}O and ^{18}O. The dissimilatory sulfate-reducing bacteria are well known to fractionate the stable isotopes of sulfate ($^{34}S/^{32}S$, $^{18}O/^{16}O$) during their metabolism, by preferential use of the lighter isotope (^{32}S, ^{16}O) causing an enrichment of the heavier isotope species (^{34}S, ^{18}O) in the residual sulfate (Thode et al., 1951; Harrison and Thode, 1957; Jones and Starkey, 1957; Kaplan and Rittenberg, 1963; Mitzutani and Rafter, 1969; Mitzutani and Rafter, 1973; Krouse, 1988).

The combined isotope approach of sulfur and oxygen can be used as a good approach to assess bacterial sulfate reduction and to trace origin and geochemical history of sulfate (Claypool et al., 1980; Caron et al., 1986; Fritz et al., 1989; Canfield, 1991; Böttcher et al., 1998; Böttcher et al., 1998; Habicht et al., 1998; Alewell, 1999; Brüchert and Pratt, 1999; Ku et al., 1999; Aharon and Fu, 2000; Canfield et al., 2000; Brandt et al., 2001; Aharon and Fu, 2003; Brüchert et al., 2003; Farquhar et al., 2003; Kirste, 2003; Mandernack et al., 2003; Einsiedl and Mayer, 2005; Goldberg et al., 2005; Cerling et al., 2007).

If sulfate is only available in limited concentrations, all sulfur molecules are used up for the reaction and the isotope signature of the produced sulfide is approaching the one from the initial sulfate. Hence, no fractionation is observable any more. In case of non limiting sulfate supply and if sulfate reduction occurs in a closed system, it is becoming a function of the proceeding reaction and can be characterized by the Rayleigh equation (see Equation 1.5). Furthermore, stable isotopes are also a useful tool to elucidate the different reduction steps on a biochemical basis. During the past 50 years, much effort has been made to understand the reduction pathway of bacterial sulfate reduction. Rees (1973) summarized the findings of earlier studies on stable isotope fractionation during bacterial sulfate reduction from Harrison and Thode (1957), Peck (1959), (1961; 1962), Kaplan and Rittenberg (1963), and Kemp and Thode (1968) and worked out a model on the reaction steps and their respective isotope effects in the following called “the Rees-model”, which can be seen in Figure 1.3.

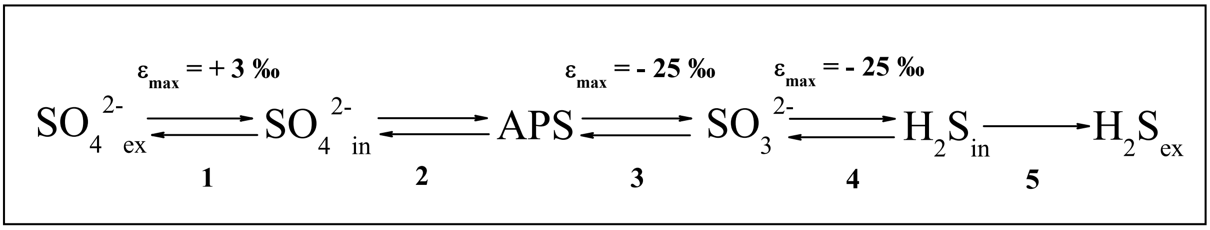

Figure 1.3: The Rees model

Rees (1973) concluded that the dissimilatory sulfate reduction pathway has to take place in mainly four enzyme catalyzed reduction steps (1 - 4), as follows:

Step (1): External sulfate is taken up into the cell via a cell wall enzyme
Step (2): Internal sulfate reacts with ATP (adenosine-triphosphate) to form APS (adenosine-5`-phosphosulfate) mediated by the enzyme ATP sulfurylase
Step (3): APS is reduced to sulfite catalyzed by APS reductase
Step (4): Sulfite is finally reduced to sulfide by the dissimilatory sulfite reductase

It was supposed that under normal environmental conditions, this last step from the intermediate sulfite to external sulfide is probably very fast without the formation of sulfur oxide intermediates as created by the trithionate pathway (mentioned above). Since this step is presumed to be a single reduction step, it is the only exception in the model as being irreversible; all other steps are thought to occur in forward and backward flows.

Additionally, Rees (1973) presumed that every reduction step causes an isotope effect, which is generally very small and can therefore be neglected. However, two exceptions were postulated, where larger isotope effects can occur.

The first exception is, that the uptake of sulfate into the cell can cause an inverse isotope effect, whereas the second exception includes all steps, which involve the breaking of S-O bonds. This occurs in step 3, the reduction of APS to sulfite and step 4, the reduction of sulfite to sulfide (see Figure 1.3).
The total isotope effect for sulfur consists therefore mainly of the ratio of the backward over the forward fluxes (X) and the respective enrichment factors for the exceptional reactions as follows in Equation 1.6:

$$\varepsilon_{\mathrm{S\ (total)}} = \varepsilon_{\mathrm{S\ (sulfate\ uptake)}} + X_1 \cdot X_2 \cdot \varepsilon_{\mathrm{S\ (reduction\ of\ APS)}} + X_1 \cdot X_2 \cdot X_3 \cdot \varepsilon_{\mathrm{S\ (reduction\ of\ sulfite)}} \tag{1.6}$$

Rees (1973) suggested two cases, which can explain the observed isotope effects during bacterial sulfate reduction. The first case excludes backward fluxes, which do not occur, when sulfate concentrations are limiting (below 0.1 mM) and the forward reactions dominate the reactions. The ratio of the backward over the forward reactions is then zero and the total ε_S equals the ε_S of sulfate uptake, which was proposed to have a maximum value of + 3 ‰ (Harrison and Thode, 1957).
The second case presumes equilibrium of fluxes. This means that the ratios of the backward fluxes over the forward fluxes are close to unity. The enrichment factor for the reduction of APS and sulfite (step 3 and 4, see Figure 1.3) were estimated by Rees (1973) to be maximum -25 ‰, respectively. Altogether, the total isotope fractionation can be therefore written as:

$$\varepsilon_{\mathrm{S\ (total)}} = +\,3\,‰ - X_1 \cdot X_2 \cdot 25\,‰ - X_1 \cdot X_2 \cdot X_3 \cdot 25\,‰ \tag{1.7}$$

The total isotope fractionation is the interplay of the respective kinetic isotope fractionation of every single reaction until the rate limiting step is reached and a total sulfur isotope fractionation of approximately - 50 ‰ was reported by different authors (Harrison and Thode, 1957; Kaplan and Rittenberg, 1963; Kemp and Thode, 1968; Rees, 1973; Peck Jr and LeGall, 1982).

While the enrichment factors are central to the concept of isotope fractionation during bacterial sulfate reduction an improved understanding is needed whether equilibrium fractionation or kinetic fractionation controls the isotope effect of sulfur.

Sulfur isotope fractionation has been determined for more than 40 species of sulfate-reducing microorganisms (Janssen et al., 1996; Habicht et al., 1998; Bolliger et al., 2001; Detmers et al., 2001; Johnston et al., 2005). In the studies with pure cultures a large variety of sulfur isotope fractionation was observed with isotope enrichment factors (ε_S) ranging from 0 to - 47 ‰ (Kaplan and Rittenberg, 1963; Bolliger et al., 2001; Detmers et al., 2001)

Various factors have been suggested in the literature that may control observable isotope fractionation. The type of carbon source and the microbial ability of oxidizing the carbon source completely to CO_2 or incompletely to acetate were reported by Detmers et al. (2001). The latter metabolic pathway is assumed to cause generally lower fractionations (Canfield, 2001; Detmers et al., 2001).
Furthermore, Bollinger et al. (2001) found a relationship between the pathway of the organic electron donor and the isotope fractionation during sulfate reduction. It was proposed that isotope enrichment factors of sulfur ≥ - 18 ‰ are characteristic for incomplete-oxidizing sulfate reducers, whereas sulfate-reducing microorganisms that oxidize the organic substrate completely to CO_2 (Rabus et al., 2000) show isotope enrichment factors of sulfur between - 18 and - 47 ‰ (Detmers et al., 2001).
Habicht and Canfield (1997), Harrison and Thode (1957), and Kaplan and Rittenberg (1963) also found evidence that the concentration of the electron donor controls the isotope fractionation during BSR.
It was shown that sulfate concentrations below 1 mM cause low sulfur isotope fractionations (Harrison and Thode, 1957; Canfield, 2001), but contrariwise, Canfield (2001) also reported that limiting substrate concentrations increase the isotope fractionation of sulfur during BSR.

Canfield et al. (2006) and previous results reported by Scherer and Neuhaus (2002) suggested in addition that temperature effects are also linked to isotope fractionation.
Canfield et al. (2006) observed temperature dependent isotope fractionation effects in laboratory experiments. Contrary to this observation, Brüchert et al. (2001) suggested in investigations with strains capable of growth at all experimental temperatures that the latter has no effect on isotope fractionation.

Kaplan and Rittenberg (1963) as well as Böttcher et al. (1999) presumed that isotope fractionation by sulfate-reducing bacteria varies as a function of the specific rate of sulfate reduction (mass cell^{-1} time^{-1}). Moreover, Habicht and Canfield (1997) assumed a correlation between the cell specific reduction rate (mass cell^{-1}time^{-1}) and the isotope fractionation of sulfur. They hypothesized that isotope fractionation of sulfate is regulated by the interaction between the cell specific reduction rate and the sulfate supply. If the sulfate concentration is low and the cell specific reduction rate is high, low sulfur isotope fractionation (ε_S > - 22 ‰) is obtained.

Most studies on BSR focus on sulfur isotope fractionation. However, little is known about oxygen isotope effects during bacterial sulfate reduction. Some laboratory and field studies indicated that with sulfate consumption the sulfur isotope ratio steadily increased in the residual sulfate, while the oxygen isotopes approached a constant value (Mitzutani and Rafter, 1973; Basharmal 1985; Fritz et al., 1989; Aharon and Fu, 2000; Böttcher et al., 2001; Aharon and Fu, 2003). While some authors found indications that kinetic isotope fractionation drives the oxygen isotope shift (Aharon and Fu, 2000; Aharon and Fu, 2003; Mandernack et al., 2003; Knöller et al., 2006), Fritz et al. (1989) and Mitzutani and Rafter (1973) found evidence that the oxygen isotope effect is governed by an equilibrium isotope exchange with sulfur intermediates formed during the reduction pathway.

Two general processes for an isotope exchange during BSR are presented by Fritz et al. (1989) and are summarized in Figure 1.4. Since the rate of isotope exchange of oxygen between sulfate and water under natural conditions is very low, two possible processes are differentiated in literature. Process one is the exchange between water and an intermediate sulfur compound such as sulfite (Lloyd, 1968), probably implicating the splitting of H-O bonds. Process two is an exchange between water and the activated sulfate in the enzyme bound sulfate (APS) as favoured by Fritz et al. (1989).

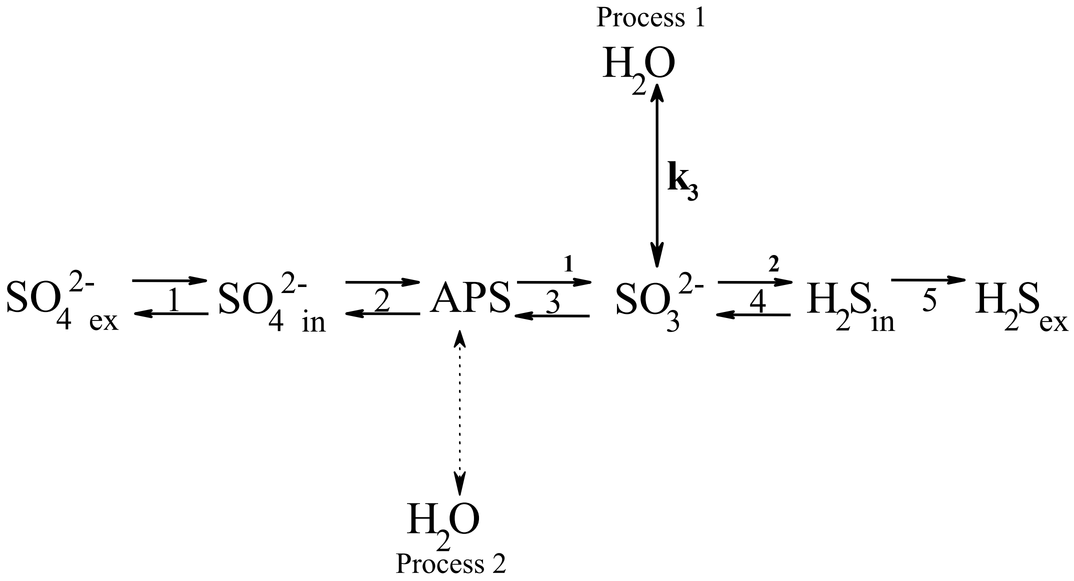

Figure 1.4: The two suggested processes of oxygen isotope exchange modified after Fritz et al. (1989)

1.3 THE BIOGEOCHEMICAL CYCLE OF NITRATE

The global cycling of nitrogen is one of the most important processes on earth. Nitrogen is found in all living organism (up to 16 % of all biomass (Lengler et al., 1999)) as it is a major component of biological tissues such as proteins and nucleic acids.

The biogeochemical cycle of nitrogen includes therefore fundamental processes for life. Nitrogen occurs in several molecular species on earth with oxidation states ranging from +5 as the most oxidized and -3 as the most reduced species. In contrast to the cycling of other elements, some steps of the nitrogen cycle are only reversible by additional processes, where procaryotes play a dominant role.

Nitrogen gas (N_2) is readily available from the atmosphere, which constitutes the largest nitrogen reservoir, but one biological procedure is able to perform nitrogen fixation via reduction to ammonia, which then can be incorporated by reduction into organic tissue. Nitrogen fixation is exclusively done by nitrogen fixing bacteria and is therefore together with photosynthesis the key to the nitrogen cycle, making the element bio-available (see Figure 1.5).

Once nitrogen is fixed into organic matter, processes convert it again into its mineral forms ammonia and nitrate. The mineralization is carried out as multi step reactions by different groups of bacteria. During ammonificaton, organic nitrogen from organic tissue is converted to ammonia or ammonium. These are afterwards oxidized to nitrate either chemically in the presence of oxygen, or biologically by bacterial nitrification.

Afterwards produced nitrate can be reduced exclusively biologically either by assimilatory or dissimilatory processes. During nitrogen assimilation bacteria, fungi, and green plants are able to reduce nitrate back to ammonia and incorporate the reduced nitrogen into their cells. The dissimilatory reduction of nitrate is a solely respiratory process, where nitrate is used as a terminal electron acceptor for energy conservation releasing as main product molecular nitrogen, as described further below.

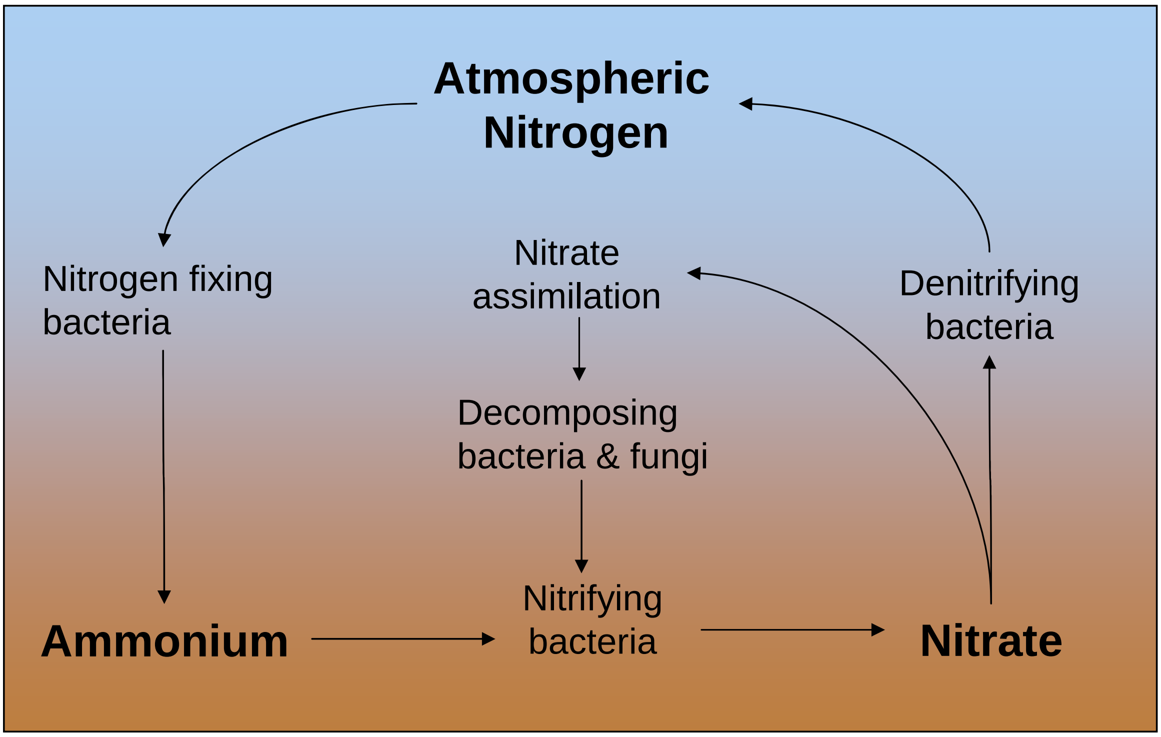

Figure 1.5: The biogeochemical nitrogen cycle

1.3.1 The Dissimilatory Nitrate Reduction

Two nitrate reducing pathways can be differentiated: On the one hand there is the dissimilatory ammonification, where nitrate is respired to ammonia and on the other hand the dissimilatory nitrate reduction. The focus in this study lies on the dissimilatory nitrate reduction or denitrification. The term “denitrifying” bacterium includes all microorganisms, which possess the ability to reduce nitrate to gaseous end products by nitrate respiration. In the late 18th century Gayon and Dupetit (1882) noted that nitrogen disappeared during their experiments and invented the term denitrifiers.

Many denitrifying bacteria belong to the gram-negative bacteria and they all have in common that nitrate is used for ATP synthesis via electron transport chains (Shapleigh, 2006). Most denitrifiers are facultative anaerobe organisms and one has to differentiate between two ways of denitrification. On the one hand the denitrification by heterotroph microorganisms, where organic substance is oxidized to CO_2 or other metabolites. And on the other hand, the chemo-lithotrophic denitrification, where electron sources such as Mn^{2+}, Fe^{2+}, sulfide and CH_4 are used without the presence of an organic carbon source.

The dissimilatory nitrate reduction is a multistep reduction similar to the bacterial sulfate reduction, where each step is performed by a respective enzyme (Figure 1.6). The initial step is the reduction of nitrate to nitrite, which is mediated by the cell membrane bound nitrate reductase.

During the next step, nitrite is reduced to gaseous nitric oxide by nitrite reductase, which is then further reduced to nitrous oxide by nitric oxide reductase. These enzymes are unlike to the nitrate reductase situated in the periplasm, between the cytoplasmic membrane and cell wall. The reduction process finally ends in the production of nitrogen gas by the enzyme nitrous oxide reductase.

$$NO_3^- \longrightarrow NO_2^- \longrightarrow NO \longrightarrow 0.5\ N_2O \longrightarrow 0.5\ N_2$$

Figure 1.6: The denitrification pathway

Denitrification is the most important process in the nitrogen cycle, where molecular nitrogen is released back into the atmosphere and is therefore responsible for large nitrogen loss in soils and aqueous systems, but also prevents accumulation of high nitrate concentrations.
Moreover, denitrifying bacteria are used in combination with nitrifying bacteria to get rid of the high nitrogen contamination in wastewater treatments, but also for degradation of organic contaminants in the subsurface.

Depending on environmental conditions and bacterial species either a complete reduction to nitrogen gas comes about, or the reduction is performed incompletely with an accumulation of nitrogen oxide intermediates such as nitrite or nitric and nitrous oxides. This excretion of nitrogen oxides is mainly caused when nitrate is not limiting (Lengler et al., 1999) and can have severe consequences for atmospheric and biologic chemistry. Nitrate itself is of minor toxicity for higher organisms, but it is reduced to harmful nitrite in the intestinal tract and can cause especially for babies death trapping diseases, like methemoglobinemia. Another harmful aspect of nitrite is the formation of nitrosamines in the gastrointestinal tract of humans, which are considered to be highly carcinogenous.
High productions of nitric and nitrous oxides affect the atmospheric chemistry. Nitrous oxide as a greenhouse gas highly contributes to the destruction of stratospheric ozone (Betlach and Tiedje, 1981; Mosier et al., 1990; Arah et al., 1991; Wrage et al., 2001).

1.3.2 Stable Isotope Fractionation during Bacterial Dissimilatory Nitrate Reduction

Nitrate consists of the isotopes of nitrogen and oxygen, whereas the oxygen isotope abundances on earth have already been discussed above in Chapter 1. 2. 2.

Nitrogen has two stable isotopes, ^{14}N and ^{15}N, whose relative abundances on earth are 99.64 %´and 0.36 %, respectively (Hoefs, 1997).
Denitrification generally causes changes in the isotopic composition of residual nitrate, as the lighter isotopes (^{14}N, ^{16}O) are preferentially metabolized and the heavy isotopes ^{15}N and ^{18}O get enriched with decreasing nitrate concentrations (Mariotti et al., 1981; Böttcher et al., 1990; Aravena et al., 1993; Kellman and Hillaire-Marcel, 1998; Silva et al., 2000; Sigman et al., 2001; Casciotti et al., 2002). This kinetic isotope fractionation is mediated by the various enzyme catalyzed reduction steps (Amberger and Schmidt, 1987; Böttcher et al., 1990) as described above. Since the middle of the past century many studies on isotope fractionation during denitrification concluded in various nitrogen isotope enrichment factors ranging from 0 to less than -40 ‰ (Richards and Benson, 1961; Delwiche and Steyn, 1970; Cline and Kaplan, 1975; Mariotti et al., 1981; Amberger and Schmidt, 1987; Böttcher et al., 1990; Aravena et al., 1993; Altabet et al., 1995; Brandes and Devol, 1997). However, for a quantitative approach the nitrogen isotope enrichment factor ε_N is substantial to calculate the *in situ* degradation of nitrate for different environmental conditions, as shown for many organic compounds (Aravena *et al.*, 1993)

In general, laboratory studies showed higher fractionations for nitrogen than studies on natural waters. However, one must take into account that nitrite is produced during denitrification and can be accumulated. If this is the case, $\delta^{15}N$ values could be severely affected by measuring a mixture of nitrite derived nitrogen and nitrate. The consequence would be an shift of the isotope enrichment factor towards more negative values by having a steepening of the slope in a Rayleigh plot between the isotope values and the supposed nitrate concentration (Bates et al., 1998).
As the single isotope approach of measuring only the nitrogen isotopes makes it often difficult to differentiate between denitrification and other processes, the coupled measurement of $\delta^{15}N$ and $\delta^{18}O$ of nitrate has been proven to be supporting for determination of denitrification in nature (Amberger and Schmidt, 1987; Aravena et al., 1993; Silva et al., 2000; Lehmann et al., 2003; Chen and MacQuarrie, 2005; Singleton et al., 2005).

As mentioned above, the isotope enrichment factors for nitrogen (ε_N) during denitrification were determined in laboratory and field studies and ranged between 0 and - 36 ‰ (Delwiche and Steyn, 1970; Cline and Kaplan, 1975; Mariotti et al., 1981; Mariotti et al., 1988; Böttcher et al., 1990; Brandes and Devol, 1997; Barford et al., 1999; Lehmann et al., 2003; Chen and MacQuarrie, 2005), whereas oxygen isotope enrichment factors (ε_O) of nitrate have been observed between - 8 and - 18.3 ‰ (Böttcher et al., 1990; Mengis et al., 1999; Lehmann et al., 2003).

For a long time a characteristic ratio of 2:1 for the isotope enrichment of ^{15}N to ^{18}O was considered (Aravena and Robertson, 1998). Recent studies in various aquatic systems, however, showed different ratios of ^{15}N to ^{18}O enrichment between 1:3 to 2:1 (Cey et al., 1999; Mengis et al., 1999; Fukada et al., 2003; Lehmann et al., 2003; McMahon et al., 2004). Although the variation in the ratio of nitrogen and oxygen isotope enrichment was contributed mainly to misinterpretation of field data and analytical errors, the question remains still open if the oxygen isotopes of residual nitrate are additionally influenced somehow during the denitrification pathway. This influence would imply a constant increase in the nitrogen isotopes of residual nitrate while the oxygen isotopes approach a constant value. During the whole process of denitrification nitrogen remains preserved while oxygen gets constantly lost. Therefore, the oxygen isotopes are constantly exposed to kinetic fractionation even if the whole nitrate is used up, where no isotope fractionation is visible in the nitrogen isotopes (Casciotti et al., 2002).

Generally the similar parameters that seem to have an isotope effect on residual sulfate during bacterial sulfate reduction are also suggested to have an influence on the residual nitrate during denitrification. Hence, an increase of temperature has an effect on the denitrification rate, which again has a direct effect on the isotope enrichment. By elevating the temperature, also the rate of denitrification is getting higher, showing a direct correlation to the isotope enrichment factor (Bates et al., 1998).

Brunner et al. (2005) speculated that one reason for the variation of the $\delta^{15}N/\delta^{18}O$ ratio during denitrification might be due to an oxygen isotope exchange between metabolic intermediate compounds such as nitrite and ambient water similar to that observed for sulfate reduction (Mangalo et al., 2007). Furthermore, it has also been suggested that stable isotope fractionation of nitrogen during denitrification is rate dependent with the largest isotope enrichments for nitrogen obtained with lowest reduction rates (Mariotti et al., 1981; Mariotti et al., 1988; Lehmann et al., 2003). Also the concentration of the carbon source and temperature were speculated to have an effect on isotope fractionation during denitrification (Bates and Spalding, 1998).

As denitrification occurs in several reduction steps, it requires an array of enzymes to catalyze the individual reactions. The first step is the reduction of nitrate to nitrite, which is then further reduced to nitric oxide. Ye et al. (1991) observed in laboratory studies an oxygen isotope exchange of ambient water with nitrite formed as an intermediate compound during the nitrate reduction pathway. Therefore, an isotopic exchange between ambient water and the metabolic intermediate nitrite and a subsequent reoxidation to nitrate during denitrification is hypothesized to explain the variation of oxygen isotope enrichment factors during dentrification similar to sulfate reduction (Mangalo et al., 2007). Experimental studies using both isotopes of nitrate, $\delta^{15}N$ and $\delta^{18}O$, are rare and the number of field studies on stable isotope fractionation during denitrification is definitely larger than laboratory studies on pure cultures.

1.4 OBJECTIVES & METHODOLOGY

Stable isotope analysis has been successfully used in environmental studies to gain quantitative information on the microbial activity of an ecosystem. However, to explain the isotope effects on a macroscale, an improved knowledge of the isotope fractionating processes in the microscale is needed to apply stable isotope analysis in the environment.

The focus of this work was to gain more fundamental knowledge on the principles of stable isotope fractionation during bacterial anaerobic respiration processes. The interpretation of isotope data also allows drawing conclusions on the biochemical reduction pathways and contributes also to the fundamental knowledge of the biochemistry of microbial respiration pathways. To follow this goal, experiments on bacterial sulfate reduction and denitrification were performed with pure cultures and the thesis can be divided into three parts.

Chapter 1 adresses the principle mechanisms of stable isotope fractionation during bacterial sulfate reduction. There has been much research done to characterize the mechanisms of sulfur isotope fractionation during bacterial sulfate reduction, but only a few studies include microbial mediated oxygen isotope effects. Due to higher geochemical and biological impact on the oxygen isotopes in sulfate during biogeochemical cycling, the significance of oxygen isotopes for an understanding of cell internal processes have been unattended in most laboratory and field studies.

Therefore, a major aspect of this work is to characterize the nature of oxygen isotope effects on residual sulfate during bacterial sulfate reduction. Since long, this is a matter of debate in literature, as several mechanisms are suggested, causing oxygen isotope exchange reactions with ambient water. Additionally, the oxygen isotope effect in residual sulfate is in all probability caused by cell internal reoxidation processes, which would have also an effect on the sulfur isotope fractionation. A series of experiments on oxygen and sulfur isotope effects caused by BSR in the remaining sulfate was therefore performed with a special focus on the previously postulated rate dependency of sulfate isotope fractionation.

The batch culture experiments were conducted with $\delta^{18}O$ labeled water to assess the oxygen isotope exchange to a larger extent and the study was accomplished with four sulfate-reducing strains, oxidizing different carbon sources and showing a variety of sulfur isotope enrichment factors. The stable isotope exchange of oxygen from ambient water with sulfate intermediate compounds was here used to study reoxidation processes of intermediates formed during bacterial sulfate reduction. These, in turn, are probably major factors for observed differences in sulfur and oxygen stable isotope fractionation studies on bacterial sulfate reduction.

The second chapter is closely related to the results of the first one, which revealed the main conclusion that the kinetics of the rate constants of the individual reduction steps control the isotope enrichment for sulfur and oxygen. It was suggested on the one hand that the isotope fractionation of the reduction of the high energy compound APS to sulfite, which is catalyzed by the enzyme APS reductase is controlled by kinetic fractionation (Rees, 1973; Brunner and Bernasconi, 2005; Canfield et al., 2006), whereas Mangalo et al. (2007) proposed an equilibrium fractionation for this step. On the other hand, the reduction of sulfite to sulfide is suggested to be accompanied by a kinetic fractionation (Rees, 1973; Mangalo et al., 2007). Mangalo et al. (2007) hypothesized that the dissimilatory sulfite reductase (DSR) controls the residence time of the metabolic intermediate sulfite in the cell and therefore, the magnitude of the overall sulfur isotope fractionation during BSR.

To support this hypothesis, experiments with the sulfate-reducing bacterium *Desulfovibrio desulfuricans* were conducted with the purpose to change the kinetic regulation of the enzyme mediated reduction step between sulfite and sulfide. Therefore, this last step of sulfate reduction was inhibited, in order to increase the magnitude of residence time and consequently also the reoxidation of sulfuroxy-intermediates. Dissolved nitrite inhibits the production of DSR and therefore influences the final reduction process of sulfite to sulfide (Greene et al., 2003; Greene et al., 2006).

Chapter 3 is dealing with isotope effects during the bacterial dissimilatory nitrate reduction (denitrification). Little is known about the processes that control isotope enrichment of nitrogen and oxygen of residual nitrate during denitrification from field experiments and even fewer laboratory studies were performed. Similar to the experiments on bacterial sulfate reduction, also an oxygen isotope exchange between nitrate and ambient water is supposed, which gets expressed in the residual nitrate via reoxidation processes of metabolic intermediates. Batch experiments with the pure culture *Thauera aromatica* are reported in this study in which the nitrogen isotope enrichment factors ε_N were determined for growth on two different carbon sources. In order to find out more about the effect of the type of carbon source on both isotopes of nitrate, nitrogen and oxygen, two different electron donors were supplied: On the one hand easily degradable acetate, on the other hand an aromatic hydrocarbon, toluene, which is more difficult for the bacterium to degrade.

Additionally, the oxygen isotope effect for denitrification during growth on toluene was monitored by using ^{18}O-labeled and unlabeled water for the growth media to compare the potential oxygen isotope effect in the residual nitrate.

Moreover, this study links for the first time a hypothetic reoxidation of the metabolic intermediate nitrite to nitrate directly to an investigation of isotope exchange of ^{18}O in nitrate and the isotope enrichment factor of nitrogen.

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2. STABLE ISOTOPE FRACTIONATION DURING BACTERIAL SULFATE REDUCTION IS CONTROLLED BY REOXIDATION OF INTERMEDIATES

2.1 INTRODUCTION

Dissolved sulfate is a major anion in terrestrial and marine environments and a very important electron acceptor in anoxic habitats. The stable isotope ratios of sulfur and oxygen have been successfully used to elucidate sources and processes affecting sulfate particularly if both $\delta^{34}S$ and $\delta^{18}O$ values of sulfate were determined (Basharmal 1985; Clark and Fritz, 1997; Böttcher et al., 1998; Aharon and Fu, 2000; Canfield et al., 2000; Spence et al., 2001; Einsiedl and Mayer, 2005). During the last 50 years the effect of bacterial sulfate reduction (BSR) on the isotopic composition of residual sulfate has been intensively discussed (Ford, 1957; Kaplan et al., 1960; Rees, 1973; Fry, 1986; Fritz et al., 1989; Canfield et al., 2000; Brüchert et al., 2001; Farquhar et al., 2003; Brunner et al., 2005). During BSR the lighter isotopes ^{32}S and ^{16}O are preferentially metabolized by microorganisms causing an enrichment of the heavy isotopes ^{34}S and ^{18}O in the remaining sulfate as sulfate concentrations decrease (Kaplan and Rittenberg, 1963).

Laboratory and field studies have shown that sulfur isotope fractionation ranges between 0 and - 46 ‰ during BSR (Harrison and Thode, 1957; Kaplan and Rittenberg, 1963; Kemp and Thode, 1968). The standard Rees model (Rees, 1973) is often used to describe the isotope fractionation during sulfate reduction (Fritz et al., 1989; Habicht and Canfield, 1997; Farquhar et al., 2003; Canfield et al., 2006) (see Figure 2.1):
After transport inside the cell (step 1), sulfate is activated as adenosine-5`-phosphosulfate (APS). Subsequently, APS is reduced to sulfite (step 3). There are two possible pathways suggested for the next step (step 4). Either sulfite is directly reduced to sulfide or sulfite is reduced by the trithionate pathway to finally form sulfide (Akagi, 1981; Peck Jr and LeGall, 1982; Akagi, 1995; Cypionca, 1995). It is assumed that step 1, 2, and 3 are reversible, whereas step 4 is irreversible. The individual steps are accompanied by isotope fractionations as follows: By the first step only a small inverse sulfur isotope enrichment of around + 3 ‰ is assumed (Harrison and Thode, 1957; Rees, 1973). No fractionation of sulfur isotopes is assumed in the second step where sulfate is activated to form the high-energy compound APS, whereas a sulfur isotope enrichment of - 25 ‰ for each of the steps of APS to sulfite and sulfite to sulfide is assumed (Krouse and Grinenko, 1991). The total isotope fractionation is the interplay of the respective kinetic isotope fractionation of every single reaction until the rate limiting step is reached and a total sulfur isotope fractionation of approximately - 50 ‰

was reported by different authors (Harrison and Thode, 1957; Kaplan and Rittenberg, 1963; Kemp and Thode, 1968; Rees, 1973; Peck Jr and LeGall, 1982).

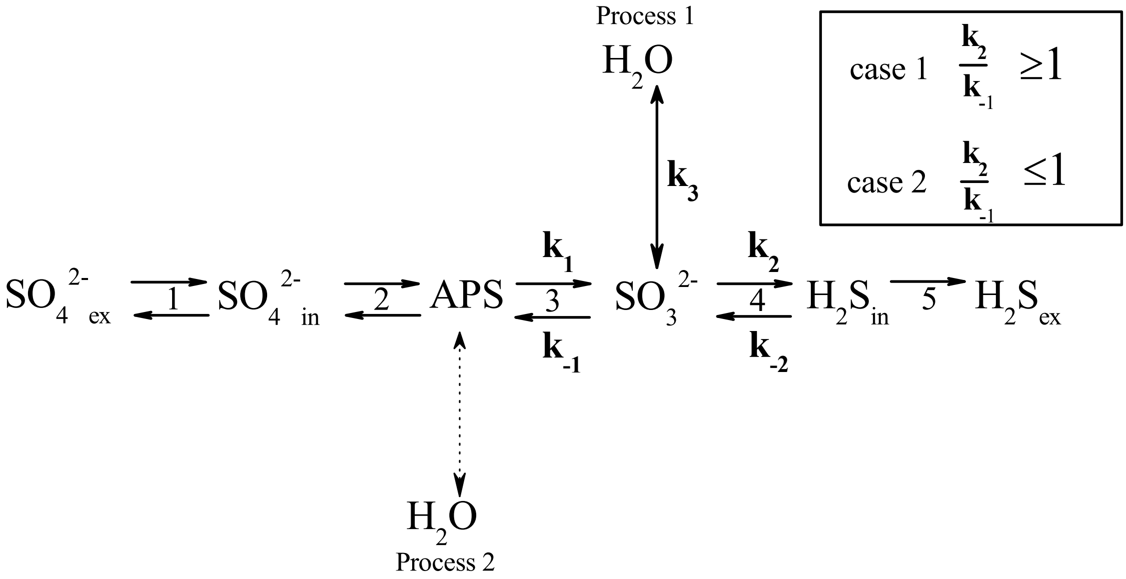

Figure 2.1: The pathway of bacterial (dissimilatory) sulfate reduction modified after Rees (1973). $SO_4^{2-}{}_{ex}$ means external sulfate, which is transported into the cell ($SO_4^{2-}{}_{in}$) (step 1). Inside the cell internal sulfate reacts with ATP (adenosine-triphosphate) to form APS (adenosine-5`-phosphosulfate) (step 2), which thereafter is reduced directly to sulfite (step 3). The final step of the biochemical reaction involves the reduction of sulfite to sulfide (step 4), which is afterwards excreted from the cell (H_2S_{ex}, step 5). The numbers denote the different reduction steps 1-5. The reaction rates are given in k_1, k_2, and k_3. The reaction k_3 is constant, while k_1 and k_2 vary between the bacterial groups A and B.

As discussed above, while enrichment factors are central to the concept of isotope fractionation during bacterial sulfate reduction an improved understanding is needed whether equilibrium fractionation or kinetic fractionation controls the isotope effect of sulfur.

Various factors have been suggested in the literature that may control observable isotope fractionation. The type of carbon source and the microbial ability of oxidizing the carbon source completely to CO_2 or incompletely to acetate were reported by Detmers et al. (2001). The latter metabolic pathway is assumed to cause generally lower fractionations (Canfield, 2001; Detmers et al., 2001). Habicht and Canfield (1997), Harrison and Thode (1957), and Kaplan and Rittenberg (1963) found evidence that the concentration of the electron donor controls the isotope fractionation during BSR. Canfield et al. (2006) and previous results reported by Scherer and Neuhaus (2002) suggested that temperature effects are also linked to isotope fractionation. Moreover, Habicht and Canfield (1997) assumed a correlation between the cell specific reduction rate (mass $cell^{-1}time^{-1}$) and the isotope fractionation of sulfur. They hypothesized that isotope fractionation of sulfate is regulated by the interaction between the cell specific reduction rate and the sulfate supply. If the sulfate concentration is low and the cell specific reduction rate is high, low sulfur isotope fractionation ($\varepsilon_S > -22$ ‰) is obtained.

Most studies on BSR focus on sulfur isotope fractionation. However, little is known about oxygen isotope effects during bacterial sulfate reduction. Some laboratory and field studies indicated that with sulfate consumption the sulfur isotope ratio steadily increased in the residual sulfate, while the oxygen isotopes approached a constant value (Mitzutani and Rafter, 1973; Basharmal 1985; Fritz et al., 1989; Aharon and Fu, 2000; Böttcher et al., 2001; Aharon and Fu, 2003). While some authors found indications that kinetic isotope fractionation drives the oxygen isotope shift (Aharon and Fu, 2000; Aharon and Fu, 2003; Mandernack et al., 2003; Knöller et al., 2006), Fritz et al. (1989) and Mitzutani and Rafter (1973) found evidence that the oxygen isotope effect is governed by an equilibrium isotope exchange with sulfur intermediates formed during the reduction pathway. Two general processes for an isotope exchange during BSR are presented by Fritz et al. (1989) and are summarized in Figure 2.1. Since the rate of isotope exchange of oxygen between sulfate and water under natural conditions is very low, two possible processes are differentiated in literature. Process one is the exchange between water and an intermediate sulfur compound such as sulfite (Lloyd, 1968), probably implicating the splitting of H-O bonds. Process two is an exchange between water and the activated sulfate in the enzyme bound sulfate (APS) as favoured by Fritz et al. (1989).

Here, additional insight is provided in the mechanism of isotope fractionation during BSR. A series of experiments on oxygen and sulfur isotope effects caused by BSR in the remaining sulfate was performed with a special focus on the previously postulated rate dependency of sulfate isotope fractionation. This was done with a broad spectrum of microorganisms, with different substrate utilization and fractionation properties, respectively.

We used the oxygen stable isotope exchange to study reoxidation processes of intermediates formed during bacterial sulfate reduction. These, in turn, are probably major factors for observed differences in sulfur and oxygen stable isotope fractionation studies on bacterial sulfate reduction.

2.2 MATERIALS & METHODS

2.2.1 Cultures

Desulfovibrio desulfuricans (DSMZ 642), *Desulfobacca acetoxidans* (DSMZ 11109), and *Desulfonatronovibrio hydrogenovorans* (DSMZ 9292) were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ). Strain TRM1 was isolated from a tar oil-contaminated aquifer near Stuttgart, Germany (Meckenstock, 1999). All strains are freshwater strains and capable of dissimilatory sulfate reduction.

2.2.2 Growth Conditions

Batch experiments with the aforesaid four different bacterial strains were conducted with ^{18}O-enriched and ^{18}O-depleted water to characterize the isotope exchange of oxygen in the remaining sulfate with ambient water. For each of the four stains a set of six bottles (n = 24) was inoculated at a ratio of 1: 10 with precultures in the respective mineral media. One half of the bottles (n = 3) was treated with 1 ml of ^{18}O-enriched water (^{18}O content: 10.1 %, Hyox Rotem GmbH) to gain a final isotope composition in the water of about + 700 ‰. For the other half of the bottles (n = 3) water from deep ice cores of Antarctica with a δ^{18}O value of approximately - 40 ‰ was used as water for the mineral media (Graf et al., 2002).
Each strain was grown with a carbonate-buffered (30 mM) mineral medium with a single carbon source under strictly anaerobic conditions (see table 2.1).

Desulfovibrio desulfuricans and strain *TRM1* were cultivated in anoxic basal freshwater medium described by Widdel and Bak (1991). *Desulfobacca acetoxidans* and *Desulfonatronovibrio hydrogenovorans* were grown in the suggested media 728 (DSMZ) and 742 (DSMZ). Lactate, acetate, and formate were added as carbon sources from 1M stock solutions. Aromatic hydrocarbons (toluene) were added directly with a syringe through the stopper to a final concentration of about 1000 µM. Sulfate was also added from a 1 M stock solution to give the respective final concentrations (Table 2.1).
The batch experiments were performed in half-filled 100 ml serum bottles. The headspace was completely replaced by a N_2:CO_2 gas mixture (80:20) [vol/vol]. All experiments were conducted at 30° C in the dark. BSR was monitored by measuring accumulating sulfide as described by Cline (1969).

Table 2.1: Conditions applied for the growth experiments in batch cultures with the respective organisms.

Microorganism	**pH**	**Sulfate (mM) final concentration**	**Electron donor (mM)**
Desulfovibrio desulfuricans	7.2	~20	Lactate ~20
Desulfonatronovibrio	9.7	~30	Acetate

hydrogenovorans			~20
Desulfobacca acetoxidans	7.0	~20	Formate ~20
TRM1	7.2	~10	Toluene ~1

2.2.3 Determination of Sulfur and Oxygen Isotope Ratios in Residual Sulfate

To prevent reoxidation of sulfide during the sampling procedure microbially produced sulfide was removed from water by adding up to 5 ml 20 % Zn-Acetate and filtration through 0.45 µm pore size Millipore syringe filters depending on the sulfide concentration. The samples were acidified to pH < 4 to eliminate HCO_3^-.

Sulfate for isotope analysis was afterwards precipitated as $BaSO_4$ with 3 to 5 ml 10 % $BaCl_2$. The precipitate was recovered by centrifugation, carefully washed and dried prior to isotope analyses. Isotope analysis for sulfur was performed by isotope ratio mass spectrometry (IRMS Thermo Electron MAT 253) coupled with an elemental analyzer (EA-EuroVector) after complete conversion of $BaSO_4$ to SO_2 via high temperature combustion (1000ºC) with WO_2 and V_2O_5. For the measurements of $\delta^{18}O$ in sulfate, CO through pyrolysis of $BaSO_4$ at 1450°C in a discharge chamber (ConFloIII-Interface) and isotope analysis was conducted by subsequent IRMS (Holt, 1991).

Sulfur and oxygen isotope ratios are reported in parts per thousand (per mil) using the conventional delta-notation (δ) (Equation 2.1):

$$\delta R = \left(\frac{R_{sample}}{R_{standard}} - 1 \right) \times 1000 \quad [‰] \tag{2.1}$$

R denotes the ratio of the concentrations of heavy over light isotope. R_{sample} and $R_{standard}$ are the isotope ratios of the sample and the standard, respectively. The $\delta^{34}S$ is reported relative to the Vienna Canon Diablo Troilite standard (V-CDT) and the $\delta^{18}O$ refers to Vienna Standard Mean Ocean Water(V-SMOW). An international standard NBS 127 and several internal standards were used to ensure accurate measurements. The standard deviation of all measurements was below ± 0.2 ‰ for $\delta^{34}S$ and± 0.4 for $\delta^{18}O$. For high $\delta^{18}O$ values above + 60 ‰ the accuracy decreases, since no standard reference material was available with such a high $\delta^{18}O$ value. Each sample was measured twice. Since all batch experiments were conducted in a closed system, sulfur isotope fractionation during sulfate reduction can be described by the Rayleigh equation (Equation 2.2).

$$R_t / R_0 = (C_t / C_0)^{(\alpha - 1)} \tag{2.2}$$

R_t and R_0 denote the stable isotope ratios of sulfate at times t and zero. C_t and C_0 represent the respective concentration of the residual sulfate, and α is the stable isotope fractionation factor. The fractionation factor α was converted to the isotope enrichment factor (ε) according to Equation 2.3:

$$\varepsilon = (\alpha - 1) \times 1000 \quad [‰] \tag{2.3}$$

2.3 RESULTS

2.3.1 *Growth Experiments with ^{18}O-Enriched and ^{18}O-Depleted Water*

All data from the batch experiments can be followed in the electronic annex.
Strain *Desulfovibrio desulfuricans* had an initial sulfate supply of 20 mM and reduced around 50 % to sulfide within 151 hours at most.
Desulfonatronovibrio hydrogenovorans consumed around 60 % of the initial supplied sulfate (30 mM) and *Desulfobacca acetoxidans* reduced around 80 % of the 20 mM sulfate start concentration within 54 days, whereas strain *TRM1* respired around 60 % of 10 mM sulfate within 69 days.

2.3.1 *Isotope Composition of Sulfate*

2.3.2.1 Oxygen Isotope Fractionation during Sulfate Reduction

In all experiments with ^{18}O-depleted water, the oxygen isotope composition showed fairly constant values (Figure 2.2b, d, f). Only for strain *TRM1* (Figure 2.2h) a trend of decreasing δ^{18}O values with increasing δ^{34}S values could be observed. The average maximum δ^{18}O value of each bacterial strain in the experiments with ^{18}O-enriched water is given in Table 2.2.

The oxygen isotope composition of residual sulfate with ^{18}O-enriched water and strain *Desulfovibrio desulfuricans* (Figure 2.2a) increased from 10.8 ‰ (n = 1) to 47.2 ± 9.9 ‰ (n = 2). *Desulfonatronovibrio hydrogenovorans* (Figure 2.2c) showed δ^{18}O values from 13.0 ‰ up to 66.2 ± 7.2 ‰ (n = 3).

In the experiments with ^{18}O-enriched water, the δ^{18}O values of sulfate in the batch cultures with *Desulfobacca acetoxidans* (Figure 2.2e) and strain *TRM1* (Figure 2.2g) were generally higher than those observed for *Desulfovibrio desulfuricans* (Figure 2.2a) and *Desulfonatronovibrio hydrogenovorans* (Fig. 2.2c). For *Desulfobacca acetoxidans* and strain *TRM1* δ^{18}O values of 654.7 ‰ (n = 1) and 585.4 ± 53.59 ‰ (n = 3) were measured in the remaining sulfate respectively. The data are close to the oxygen isotope content of the enriched water used for the experiments (+ 700 ‰).

2.3.2.2 Sulfur Isotope Fractionation during Sulfate Reduction

In all cultures, the δ^{34}S values of the residual sulfate increased from an average starting value of approximately 4.8 ± 0.2 ‰ and became enriched during BSR (Figure 2.3). At the end of the experiment with *Desulfovibrio desulfuricans* (Figure 2.3a) the δ^{34}S values were between 10.6 ‰ (n = 1) in the experiments with ^{18}O-depleted water and 8.8 ± 0.1 ‰ (n = 2) in ^{18}O-enriched water, respectively. With *Desulfonatronovibrio hydrogenovorans* (Figure 2.3b) the sulfur isotopes of residual sulfate shifted up to 16.2 ± 0.7 ‰ (n = 3) in the ^{18}O-depleted water and to 14.1 ± 0.1 ‰ (n = 3) in the ^{18}O-enriched water. The highest δ^{34}S values were observed for *Desulfobacca acetoxidans* (Figure 2.3c) which increased the isotopic composition of sulfate up to 62.6 ‰ (n = 1) and 57.9 ‰ (n = 1), respectively.

δ^{34}S values for strain *TRM1* (Figure 2.3d) were around 23.2 ± 0.9 ‰ (n = 3) in the ^{18}O-depleted media and 23.5 ± 3.7 ‰ (n = 3) in the ^{18}O-enriched ones.

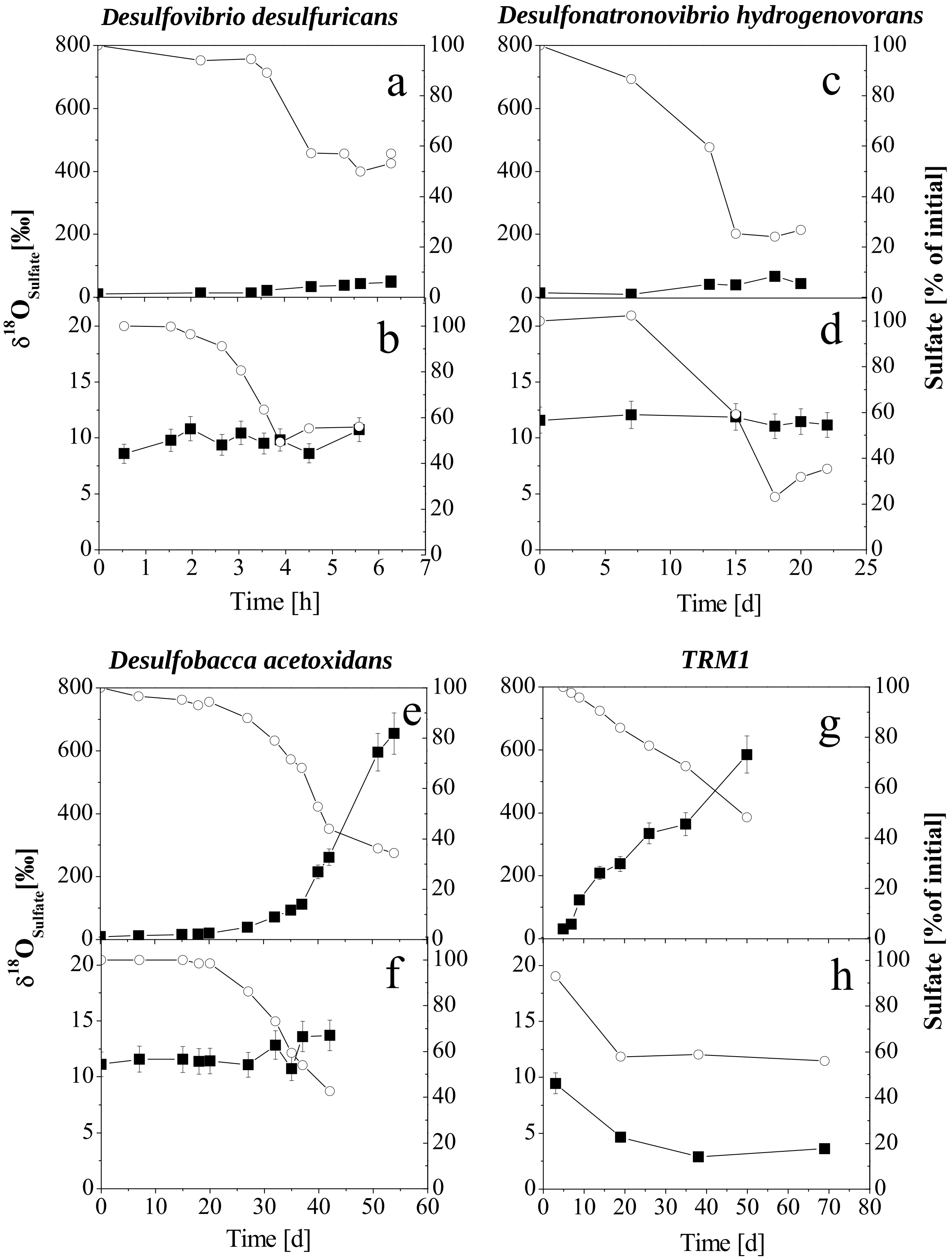

Figure 2.2: Oxygen isotope fractionation during sulfate reduction in batch cultures. The open circles point out the sulfate consumption calculated from the H_2S concentrations. The right y-axis refers to the percent sulfate consumed during bacterial sulfate reduction. $\delta^{18}O$ values of residual sulfate (closed squares) obtained from the experiments with ^{18}O-enriched water (upper panels) and ^{18}O-depleted water (lower panels), for *D. desulfuricans* (a, b), *D. hydrogenovorans* (c, d), *D. acetoxidans* (e, f) and strain *TRM1* (g, h). Error bars indicate the standard deviation of parallel growth experiments (n = 2-3).

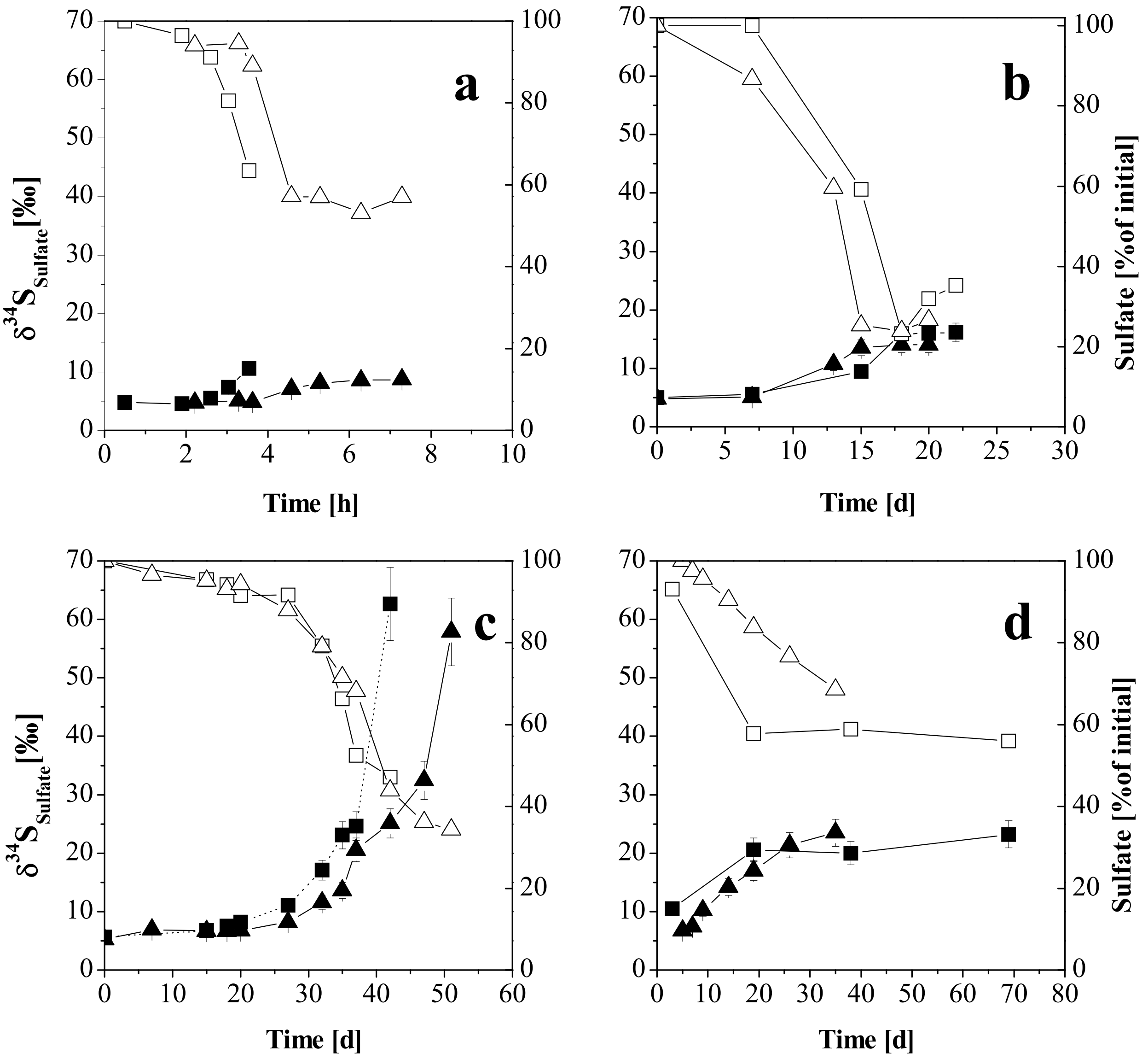

Figure 2.3: Sulfur isotope fractionation during sulfate reduction in batch cultures. δ^{34}S values of residual sulfate from experiments with ^{18}O-depleted water (squares) and ^{18}O-enriched water (triangles) for *D. desulfuricans* (a), *D. hydrogenovorans* (b), *D. acetoxidans* (c) and strain *TRM1* (d). The open symbols give the respective sulfate concentrations in percent of initial. Error bars depict the standard deviation of two or three replicates.

2.3.3 Calculations

In order to reveal the relationship between the apparent sulfur isotope enrichment factor and the $\delta^{18}O$ values of residual sulfate, the the isotope enrichment factor of sulfur (ε_s) was calculated for the investigated sulfate reducers using Equations 2.1 and 2.2 and by plotting $\ln(C_t/C_0)$ over $\ln(R_t/R_0)$. The slope of the linear regression is equal to (α-1), which was converted to the enrichment factor ε as described in Equation 2.3. The results are summarized in figure 2.4 and table 2.2. Due to limited sample amounts for isotope measurements, we did not get full triplicate data sets for all sampling points to calculate the standard deviation for the sulfur isotope enrichment factors ε_S. Therefore, the ε_S for each strain were calculated from the mean $\delta^{34}S$ values obtained from each of the experiments with ^{18}O-depleted and enriched water.

Strain *Desulfovibrio desulfuricans* (Figure 2.4a) yield enrichment factors for sulfur isotopes between - 13.2 ‰ and - 6.7 ‰ in the batch experiments with ^{18}O-depleted and enriched water, respectively. *Desulfonatronovibrio hydrogenovorans* (Figure 2.4b) revealed sulfur isotope fractionation in the ^{18}O-enriched water of - 4.7 ‰ and in the ^{18}O-depleted water of - 5 ‰. *Desulfobacca acetoxidans* (Figure 2.4c) and strain *TRM1* (Figure 2.4d) showed significant higher sulfur enrichment factors in both waters. For strain *Desulfobacca acetoxidans* an enrichment factor for sulfur isotopes of - 25.9 ‰ was calculated from the experiments with ^{18}O-depleted water. The experiments with ^{18}O-enriched water yield a sulfur isotope enrichment factor of - 22.7 ‰. Strain *TRM1* revealed sulfur fractionations between - 38.7 ‰ (^{18}O-depleted water) and - 40.6 ‰ (^{18}O-enriched water).

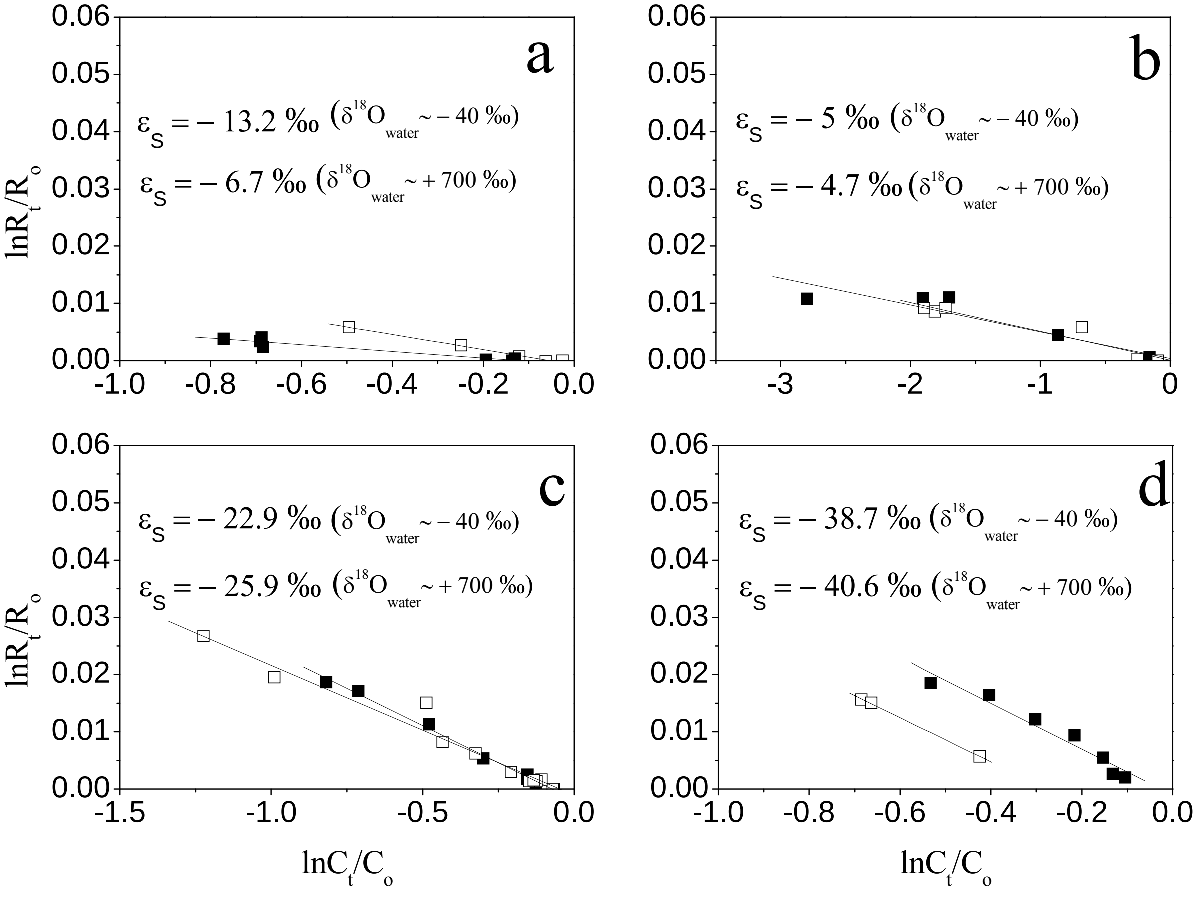

Figure 2.4: Calculation of the total sulfur isotope enrichment factors ε_S from the experiments with ^{18}O-depleted water (open squares) and ^{18}O-enriched water (closed squares) respectively for each bacterial strain: *D. desulfuricans* (a), *D. hydrogenovorans* , (b) *D. acetoxidans* (c) and *TRM1* (d).

Table 2.2 contains the average enrichment factors for all strains and experiments.Since the average sulfur isotope enrichment factor of *Desulfovibrio desulfuricans* (Figure 2.4a) and *Desulfonatronovibrio hydrogenovorans* (Figure 2.4b) was more than 14 ‰ lower compared to those of *Desulfobacca acetoxidans* (Figure 2.4c) and *TRM1* (Figure 2.4d), the data set can be clustered into two groups. On the one hand, the strains *Desulfovibrio desulfuricans* and *Desulfonatronovibrio hydrogenovorans* represent group “A”, showing sulfur isotope enrichment factors $\varepsilon_S \geq$ - 13.2 ‰ while on the other hand group “B” (*Desulfobacca acetoxidans, TRM1*) yield isotope enrichment factors more negative than - 24.3 ‰. The calculated values of the sulfate reduction rates ranged from 1.4 to zero and the respective enrichments factors for sulfur of each respective time step varied between + 3.2 to - 5.2 ‰ (Fig. 2.5).

Table 2.2: Average sulfur isotope enrichment factors (ε_s) of the respective organisms and the maximum $\delta^{18}O$ values of the experiments with $\delta^{18}O$-enriched water.

Microorganism	**ε (average)**	**$\delta^{18}O_{max}$**	
Desulfovibrio desulfuricans	-9.9 ‰	47.2 ‰	**Group A**
Desulfonatronovibrio hydrogenovorans	-4.8 ‰	66.2 ‰	
Desulfobacca acetoxidans	-24.3 ‰	654.7 ‰	**Group B**
TRM1	-39.6 ‰	585.4 ‰	

Based on the results of the oxygen and sulfur isotope data, the microorganisms can be clustered into two groups A and B.

For group "A", characterized by low sulfur isotope fractionation, the $\delta^{18}O$ of residual sulfate increased in the experiments with ^{18}O-enriched water only from 12 ‰ to around 50 ‰. In contrast, group "B" showed a nearly complete exchange between $\delta^{18}O$ of water and sulfate, as expressed by absolute isotope values of nearly + 700 ‰ in the $\delta^{18}O$ of the residual sulfate at the end of the reaction (see Figure 2.2). The sulfur isotope enrichment factors from the experiments were in close agreement with published data. The data set from *Desulfovibrio desulfuricans* coincides with experiments performed by Kaplan and Rittenberg (1963) and Thode et al. (1951), where the same strain reached sulfur fractionations in sulfate between -10 and - 12 ‰. Enrichment factors obtained for *Desulfonatronovibrio hydrogenovorans* varied only a little between - 4.7 ‰ and - 5 ‰. This pure culture showed similar enrichment factors as previously reported by Detmers et al. (2001) who denoted an average enrichment factor of - 5.5 ‰.

Our results for strain *Desulfobacca acetoxidans* showed considerably more negative sulfur isotope enrichment factors than reported in a previous study by Detmers et al. (2001) who found a maximum ε_S value of - 18 ‰. However, their experimental conditions differed slightly from the present experiments, by using different media and higher incubation temperatures (37°C).

High sulfur isotope fractionations for sulfate reducers using toluene as electron donor, such as strain *TRM1*, were also observed by Bolliger et al. (2001) for a hydrocarbon-contaminated groundwater system and Knöller et al. (2006) in microcosm experiments.

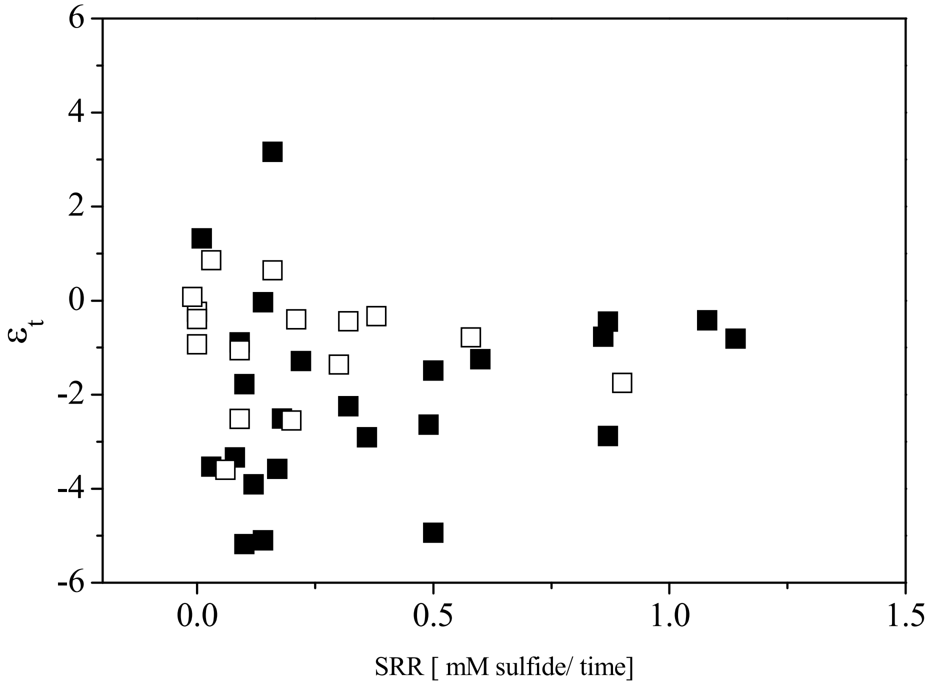

Figure 2.5: Relationship between the sulfate reduction rates for each sampling point of all batch cultures from Figure 2 (sulfide production per time) and the respective sulfur isotope enrichment factor for each time step of the experiments. Group A (closed squares) containing *Desulfovibrio desulfuricans* and *Desulfonatronovibrio hydrogenovorans* and group B (open squares) including *Desulfobacca acetoxidans* and strain *TRM1*.

2.4 DISCUSSION

2.4.1 Oxygen Isotope Exchange

Different authors reported that oxygen isotope fractionation during sulfate reduction is controlled by kinetic fractionation (Aharon and Fu, 2000; Mandernack et al., 2003). If this was really the case a linear slope would be expected when the δ^{18}O values and the δ^{34}S values of sulfate during sulfate reduction would be plotted against each other. However, laboratory and field studies showed that with increasing δ^{34}S values the oxygen isotopes approached a constant value (Mitzutani and Rafter, 1973; Fritz et al., 1989; Böttcher et al., 1998; Böttcher et al., 2001; Aharon and Fu, 2003).

Brunner et al. (2005) and Fritz et al. (1989) found some evidence in experiments with sulfate reduction that oxygen isotope fractionation is controlled by isotope exchange with ambient water and therefore the $\delta^{18}O$ of the residual sulfate should approach a constant value. There are two possible processes described in literature to explain, how the water oxygen atoms could exchange with sulfuroxy-intermediates during sulfate reduction. On the one hand, an oxygen isotope exchange could happen between water and the sulfate-enzyme complex (APS) (Fritz et al., 1989) and react back to sulfate, and on the other hand, sulfite can definitely exchange oxygen with ambient water at a relatively fast rate (Lloyd, 1968) and this could also somehow react back to sulfate. An abiotic oxygen isotope exchange with sulfate can be neglected under the experimental conditions, as it is a slow process over geologic timescales (10^6 years) (Lloyd, 1968; Mitzutani and Rafter, 1973).

It has been suggested that sulfur intermediate compounds such as sulfite formed during the sulfate reduction pathway can exchange oxygen atoms with ambient water. To verify this hypothesis ^{18}O-enriched and depleted water was to used in experiments with sulfate-reducing strains in order to analyze an oxygen isotope exchange with sulfite and to see if a reoxidation of the sulfur intermediates such as sulfite could be demonstrated to a larger extent. The data set for the experiments with ^{18}O-enriched water clearly indicated that a microbial mediated oxygen isotope exchange occurred during sulfate reduction. The experiments with *Desulfobacca acetoxidans* and strain *TRM1* showed $\delta^{18}O$ values of the residual sulfate close to + 700 ‰ and equilibrium between the oxygen isotopes of water and sulfate was almost approached. These results are in line with the findings of Mizutani and Rafter (1973), Fritz et al. (1989), Brunner et al. (2005), and Knöller et al. (2006) that the isotope effect for the $\delta^{18}O$ of sulfate during bacterial sulfate reduction is affected by isotope exchange with water.

However, an oxygen isotope exchange between the intermediate APS as suggested by Fritz et al (1989) and ambient water could not be supported by the present results. No further oxygen isotope exchange could be observed in the stationary growth phase, where sulfate reduction comes to an end, due to complete substrate consumption. However, the enzyme APS reductase, which is catalyzing the reduction of APS to sulfite is still present.

In contrast, the results reflect the isotope exchange between the metabolic intermediates thiosulfate or sulfite and the reoxidation of thiosulfate or sulfite to sulfate during sulfate reduction. This findings are also supported by earlier studies, where Spencer et al. (1957), Cloves et al. (1977), and recently Bao and Kohl (2006) found no evidence of an isotope exchange between APS and ambient water.

The used organisms can be clustered into two groups. Group A shows low isotope exchange of sulfate with water and group B shows a high exchange in the experiments with ^{18}O-enriched water.

In the experiments with δ^{18}O-depleted water, the oxygen isotope values of residual sulfate with organisms of group "A" (Table 2.2) showed fairly constant δ^{18}O values. For group "B", (Table 2.2) with the highest δ^{18}O values in the experiments with enriched water, a trend of decreasing δ^{18}O values with the remaining sulfate was observed for strain *TRM1* in the experiments with depleted water. The theoretical isotope composition of residual sulfate (Equation 2.4) was calculated, based on the equation published by Böttcher and Thamdrup (2001):

$$\begin{aligned}\delta^{18}O_{sulfate} &= (3\times(\delta^{18}O_{water}+25‰)+1\times(\delta^{18}O_{water}+4‰))/4 \\ &= \delta^{18}O_{water}+19.8‰\end{aligned} \tag{2.4}$$

By adding one oxygen molecule from water ($\varepsilon = +4$ ‰ (Taylor et al., 1984)) with an δ^{18}O value of - 40 ‰ and assuming an oxygen isotope equilibrium value of sulfate with water of + 25 ‰ (Fritz et al., 1989), the isotope content in the newly formed sulfate should result in a value of about - 20 ‰. In contrast, the δ^{18}O values in the experiments with depleted water were around + 12 ‰ in the residual sulfate. This denotes that the reaction of oxygen exchange between the sulfuroxy-intermediate with depleted water is probably masked by increasing δ^{18}O values due to an additional kinetic isotope effect. Based on the principles that backward and forward fluxes control kinetic isotope effects, it is suggested that the backward reaction of the intermediate or a disproportionation is associated with stable isotope enrichment in the δ^{18}O and is a controlling factor for the observed δ^{18}O values of sulfate.

Since the δ^{18}O values of the remaining sulfate varied between the experiments with depleted and enriched water, an isotope exchange with ambient water seems, however, to be the dominant mechanism controlling oxygen isotope enrichment during BSR, but additionally a second isotope effect has to be present, influencing the total stable isotope enrichment of δ^{18}O in sulfate. Böttcher et al. (2001) and Brunner et al. (2005) discussed the influence of the isotope enrichment factor and the exchange with ambient water on the δ^{18}O value of residual sulfate. They concluded that their results can be explained by a direct incorporation of an oxygen atom from water and reaching the equilibrium value of around - 25 ‰. Therefore they regarded the oxygen isotope exchange with ambient water as the driving process for oxygen isotope enrichment. The experimental results indicated that higher δ^{18}O values in the residual sulfate are caused by oxygen isotope exchange between water and intermediates and a subsequent reoxidation. However, this reoxidation or disproportionation process as mentioned above is probably accompanied by an additional kinetic isotope fractionation effect influencing the stable isotope enrichment of oxygen and sulfur.

2.4.2 Sulfur stable Isotope Enrichment governed by Reoxidation of Intermediates

Many efforts have been made to understand the broad variation of sulfur isotope enrichment factors observed in lab and field studies. Different parameters such as temperature, carbon source, and sulfate supply were suggested to influence the bacterial isotope fractionation. The oxygen isotope enrichment during sulfate reduction depends mostly on the backward and forward fluxes of the intermediate. To support the hypothesis that the reoxidation of intermediates governs the isotope enrichment factor of sulfur during bacterial sulfate reduction the stable isotope data of oxygen in sulfate was used to elucidate the reduction pathway and to follow cell internal isotope fractionation steps.

This study also revealed results to emphasize a relation of isotope fractionation with the reaction rate. This relation is however only indirect, via the stability and the life-time of intermediate sulfur compounds such as sulfite or thiosulfate as speculated previously by Böttcher et al. (2001) in the content of the microbial disproportionation of elemental sulfur.The experiments with enriched water retrieved highly enriched $\delta^{18}O$ values in the residual sulfate. This clearly shows that intermediates are reoxidized during sulfate reduction, delivering recycled sulfate to the residual sulfate pool. Furthermore, a high degree of reoxidation of e.g. sulfite can only occur if the limiting step in the reduction pathway is after the production of sulfite (see Figure 2.1).

Then, a pronounced isotope effect is obtained because both the isotope fractionations of the enzymatic steps before APS reductase and after sulfite production (dissimilatory sulfite reductase) contribute to the overall isotope effect in the residual sulfate. If the enzymatic step after sulfite production is not rate limiting only the first step (APS-reductase) will contribute to the isotope effect that is measurable in sulfate and thus the isotope enrichment will be much smaller. The results with enriched water of increasing $\delta^{18}O$ values and more negative isotope enrichment factors of sulfur show that the reoxidation process may in turn strongly affect the stable isotope enrichment factors for sulfur (ε_S) that can be measured during sulfate reduction.

The model of Rees (1973) was used in this study and it is assumed that equilibrium fractionation processes between APS and sulfite and a kinetic isotope fractionation between sulfite and sulfide control stable isotope enrichment during the sulfate reduction pathway. Rees (1973) suggested that if there is no reoxidation of sulfite to sulfate during the reduction pathway, this may result in sulfur isotope enrichment factors more positive than - 22 ‰. In contrast, a sulfur isotope enrichment factor more negative than - 22 ‰ would be indicative of a high recycling of sulfite to sulfate. Therefore, sulfur enrichment factors lower than - 22 ‰ may indicate that step 4 of the model (figure 2.1) was rate limiting and irreversible. As already mentioned above, the organisms used in the experiments were divided in two groups A and B.

Sulfur isotope enrichment factors more negative than - 22 ‰ were observed in group "B". Furthermore, the $\delta^{18}O$ values of group "B" in the remaining sulfate approached those of the ambient water and therefore indicate a strong influence of sulfite-derived sulfate in the remaining fraction.

On the other hand, group "A" showed less negative isotope enrichment factors for sulfur as well as low $\delta^{18}O$ values of residual sulfate indicating little reoxidation. One possible explanation for the different reoxidation behaviour of intermediates during bacterial sulfate reduction might be the location of the limiting step in the pathway. The reaction pathway was simplified according to Rees (1963) however, we are aware that the rate constant k is the product of the maximum velocity and the sulfate concentration in relation to the sum of the sulfate concentration and the Michaelis constant. It is assumed that the reduction of sulfite to sulfide (Figure 2.1) is fast in some organisms ($k_2 > k_{-2}$) and slower in others ($k_2 < k_{-2}$). Furthermore, it is presumed that sulfite undergoes an oxygen isotope exchange with ambient water with a constant first order rate constant (k_3). Then two possible scenarios can be differentiated, generating the high and low $\delta^{18}O$ values for both groups A and B:

For group A organisms, exchange of sulfate oxygen and ambient water occurs with the rate constant k_3 and the backward reaction rate constant of sulfite to APS (k_{-1}) is small. Therefore, only a small amount of sulfite recycling can be observed in the remaining sulfate, as the intermediate will pass on to the next forward step (step 4, Figure 2.1), rather than to the backward step.

For group B organisms, the intermediate sulfite also exchanges its oxygen isotopes with ambient water with a constant rate k_3 but k_{-1} is fast. Only a small amount of sulfite will be passed to the next step, but a higher amount of sulfite derived will be recycled to sulfate. This findings lead to the hypothesis that reoxidation processes of sulfite to sulfate will only be observed to a larger extent if step 4 is slower ($k_2 < k_{-2}$), which may lead to apparent rate dependencies of fractionation. This would explain the high $\delta^{18}O$ values of residual sulfate for group "B" as well as the elevated enrichment factors for sulfur.

This reoxidation process seems to influence the stable isotope enrichment factor in a rate dependent manner. However, this effect is probably only indirect. The isotope fractionation itself is probably not rate dependent.

Habicht and Canfield (1997) presented experimental evidence that the fractionation factor of sulfur during bacterial sulfate reduction is governed by specific sulfate reduction rates. Therefore, they assumed that high fractionations are caused by a rate limitation of the reduction of sulfite to sulfide (step 4, Figure 2.1). If sulfate reduction is limited by the sulfate supply to the cell (step 1, Figure 2.1), low fractionations will occur. Many studies have been performed to demonstrate that the fractionation is controlled by the sulfate reduction rate (mass volume^{-1} time^{-1}) or the specific sulfate reduction rate (mass cell^{-1} time^{-1}) (Harrison and Thode, 1957; Jones and Starkey, 1957; Kaplan and Rittenberg, 1963). Habicht and Canfield (1997) stated that high fractionations (ε_S < - 22 ‰) can only be achieved with unlimited sulfate supply. Low fractionations (ε_S > - 22 ‰) are controlled by limiting sulfate concentrations and /or low specific sulfate reduction rates. Bolliger et al. (2001) and Detmers et al. (2001) suggested that neither the specific sulfate reduction rate nor the sulfate reduction rate appeared to control the enrichment factor of sulfur. The experiments were all conducted under non-limiting sulfate concentrations, but they also obtained enrichment factors more negative than - 22 ‰. In addition, no correlation between the sulfate reduction rates and the respective enrichment factors could be ascertained.

The bacterial strains used in our experiments showed also different properties in the complete or incomplete electron donor oxidation. Detmers et al. (2001) observed that sulfate reducers releasing acetate (e.g. *Desulfovibrio desulfuricans*) show generally lower fractionations (ε_S > - 18.7 ‰) compared to those oxidizing the carbon source completely to CO_2. The incomplete oxidizing strain *Desulfovibrio desulfuricans* indeed showed low fractionations. However, this was also found low ones with the complete oxidizing bacteria *Desulfonatronovibrio hydrogenovorans*. Compared to Detmers et al. (2001) correlation between the sulfur isotope fractionation of residual sulfate and the electron donor oxidation could not be confirmed with this experiments.

Following the experimental setup a correlation between the ratio of the rate constants and the stable isotope enrichment factors for oxygen and sulfur could be observed during bacterial sulfate reduction.

2.5 CONCLUSION

The results of this study reflect the reoxidation of metabolic intermediate sulfite to sulfate, which can vary between different bacterial species.

This reoxidation affects the isotopic composition of residual sulfate. The commonly used model of Rees (1973) describing the isotope fractionation processes during bacterial sulfate reduction can be confirmed with the provided data. It was also observed that there is no linear relationship between the increase of $\delta^{34}S$ values and $\delta^{18}O$ values of sulfate during sulfate reduction. Different slopes in $\delta^{18}O$ versus $\delta^{34}S$ plots observed in the experiments with enriched and depleted water favour an equilibrium isotope exchange with water as the dominant process influencing the oxygen isotope values.

It is also evaluated in the presented work, whether the observations in the batch experiments with depleted and enriched water are consistent with field data. Since fairly constant $\delta^{18}O$ values were observed in the experiments with depleted $\delta^{18}O$ values of water up to - 40 ‰ it is assumable that under environmental conditions ($\delta^{18}O$-groundwater: ~ - 10 ‰) the effect of isotope exchange between water and residual sulfate is close to the analytical uncertainty of this parameter. For bacterial sulfate reduction dominated by sulfate reducers with high fractionation factors and high backward fluxes (Group B), decreasing $\delta^{18}O$ values with decreasing sulfate concentrations and increasing $\delta^{34}S$ values are expected. Generally, in a closed system the *Rayleigh* equation can be used for the quantitative examination of in situ sulfate reduction.

The dual isotope approach in the performed experiments may allow an estimation of the enrichment factor of sulfur. It can be assumed that sulfate-reducing microorganisms characterized by sulfur isotope enrichment factors between - 25 ‰ and - 47 ‰ control bacterial sulfate reduction, if the exchange between water and an intermediate was the major process responsible for the trend of decreasing $\delta^{18}O$ values in the residual sulfate. Therefore, if the initial isotope value of sulfate in the system is known, the source concentration of sulfate can be estimated using an isotope enrichment factor of approximately - 30 ‰.

Since we rarely know exactly which organisms are performing the reactions in situ, it is probably not possible to give exact oxygen isotope enrichment factors for sulfate reduction in the environment. Nonlinear $\delta^{18}O$ values with increasing $\delta^{34}S$ values were already reported by Böttcher et al. (2001) and Fritz et al. (1989) in marine environments and a deep groundwater system. The observed trend of decreasing $\delta^{18}O$ values with increasing $\delta^{34}S$ values during bacterial sulfate reduction may be explained by incorporation of water oxygen in the intermediates of the sulfate reduction pathway controlled by sulfate reducers with high fractionation factors over geological time scales.

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3. EFFECTS OF NITRITE ON THE STABLE ISOTOPE FRACTIONATION DURING BACTERIAL SULFATE REDUCTION

3.1 INTRODUCTION

Bacterial sulfate reduction (BSR) represents a significant part of the sulfur cycle in marine and terrestrial habitats and can be identified by stable isotope analysis (Jörgensen and Bak, 1991; Böttcher et al., 1998; Aharon and Fu, 2003; Einsiedl and Mayer, 2005; Goldberg et al., 2005). Sulfate-reducing bacteria preferentially utilize the lighter ^{32}S isotope of sulfate. As a consequence, the residual sulfate gets enriched in the heavier ^{34}S isotope over time (Harrison and Thode, 1957; Kaplan and Rittenberg, 1963; Chambers and Trudinger, 1975). Sulfur isotope fractionation has been determined for more than 40 species of sulfate-reducing microorganisms (Janssen et al., 1996; Habicht et al., 1998; Bolliger et al., 2001; Detmers et al., 2001; Johnston et al., 2005). In the studies with pure cultures a large variety of sulfur isotope fractionation was observed with isotope enrichment factors (ε_S) ranging from 0 to - 47 ‰ (Kaplan and Rittenberg, 1963; Bolliger et al., 2001; Detmers et al., 2001). Some laboratory studies have been performed to explain the large variability and to identify the various parameters controlling the isotope fractionation of sulfur during BSR.

On the one hand, it was shown that sulfate concentrations below 1 mM cause low sulfur isotope fractionations (Harrison and Thode, 1957; Canfield, 2001), on the other hand Canfield (2001) reported that limiting substrate concentrations increase the isotope fractionation of sulfur during BSR. Moreover, Canfield et al. (2006) observed temperature dependent isotope fractionation effects in laboratory experiments. Contrary to this observation, Brüchert et al. (2001) suggested in investigations with strains capable of growth at all experimental temperatures that the latter has no effect on isotope fractionation. Kaplan and Rittenberg (1963) as well as Böttcher et al. (1999) presumed that isotope fractionation by sulfate-reducing bacteria varies as a function of the specific rate of sulfate reduction (mass $cell^{-1}$ $time^{-1}$). Furthermore, Bollinger et al. (2001) found a relationship between the pathway of the organic electron donor and the isotope fractionation during sulfate reduction. It was proposed that isotope enrichment factors of sulfur ≥ - 18 ‰ are characteristic for incomplete-oxidizing sulfate reducers, whereas sulfate-reducing microorganisms that oxidize the organic substrate completely to CO_2 (Rabus et al., 2000) show isotope enrichment factors of sulfur between - 18 and - 47 ‰ (Detmers et al., 2001).

Rees (1973) developed a model on the pathway of BSR with special focus on the sulfur isotope fractionation effect, which is commonly used in isotope studies on sulfate reduction

(Habicht and Canfield, 1997; Farquhar et al., 2003; Brunner and Bernasconi, 2005; Canfield et al., 2006). According to this model, BSR takes place in several reduction steps as shown in Figure 3.1. External sulfate is taken into the cell, where it is subsequently activated to adenosine-5`-phosphosulfate (APS). Thereupon, it is reduced to intermediate compounds such as sulfite, which in turn are reduced to the final product sulfide and afterwards excreted from the cell. The last reduction step of APS to sulfite requires the enzyme dissimilatory sulfite reductase (DSR). However, it still remains open whether the last step of the reduction pathway to sulfide takes place in an unidirectional single step reduction or if the reduction proceeds in bidirectional multisteps via the trithionate pathway (Rabus et al., 2000). According to this concept, Rees (1973) estimated maximum isotope enrichment factor for sulfur of approximately - 25 ‰ for the APS reductase, after subtracting the experimentally found maximum isotope fractionation of also - 25 ‰ for the DSR from the averaged maximum total isotope enrichment factor of approximately - 50 ‰ (Kaplan and Rittenberg, 1963).

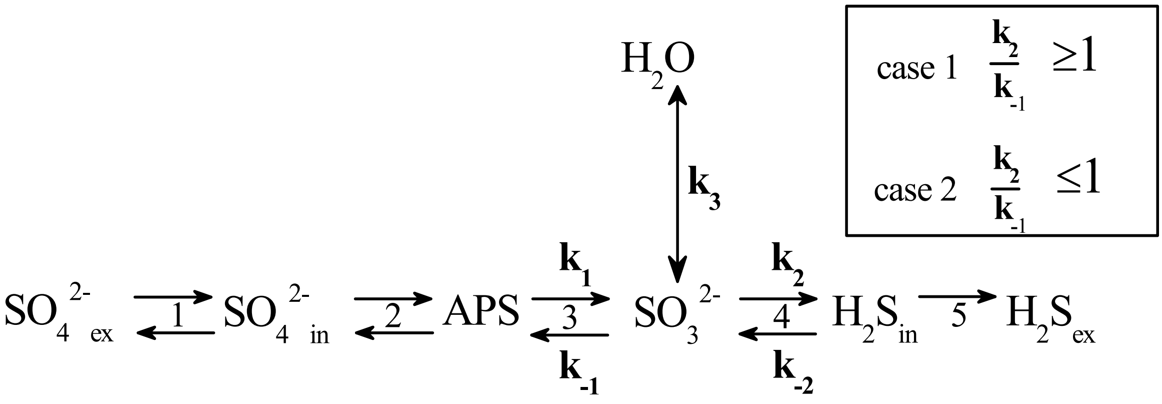

Figure 3.1: The pathway of bacterial (dissimilatory) sulfate reduction modified after Rees (1973). SO42-ex means external sulfate, which is transported into the cell (SO42-in) (step 1). Inside the cell internal sulfate reacts with ATP (adenosine-triphosphate) to form APS (adenosine-5`-phosphosulfate) (step 2), which thereafter is reduced directly to sulfite (step 3). The final step of the biochemical reaction is the reduction of sulfite to sulfide (step 4), which is afterwards excreted from the cell (H2Sex). The numbers denote the different reduction steps 1-5. The reaction rates are given as k1, k2, and k3.

It was suggested on the one hand that the isotope fractionation of the reduction of the high energy compound APS to sulfite, which is catalyzed by the enzyme APS reductase is controlled by kinetic fractionation (Rees, 1973; Brunner and Bernasconi, 2005; Canfield et al., 2006), whereas Mangalo et al. (2007) suggested an equilibrium fractionation for this step. On the other hand, the reduction of sulfite to sulfide is suggested to be accompanied by a kinetic fractionation (Rees, 1973; Mangalo et al., 2007). Mangalo et al. (2007) hypothesized that the DSR controls the residence time of the metabolic intermediate sulfite in the cell and therefore, the magnitude of the overall sulfur isotope fractionation during BSR.

To verify this hypothesis, batch experiments with *Desulfovibrio desulfuricans* were performed in the presence of different nitrite concentrations. Dissolved nitrite inhibits the production of DSR and influences the final reduction process of sulfite to sulfide (Greene et al., 2003; Greene et al., 2006). It was the aim of this study to test the hypothesis, if inhibition with nitrite would reveal the dependency of rate limitation as a regulator of isotope fractionation during BSR.

3.2. MATERIAL & METHODS

3.2.1 Growth Conditions

The pure culture *Desulfovibrio desulfuricans* (DSMZ 642) was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ).

The strain was grown in a bicarbonate-buffered (30 mM) basal freshwater mineral medium with lactate as a carbon source under strictly anaerobic conditions as described by Widdel and Bak (1991). The medium was reduced with 1 mM sulfide. Lactate and sulfate were added from 1 M sterile stock solutions to final concentrations of 20 mM.

Six batch experiments, each consisting of two parallels (n = 2) were inoculated with strain *Desulfovibrio desulfuricans* and one control experiment without microorganism (1:10 inoculum) into half-filled 100 ml serum bottles. The headspace was completely replaced by a N_2:CO_2 gas mixture (80:20) [vol/vol]. An anoxic 1 mM nitrite solution was freshly prepared and added to respective concentrations between 100 and 1000 µM. Also ^{18}O-enriched water (^{18}O content: 10 %; Hyox; Rotem GmbH) was added to a final δ^{18}O value of approximately + 700 ‰ to selected serum bottles.

Substrate utilization was determined by analysis of accumulating sulfide as described by Cline (1969). All experiments were conducted at 30°C in the dark.

3.2.2 Isotope Analysis

For sulfur and oxygen isotope analyses, microbially produced sulfide was removed from a sample aliquot (between 2 and 10 ml) with up to 5 ml of a 20 % Zn-acetate solution in order to prevent reoxidation of sulfide to sulfate. After ZnS precipitation, the samples were filtered through 0.45 µm pore size Millipore syringe filters.

All samples were acidified to pH < 4 to eliminate HCO_3^- and sulfate was afterwards precipitated as $BaSO_4$ with 3 to 5 ml of a 10 % $BaCl_2$ solution. The precipitate was recovered by centrifugation, carefully washed, and dried prior to isotope analysis, which was performed by isotope ratio mass spectrometry (IRMS Thermo Electron MAT 253) after complete conversion of $BaSO_4$ to SO_2 via high temperature combustion (1000ºC) with WO_2 and V_2O_5 in an elemental analyzer (EA- EuroVector). For the measurements of $\delta^{18}O$ from sulfate, CO was produced through pyrolysis of $BaSO_4$ at 1450°C in a discharge chamber and subsequent isotope analysis was conducted by IRMS.

Sulfur and oxygen isotope ratios are reported in parts per thousands (per mil) using the conventional delta-notation (δ) (Equation 3.1):

$$\delta R = \left(\frac{R_{sample}}{R_{\mathrm{standard}}} - 1 \right) \times 1000 \quad [‰] \tag{3.1}$$

R denotes the ratio of the absolute isotope value of the heavy over the light isotope. R_{sample} and R_{standard} are the isotope ratios of the sample and the standard. $\delta^{34}S$ values are reported relative to the Canon Diablo Troilite standard (V-CDT) and $\delta^{18}O$ refers to Vienna Standard Mean Ocean Water (V-SMOW). An international standard (NBS 127) and several internal standards were used to ensure accurate measurements. The standard deviation of all measurements was ± 0.2 ‰ for $\delta^{34}S$ and ± 0.4 ‰ for $\delta^{18}O$. Each sample was measured twice.

The Rayleigh equation was used to assess stable isotope fractionation for sulfur (Equation 3.2).

$$R_t / R_0 = (C_t / C_0)^{(\alpha - 1)} \tag{3.2}$$

R_t and R_0 denote the stable isotope ratios of sulfate at times t and zero. C_t and C_0 represent the respective concentration of the residual sulfate. The stable isotope fractionation factor α was converted to the isotope enrichment factor (ε) according to Equation 3.3:

$$\varepsilon = (\alpha - 1) \times 1000 \quad [‰] \tag{3.3}$$

3.3 RESULTS & DISCUSSION

Isotope fractionation of sulfur and oxygen during BSR has been studied in many field and laboratory experiments, however, rarely in relation to enzymatic reactions. Incubation experiments with the sulfate-reducing strain *Desulfovibrio desulfuricans* were performed to clarify if physiological and ecological aspects influence the extent of the stable isotope fractionation of sulfur during BSR. Dissolved nitrite, for example produced by nitrate reducing microorganisms, inhibits the production of dissimilatory sulfite reductase (Reinsel et al., 1996; Casey et al., 2005; Greene et al., 2006) and consequently represses sulfate reduction. Some sulfate reducing microorganism avert this effect by reducing nitrite further to ammonia via nitrite reductase and are therefore able to remove nitrite from their environment and afterwards continue sulfate reduction (Greene et al., 2003). Krekeler and Cypionka (1995) as well as Pereira et al. (1996) documented in laboratory enzymatic studies that *Desulfovibrio desulfuricans* possesses the enzyme nitrite reductase, which catalyzes the reduction of nitrite to ammonia. Greene et al. (2003) observed in laboratory studies with *Desulfovibrio* sp. strains that nitrite reduction occurs concomitant with sulfate reduction and is only completely inhibited with higher nitrite concentrations (> 5 mM nitrite).

The inhibition of bacterial sulfate reduction as well as the switching to nitrite reduction may have also an isotope effect on the residual sulfate. Therefore, this bacterium represents a suitable strain to test the hypothesis that the enzymatic activity of DSR acts as regulator for the stable isotope fractionation during BSR.

3.3.1 Growth in Batch Experiments

Substrate utilization by *Desulfovibrio desulfuricans* was indicated by sulfide production from 1 mM to 8.5 ± 0.18 mM sulfide within 191 hours. This corresponds to an averaged sulfate consumption of approximately 37 % of the initially supplied sulfate (Figure 3.2a, b). With increasing nitrite concentration (Figure 3.2c, d) an inhibition of the DSR enzyme activity was observed as indicated by lower sulfide production during BSR compared to the experiments without nitrite. In the batch experiments containing 500 and 1000 µM nitrite, sulfide concentrations below 5 mM (Figure 3.2e, f) were observed within 381 hours and 953 hours, which correlates to a sulfate consumption of around 13 and 20 %, respectively could be observed. Based on a simple electron balance calculation of available lactate and produced sulfide, sulfate reduction did not go to completion in this batch experiment even after 953 hours.

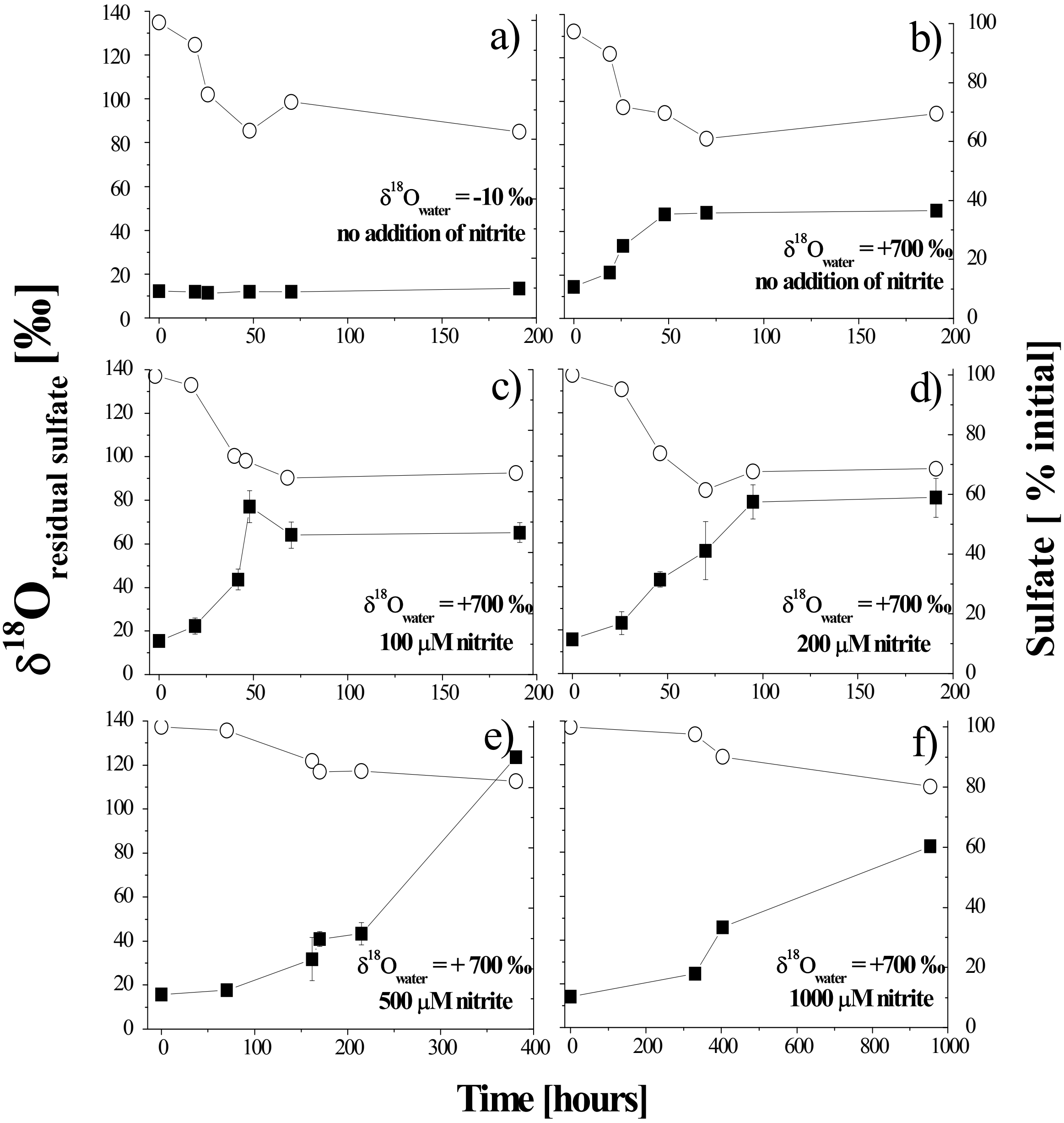

Figure 3.2: Oxygen isotope fractionation during sulfate reduction in batch cultures. $\delta^{18}O$ values of residual sulfate (filled squares) obtained from the batch experiments. Error bars indicate the standard deviation of parallel growth experiments (n = 2). The open circles denote the sulfate consumption in %.

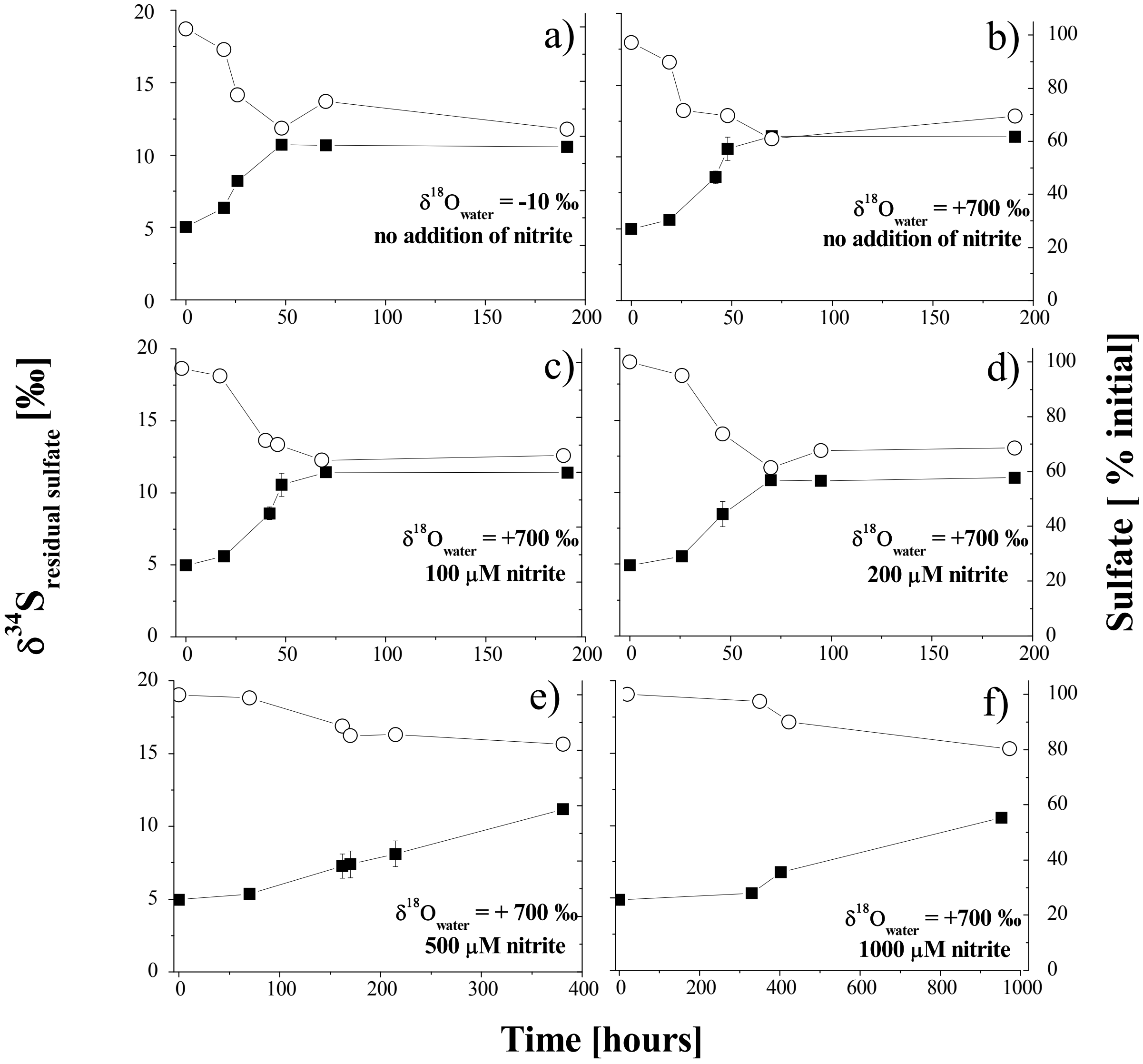

Figure 3.3: Sulfur isotope fractionation during sulfate reduction in batch cultures. δ34S values of residual sulfate (filled squares) obtained from the batch experiments. Error bars indicate the standard deviation of parallel growth experiments (n = 2). The open circles denote the sulfate consumption in %.

Also the duration of the stationary phase was affected by nitrite. With nitrite concentrations of 100 and 200 μM, the stationary phase of growth was reached after approximately 60 hours (Figure 3.2c, d). A more pronounced inhibitory effect was observed when nitrite concentrations of 500 and 1000 μM were added to the media. The stationary phase was reached after 170 hours in the experiments with 500 μM nitrite and the experiments with the highest nitrite concentrations (1000 μM) did not reach the lag phase within 900 hours (Figure 3.2e, f).

The experimental results indicate that the observed delay of sulfide production in *Desulfovibrio desulfuricans* with increasing nitrite concentrations is caused by the inhibition of sulfate reduction by nitrite and the temporary switching of the organisms to nitrite reduction for respiration. This effect has also been observed by Greene et al. (2003) for *Desulfovibrio* sp. grown with nitrite concentrations between 0.25 and 10 mM.

3.3.2 The Control of Nitrite on Stable Isotope fractionation by Desulfovibrio desulfuricans on Residual Sulfate

Our isotope results will be interpreted in the light of the sulfate reduction pathway reported by Rees (1973), Canfield et al. (2006), Farquhar et al. (2003), and Mangalo et al. (2007) and is summarized in Figure 3.1. The concept describes that the faster the reduction of sulfuroxy-intermediates (e.g. SO_3^{2-}) to sulfide takes place in the cell, the less sulfuroxy-intermediates are accumulated in the cell and consequently, the amount of backward-fluxes of the metabolic intermediates decreases. Small backward fluxes would cause only moderate changes in the isotopic composition of both, the $\delta^{34}S$ and $\delta^{18}O$ in the remaining sulfate and isotope fractionation factors of sulfur more positive than - 20 ‰ as well as little ^{18}O exchange of sulfate with water are observed (Habicht and Canfield, 1997; Mangalo et al., 2007). In contrast, the slower the cell internal sulfur oxides are reduced by DSR, the more sulfuroxy-intermediates “recycling” takes place back to sulfate (case 2). It was suggested that this re-oxidation process strongly affects the stable isotope fractionation factor for sulfur and concomitantly, the exchange of ^{18}O in oxygen of the remaining sulfate with that of ambient water.

In the experiments with $\delta^{18}O_{water}$ ~ - 10 ‰ and without nitrite, the $\delta^{18}O$ values of residual sulfate did not significantly change from 12.2 ± 0.2 ‰ to 13.4 ± 0.2 ‰ (Figure 3.2a). Furthermore, in the experiments with oxygen isotope enriched water of + 700 ‰ and also with no nitrite addition, only little backward fluxes of intermediates to sulfate were observed, as indicated by increasing oxygen isotope values from 14.4 ± 0.1 ‰ to 49.6 ± 1.8 ‰ (Figure 3.2b). It is suggested that this effect is due to the oxygen isotope exchange of a sulfuroxy-intermediate compound (e.g. sulfite) and ambient water and a re-oxidation of sulfite back towards sulfate (Mitzutani and Rafter, 1973; Aharon and Fu, 2000; Mandernack et al., 2003; Brunner et al., 2005) .

The experiments with ^{18}O-enriched water and lower nitrite concentrations showed increasing $\delta^{18}O$ values in the residual sulfate from 15.2 ± 0.1 to 68.2 ± 8.8 ‰ with 100 µM supplied nitrite and 16.0 ± 0.7 ‰ to 77.8 ± 13.3 ‰ with 200 µM nitrite concentration (Figure 3.2c, d). The $\delta^{18}O$ values of residual sulfate from the experiments with 500 µM final nitrite concentration increased from 15.7 ± 0.5 to 123.5 ± 1.4 ‰ (Figure 3.2e). When nitrite was added to a final concentration of 1000 µM, the oxygen isotopes of residual sulfate increased from 14.1 ‰ ± 0.4 to 82.9 ‰ ± 2.5 (Figure 3.2f). However, in this experiment the stationary phase of growth was not yet reached even after 900 hours and therefore it is suggested that the oxygen isotope exchange was not completed. Based on the results shown in Figure 3.2 it can be presumed that the reduction of sulfite to sulfide was faster in the experiments without addition of nitrite consistent with the hypothesis that nitrite specifically inhibits DSR. This would correspond to case 1 where a small extent of re-oxidation of sulfuroxy-intermediates to sulfate is expected, because they are readily further reduced to sulfide. With increasing nitrite concentrations also the $\delta^{18}O$ values in the residual sulfate increase and the suggestion arises that the reduction of sulfite to sulfide by the enzyme DSR was more and more inhibited and the internal cycling of sulfite back to sulfate was promoted, because the internal sulfite pool increased (case 2). The increase of the $\delta^{18}O$ values in the residual sulfate is also in line with the hypothesis mentioned above that the residence time of the internally formed intermediate is controlled by the activity of the DSR, as this residence time governs the isotope exchange between water oxygen and sulfite formed during the sulfate reduction pathway.

To support this hypothesis that the re-oxidation processes of sulfite is correlated with the stable isotope fractionation for sulfur measured during sulfate reduction (Figure 3.3) the isotope enrichment factors for sulfur (ε_S) were calculated as described in equation 3.3. In the absence of nitrite, sulfur isotope enrichment, with factors ε_S of around - 12.1 and - 12.4 ‰ could be observed (Figure 3.3a, b, 4a, b). This is in line with previous experiments with strain *Desulfovibrio desulfuricans*. Thode et al. (1951) as well as Kaplan and Rittenberg (1963) also found isotope enrichment factors for sulfur for *Desulfovibrio desulfuricans* of approximately - 12 ‰.

Slightly increasing sulfur isotope enrichment factors of - 14.7 and - 13.9 ‰ were observed in the experiments with 100 µM and 200 µM added nitrite (Figure 3.3c, d; 3.4c, d), respectively, consistent with moderate increase in the extent of ^{18}O exchange (Figure 3.2). Most importantly a doubling of the enrichment factors of sulfur was found in the experiments with 500 and 1000 µM nitrite between - 24.0 and - 25.6 ‰, respectively (Figure 3.3e, f; 3.4e, f). Overall, a difference of around -13 ‰ with the isotope enrichment factors of sulfur for the experiments without addition of nitrite (ε_S = - 12 ‰) and the experiments with high nitrite concentrations of up to 1000 µM (ε_S = - 25 ‰) could be observed in the batch experiments.

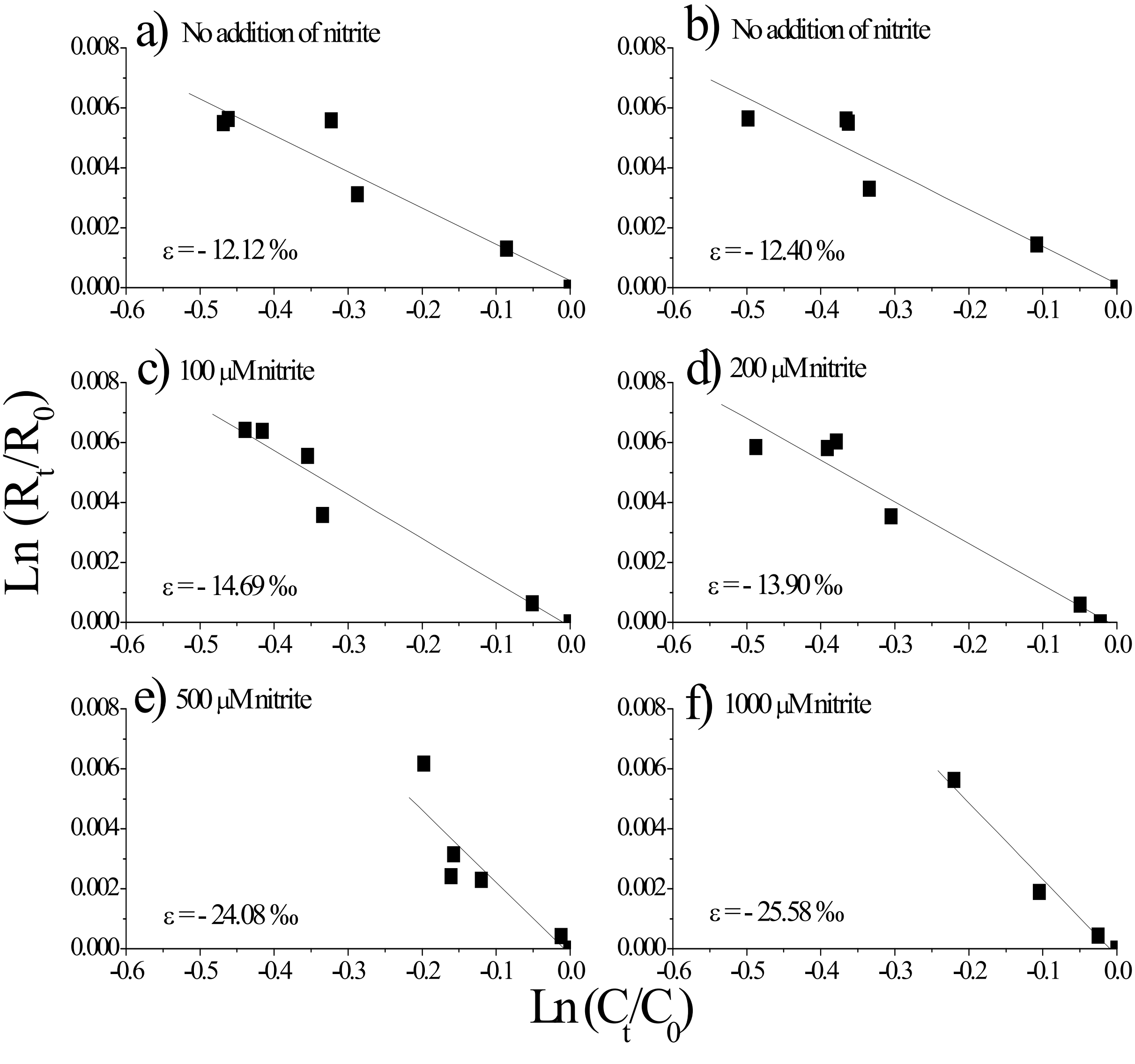

Figure 3.4: Variation of the sulfur isotope enrichment factors (ε_s) calculated by Equation 3.3 from the isotope fractionation factor α from the batch experiments. a) and b) without nitrite addition, c) with 100 µM and d) 200 µM added nitrite and e) & f) with highest nitrite concentrations of 500 to 1000 µM, respectively.

Now the question arises, which factors are regulating the isotope enrichment of sulfur during BSR. The Rees model (Figure 3.1) suggests that the sulfur isotope fractionation consists of the inverse isotope enrichment from sulfate uptake into the cell (step 1) (max. 3 ‰), the equilibrium fractionation between APS and sulfite (step 3) and an additional kinetic isotope fractionation effect between sulfite and sulfide (step 4), both of which are accompanied by enrichments with isotope enrichment factors (ε_S) of up to - 25 ‰. Recently, it was speculated that isotope fractionation during the reduction of sulfite to sulfide can actually shift the total sulfur isotope fractionation towards - 53 ‰ (Brunner and Bernasconi, 2005).

The total isotope enrichment of sulfur during BSR is therefore strongly dependent on how prominent each fractionation comes into play during sulfate reduction. Without addition of nitrite, *Desulfovibrio desulfuricans* showed low sulfur isotope enrichment factors, which indicates a fast reduction of sulfite to sulfide. Hence, in a case where the rate constant k_2 is relatively fast compared to k_{-1} the backward flux of the intermediate is low (Figure 3.1). This means that the reduction of APS to sulfite (step 3, Figure 3.1) can be expected to regulate the totally observed isotope effect for sulfur.

In contrast, with the addition of nitrite conditions were promoted under which the rate constant k_2 decreased in comparison to k_{-1}, which resulted in an increase of the backward flux. In this case, the reaction rate of DSR and its accompanying isotope effect is governing the isotope composition of residual sulfate, because it is becoming the rate limiting step.

Northrop et al. (1981) developed the conceptual framework that changes in enzymatic activity of a microorganism can cause variations in the expression of apparent isotopic effects. Brown and Drury (1967) observed during denitrification processes much higher enrichments for the $\delta^{15}N$ in the presence of e.g. silver ions (Ag(I)). Other studies demonstrated that temperature, pH, ionic activity, and the presence of trace metals can affect stable isotope fractionation in enzymatic reactions (Kaplan and Rittenberg, 1963; Habicht and Canfield, 1997; Brüchert et al., 2001; Canfield, 2001; Canfield et al., 2006; Mancini et al., 2006).

This study could demonstrate that nitrite has a strong effect on the $\delta^{34}S$ and $\delta^{18}O$ values of sulfate formed by the sulfate-reducing strain *Desulfovibrio desulfuricans* during bacterial sulfate reduction.

Nitrite is formed as an intermediate during bacterial nitrate reduction in groundwater systems (Bates and Spalding, 1998) and also accumulates at the bottom of the ocean's euphotic zone from oxidation of ammonium (Ward, 1987). It was also reported from marine systems that nitrite concentrations above 13 µM were found when nitrate concentrations in these waters were only between 10 to 30 µM (Granger et al., 2006).

The increase of the sulfur enrichment and a more pronounced isotopic exchange between water oxygen and the intermediates of the sulfate reduction pathway at high nitrite concentrations suggest that ecological aspects may have important implications for the regulation of sulfur isotope fractionation.

3.4 CONCLUSION

Our study shows that stable isotope fractionation during BSR is regulated by the kinetic conversions in the sulfate-reducing pathway, which can be influenced by different environmental parameters such as elevated nitrite concentrations.

The inhibition of the dissimilatory sulfite reductase step (DSR) generated by the addition of nitrite caused a change in the isotope fractionation ability of a sulfate-reducing microorganism and shifted the sulfur isotope enrichment factor towards more negative values.

A fundamental conclusion of the presented work is therefore that environmental factors eventually may also govern the kinetic fractionation of BSR and consequently influence the isotope effects of sulfur and oxygen of residual sulfate.

Nitrite, deriving from processes such as denitrification or ammonium oxidation seems to represent one environmental factor for the magnitude of the sulfur isotope fractionation. High nitrite concentrations can be expected in micro niches, where sulfate reduction has occurred next to denitrification. A constant input of nitrite may shift nitrite concentrations in micro niches to significantly higher levels compared to those found in marine environments.

However, other factors, which have been already described in literature such as temperature, substrate, or pH might also affect stable isotope fractionation during BSR in this way.

It was demonstrated in a defined case model how changes in the enzymatic activity affect the magnitude of isotope fractionation, although it should chemically not have any influence. Nevertheless, the experiments failed to promote isotope fractionation in *Desulfovibrio desulfuricans* to the maximum isotope enrichments (ε_S = - 47 ‰), which have been found for bacterial sulfate reduction. The explanation for this could be that even the present experimental conditions were not able to influence the kinetics in a way that the maximum isotope fractionations were expressed.

The other option might be that some microorganisms possess DSR, which produces higher isotope effects than the DSR of *Desulfovibrio desulfuricans*.

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4. STABLE ISOTOPE FRACTIONATION DURING DENITRIFICATION BY STRAIN *THAUERA AROMATICA*

4.1 INTRODUCTION

Due to extensive agricultural use nitrate concentrations in groundwater increased during the last 40 years (Casey, 1992; Jones and Smart, 2005; Flipo et al., 2007). The stable isotopes of dissolved nitrate ($^{15}N/^{14}N$, $^{18}O/^{16}O$) have been used as a powerful indicator to identify the sources and processes affecting nitrate concentrations in soils and groundwater systems (Delwiche and Steyn, 1970; Amberger and Schmidt, 1987; Böttcher et al., 1990; Aravena et al., 1993; Lehmann et al., 2003; Einsiedl and Mayer, 2006). Stable isotope fractionation of nitrogen and oxygen in nitrate can give qualitative evidence for denitrification processes as well as quantification of denitrification *in situ*.

However, for such a quantitative approach the nitrogen isotope enrichment factor ε_N is substantial to calculate the *in situ* degradation of nitrate for different environmental conditions, as shown for many organic compounds (Aravena *et al.*, 1993). So far, isotope enrichment factors for nitrogen (ε_N) during denitrification were determined in laboratory and field studies and ranged between 0 and - 36 ‰ (Delwiche and Steyn, 1970; Cline and Kaplan, 1975; Mariotti et al., 1981; Mariotti et al., 1988; Böttcher et al., 1990; Brandes and Devol, 1997; Barford et al., 1999; Lehmann et al., 2003; Chen and MacQuarrie, 2005), whereas oxygen isotope enrichment factors (ε_O) of nitrate have been observed between - 8 and - 18.3 ‰ (Böttcher et al., 1990; Mengis et al., 1999; Lehmann et al., 2003). It is proposed that the $\delta^{15}N/\ \delta^{18}O$ ratio during denitrification is close to 2 : 1 (Böttcher et al., 1990; Lehmann et al., 2003; Chen and MacQuarrie, 2005). However, recent studies found nitrogen isotope enrichment factors of up to 3 : 1 (Cey et al., 1999; Mengis et al., 1999; Fukada et al., 2003; Lehmann et al., 2003; McMahon et al., 2004).

Brunner *et al.* (2005) speculated that one reason for the variation of the $\delta^{15}N/\delta^{18}O$ ratio during denitrification might be due to an oxygen isotope exchange between metabolic intermediate compounds such as nitrite and ambient water similar to that observed for sulfate reduction (Mangalo *et al.*, 2007). Furthermore, it has also been suggested that stable isotope fractionation of nitrogen during denitrification is rate dependent with the largest isotope enrichments for nitrogen obtained with lowest reduction rates (Mariotti et al., 1981; Mariotti et al., 1988; Lehmann et al., 2003). Also the concentration of the carbon source and temperature were speculated to have an effect on isotope fractionation during denitrification (Bates and Spalding, 1998).

As denitrification occurs in several reduction steps, it requires an array of enzymes to catalyze the individual reactions. The first step is the reduction of nitrate to nitrite, which is then further reduced to nitric oxide. Ye *et al.* (1991) observed in laboratory studies an oxygen isotope exchange of ambient water with nitrite formed as an intermediate compound during the nitrate reduction pathway. Therefore, an isotopic exchange between ambient water and the metabolic intermediate nitrite and a subsequent reoxidation to nitrate during denitrification is hypothesized to explain the variation of oxygen isotope enrichment factors during dentrification similar to sulfate reduction (Mangalo *et al.*, 2007). Experimental studies using both isotopes of nitrate, $\delta^{15}N$ and $\delta^{18}O$, are rare and the number of field studies on stable isotope fractionation during denitrification is definitely larger than laboratory studies on pure cultures.

Laboratory experiments with the pure culture *Thauera aromatica* are reported in this study in which the nitrogen isotope enrichment factors ε_N were determined for growth on two different carbon sources, acetate and toluene. Additionally, the oxygen isotope effect for denitrification during growth on toluene was monitored. Moreover, this study links for the first time a hypothetic reoxidation of the metabolic intermediate nitrite to nitrate directly to an investigation of isotope exchange of ^{18}O in nitrate and the isotope enrichment factor of nitrogen. To follow this goal batch experiments with ^{18}O-enriched water (~ + 700 ‰) were performed to identify an oxygen isotope exchange and respectively the reoxidation processes of nitrite to a larger extent.

4.2 MATERIAL & METHODS

4.2.1 Growth Conditions

The denitrifying microorganism *Thauera aromatica* was grown anaerobically in a carbonate buffered medium after Widdel and Bak (1991) in half-filled 120 ml serum bottles with butyl rubber stoppers. The headspace was completely replaced by a N_2 - CO_2 gas mixture (80 : 20) [vol/vol]. The salt concentration in the medium was ten times diluted to reduce the ionic strength for chemical analysis. A 0.5 M anaerobe ascorbate solution was used as a reducing agent and the pH was adjusted between 7.0 and 7.2. A set of four batch experiments (n = 4) was performed and inoculated either with 3 mM acetate or with 1 mM toluene as final concentration of the carbon source. Dissolved nitrate was added to each experiment to a final concentration of 10 mM from a 1 M sterile stock solution. To follow the oxygen isotope effects in the residual nitrate during denitrification the water of selected experiments was labeled with ^{18}O-enriched water (^{18}O content: 10 %; Hyox; Rotem GmbH, Leipzig) to an approximately final $\delta^{18}O$ value of + 700 ‰.

Since a relatively large amount of sample volume is required for isotope measurements, each experiment consisted of a set of 7 to 8 single bottles, where each bottle was sacrificed for one sampling point. All experiments were conducted at 30 °C in the dark.

Growth was assessed by measuring an increase in the optical density at a wavelength of 380 nm using a spectrophotometer (Cary UV/ViS, Varian, Palo Alto) and nitrate concentrations were determined in the remaining solution by ion chromatography (Dionex DX 100, Dionex GmbH, Idstein).

4.2.2 Sampling Procedure

For nitrogen and oxygen isotope analysis cell growth was inhibited by addition of one drop of $CHCl_3$. Thereupon, accumulating nitrite, produced during denitrification, was removed by a modified method after Granger *et al.* (2006), preventing a reoxidation of nitrite back to nitrate during sampling: The samples were acidified to a pH ~ 3.5 with a 1 M ascorbic acid stock solution to reduce nitrite to nitric oxide gas (NO) and degassed with helium over night without any direct air contact to avoid reaction of NO with molecular oxygen.

Subsequently, the samples were filtered through 0.45 µm pore size cellulose acetate filters and aliquots were taken for the determination of nitrate concentrations as described above.

2 ml of a 10 % $BaCl_2$ solution was afterwards added to precipitate sulfate contained in the medium as $BaSO_4$, which was then removed from the sample by filtration. Nitrate was absorbed from the sample by anion exchange resin columns (BIO-RAD, AG1-X8, mesh 200 - 400) as described by Silva *et al.* (2000). Afterwards, nitrate was eluted with 15 ml 10 % HCl from the anion exchange columns and the solution was neutralized with Ag_2O to a pH around 6.5. Accumulating AgCl precipitate was removed by filtration and $AgNO_3$ for isotope analysis was then gained by freeze-drying. Samples containing toluene were treated with 2-3 ml of cyclohexane to avoid a disturbance of toluene during further sample preparation and were afterwards treated as described above. As acetate reacts with Ag_2O to $Ag(CH_3C00)_2$ which was not possible to separate from the sample, the focus in the experiments with acetate as carbon source only lies on the isotopic composition of $\delta^{15}N$ in the residual nitrate and not on oxygen. The experiment with toluene as a carbon source and ^{18}O-enriched water was too much depleted in nitrate after 40 hours growth to reveal enough nitrogen for proper isotope analysis.

4.2.3 Isotope Analysis

Stable isotope analyses were performed with an isotope mass spectrometer (IRMS Thermo Electron MAT 253) in continuous flow mode, where the nitrogen in the samples was previously converted to N_2 by decomposition in an elemental analyzer (EA). For measurements of $\delta^{18}O$ from nitrate, CO was produced by pyrolysis at 1450°C in a discharge chamber and subsequent isotope analysis was conducted by IRMS. The results are reported in parts per thousands (per mil) using the conventional delta notation (δ) (Equation. 4.1):

$$\delta^{15}N = \left(\frac{R_{sample}}{R_{\text{standard}}} - 1 \right) \times 1000 \quad [‰] \tag{4.1}$$

R denotes the isotope ratio of the heavier over the lighter isotope. R_{sample} and R_{standard} are the isotope ratios of the sample and the standard. $\delta^{15}N$ values are reported with respect to air and $\delta^{18}O$ refers to Vienna Standard Mean Ocean Water (V-SMOW). International standard and several internal standards were used to ensure accurate measurements. The overall analytical precision of all measurements was ± 0.4 ‰ for $\delta^{15}N$ and ± 0.8 ‰ for $\delta^{18}O$. Each sample was measured twice.

The Rayleigh equation was used to assess stable isotope fractionation for nitrogen (Equation 4.2).

$$R_t / R_0 = (C_t / C_0)^{(\alpha - 1)} \tag{4.2}$$

R_t and R_0 denote the stable isotope ratios of nitrate at times t and zero. C_t and C_0 represent the respective concentration of the residual nitrate. The nitrogen isotope fractionation factor α was converted to the nitrogen isotope enrichment factor (ε_N) according to Equation. 4.3:

$$\varepsilon = (\alpha - 1) \times 1000 \quad [‰] \tag{4.3}$$

4.3 RESULTS & DISCUSSION

The turnover of nitrate in groundwater systems is of general interest, because increasing human activity has dramatically increased inputs of nitrogen. Denitrification represents an important process to eliminate nitrate from groundwater and was demonstrated in many studies using the stable isotope analysis. This analytical tool has been proven to be useful to identify sources and processes affecting nitrate (Cline and Kaplan, 1975; Focht, 1978; Mariotti et al., 1981; Amberger and Schmidt, 1987; Mariotti et al., 1988; Shearer and Kohl, 1988; Böttcher et al., 1990; Aravena and Robertson, 1998; Bates et al., 1998; Bates and

Spalding, 1998; Lehmann et al., 2003; Chen and MacQuarrie, 2005). However, little progress was made to understand the processes regulating nitrogen and oxygen isotope fractionation during the denitrification pathway.

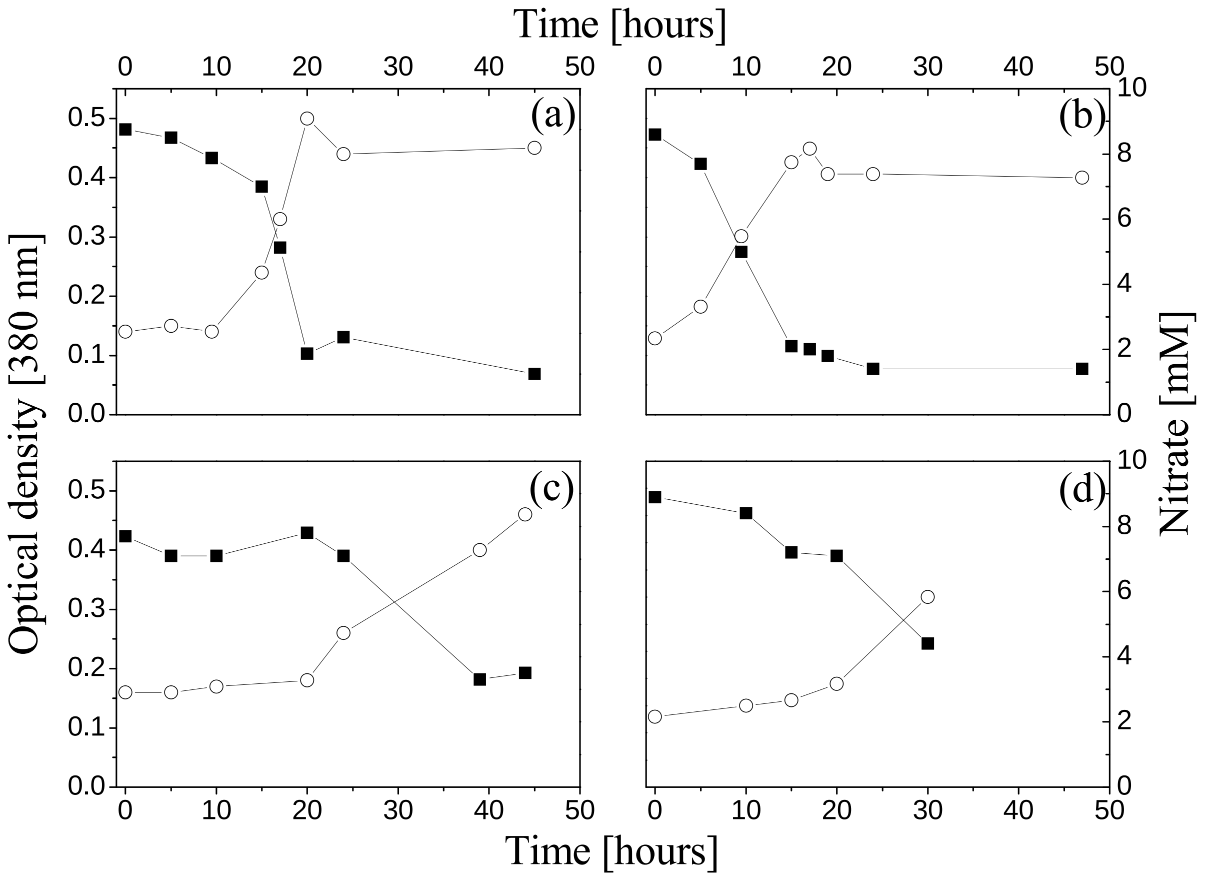
One aim of this study is to clarify, if reoxidation processes of metabolic intermediates formed during the denitrification pathway influence the stable isotope composition of nitrogen and oxygen in the residual nitrate. It has already been shown that the metabolic intermediates such as nitrite or nitric oxide exchange their oxygen isotopes with ambient water up to 100 % (Ye et al., 1993; Casciotti et al., 2002). The extent of isotope exchange, however, strongly depends on the biochemistry of the involved enzymes (Ye *et al.*, 1991). A high degree of oxygen isotope exchange was observed for bacteria possessing a heme-type nitrite reductase (Ye *et al.*, 1993). Contrary, low oxygen isotope exchange (6 %) occurs with bacteria, with the copper-type nitrite reductase (Glockner *et al.*, 1993). Nitrite reduction in *Thauera aromatica* is catalyzed by a heme-type nitrite reductase, which causes relatively large amounts of oxygen isotope exchange of 39 to 76 % (Ye et al., 1991; Song and Ward, 2003). This relatively large isotope exchange of oxygen isotopes of nitrite constitutes therefore a useful tool to identify putative reoxidation of the intermediate nitrite formed during the nitrate reduction pathway. If nitrite would reoxidize to nitrate, the oxygen isotope ratio of the nitrate produced should be similar to that of the ambient water. Such a putative reoxidation may also represent an important factor for the regulation of stable isotope fractionation of nitrogen in residual nitrate during denitrification.

To reach these goals we used two different carbon sources, toluene and acetate as well as ^{18}O-enriched ($\delta^{18}O_{water}$ ~+ 700 ‰) and unlabeled water ($\delta^{18}O_{water}$ ~ - 10 ‰) to assess the reoxidation of intermediates to a larger extent.

4.3.1 Growth

Growth of *Thauera aromatica* was accompanied by a strong increase in the optical density (Figure 1) and decreasing nitrate concentrations. The acetate-grown cultures reduced nitrate concentrations from 9 mM to values of around 1.4 mM within 20 hours (Figure. 1a, b). In contrast, with toluene as the sole carbon source, growth did not come to the stationary phase within 40 hours (Figure. 1c, d) and nitrate concentrations decreased from 8.9 mM start concentration to values below 3.5 mM.

Thauera aromatica showed significant differences in the growth rates between acetate and toluene oxidation indicating that acetate is easier degradable for *Thauera aromatica* than toluene.



Figureure 4.1: Growth of Thauera aromatica in batch cultures as indicated by the optical density at 380 nm (open circles) and the nitrate concentration (closed squares) during growth of *Thauera aromatica*: (a, b) acetate-as a carbon source, (c) the toluene-grown culture with a $\delta^{18}O_{water}$ ~ - 10 ‰, and (d) the toluene-grown culture with a $\delta^{18}O_{water}$ ~ + 700 ‰.

4.3.2 Isotope Fractionation during Denitrification

The toluene-grown cultures without ^{18}O-enriched water had an initial oxygen isotope composition of nitrate of around 18.4 ± 0.6 ‰ and the $\delta^{18}O$ values of nitrate did not change significantly until the end of the experiments (final values of 19.9 ‰ (Figure 4.2c)). Also, in the experiments with toluene and addition of ^{18}O-enriched water, the oxygen isotope composition remained fairly constant with an initial $\delta^{18}O$ value of residual nitrate of 23 ± 0.3 ‰ and 24.5 ± 1.7 ‰ after 30 hours (Figure 4.2d). We did not find any significant isotope exchange in the oxygen isotopes of the residual nitrate with ^{18}O-enriched water, although the nitrite reductase of *Thauera aromatica* most likely catalyzed an oxygen isotope exchange between ambient water and the metabolic intermediates as reported by Ye *et al* (1991) and Song & Ward (2003). However, this effect is was not visible in the residual nitrate, which should have been especially in the experiments with highly ^{18}O-enriched water. Thus, at least under the batch cultivation conditions employed here, there is probably no reoxidation process of intermediates formed during the denitrification of *Thauera aromatica* mixing "recycled" nitrate with the residual nitrate pool.

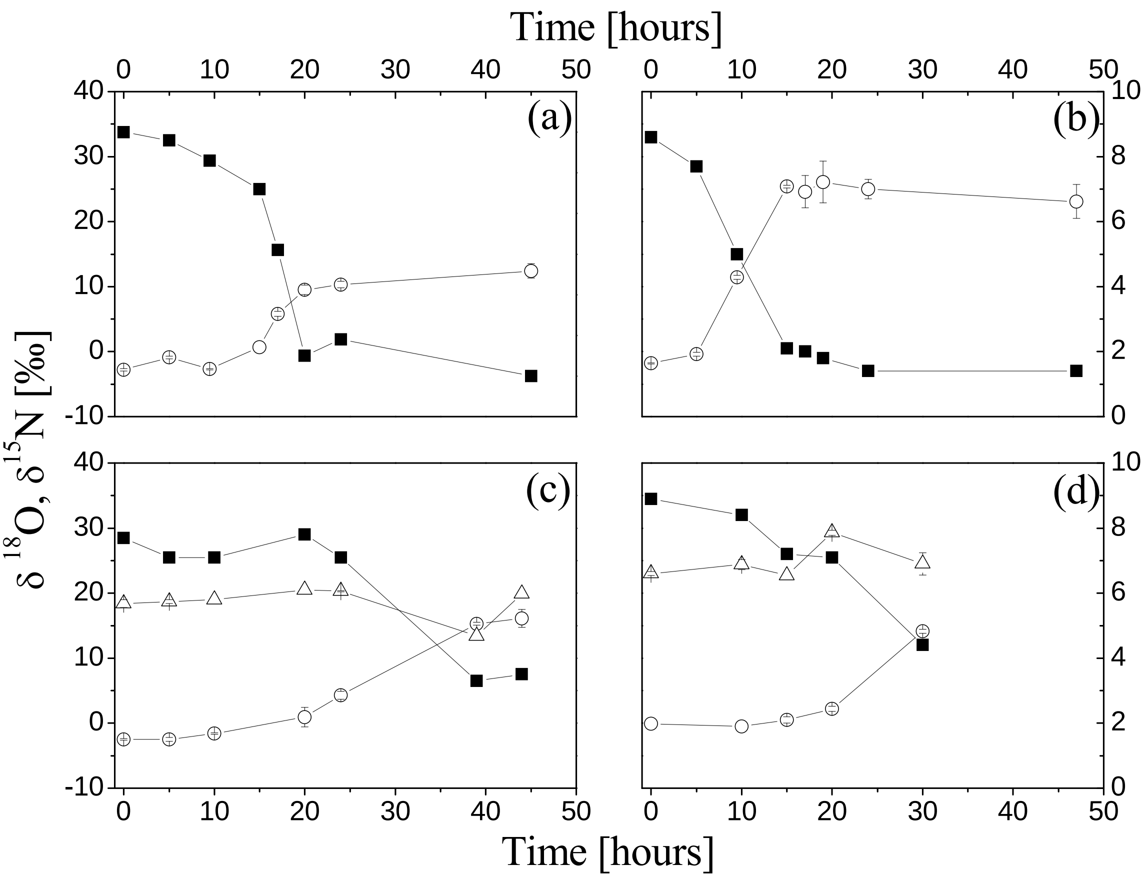


Figure 4.2: Stable isotope fractionation of oxygen and nitrogen in the residual nitrate measured in the batch experiments shown in Fig. 4.1: The $\delta^{15}N$ values of residual nitrate (open circles) and the respective nitrate concentrations (closed squares) during growth of *Thauera aromatica*: (a, b) with acetate as a carbon source and (c, d) with toluene as a carbon source. The $\delta^{18}O$ values (open triangles) for the experiments with toluene as a carbon source with unlabeled water (c), and (d) with labeled water ($\delta^{18}O_{water}$ ~ + 700 ‰).

Moreover, the lack of oxygen isotope fractionation during this experiment is in contrast to other findings: Olleros (1983), Böttcher et al. (1990) and Mengis et al. (1999) (listed in Lehmann *et al.*, 2003) reported oxygen isotope enrichment factors ranging between - 18.3 and - 8 ‰ during denitrification in pristine aquifers. This variation of the oxygen isotope enrichment has to be implemented by an oxygen isotope effect. However, the nature of this isotope effect was not further specified.

In this study, the oxygen isotope enrichment for *Thauera aromatica* during denitrification seems to be close to the analytical uncertainty of this parameter. In contrast to the oxygen isotope values of residual nitrate, nitrogen was accompanied by an enrichment in the $\delta^{15}N$ of in the residual nitrate in all experiments (Figure 4.2). The acetate-grown culture showed initial $\delta^{15}N$ values of nitrate of - 2.8 ± 0.2 ‰, which increased during growth to 12.4 ± 1.1 ‰ (Figure 4.2a). In a second experiment with acetate as carbon source the initial nitrogen isotope composition of -1.8 ± 0.1 ‰ increased to $\delta^{15}N$ values up to 23.1 ± 2.6 ‰ (Figure 4.2b). Reduction of nitrate in the experiments with toluene as carbon source reduction was accompanied by an increase in $\delta^{15}N$ values from -2.5 ± 0.1 ‰ to 16.1 ± 1.4 ‰ (Figure 4.2c) When ^{18}O-enriched water was added, the nitrogen isotopes increased from -0.1 to 13.5 ± 0.0 ‰ (Figure 4.2d).

Nitrogen isotope enrichment factors (ε_N) of - 15.1 ± 1.3 ‰ and - 13.2 ± 1.3 ‰ (Figure 3a, b) were calculated for the acetate-grown culture. When toluene was supplied as a sole carbon source, the experiments revealed nitrogen isotope enrichment factors (ε_N) of - 21.1 ± 0.6 ‰ and - 20.2 ± 0.6 ‰ (Figure 3c, d). The results obtained for the nitrogen isotope enrichment factors (ε_N) of *Thauera aromatica* are in line with observed range for nitrogen isotope fractionation during denitrification of 0 to - 36 for field experiments (Richards and Benson, 1961; Delwiche and Steyn, 1970; Cline and Kaplan, 1975; Mariotti et al., 1981; Amberger and Schmidt, 1987; Mariotti et al., 1988; Böttcher et al., 1990; Aravena et al., 1993; Altabet et al., 1995; Brandes and Devol, 1997; Aravena and Robertson, 1998).

Cell growth of *Thauera aromatica* on acetate is approximately two times faster than on toluene. Comparing the nitrogen isotope enrichment factors of all experiments, significantly more negative values (mean values $\varepsilon_N \sim$ - 20 ± 0.6 ‰) are retrieved for toluene oxidation than observed for cell growth on acetate (mean value $\varepsilon_N \sim$ -14 ± 1.3‰). This clearly indicates that the nitrogen isotope fractionation of *Thauera aromatica* is regulated by the kinetic reaction rates of the envolved enzymes for denitrification and/or on the degradation rate of the carbon source. This study could therefore support the hypothesis that maximum isotope fractionations are caused by the enzymatic catalyzed reduction steps, which get fully expressed, if the latter limit the rate of denitrification (Mariotti *et al.*, 1981).

With an easily degradable carbon source (acetate) the denitrification reactions will run at higher rates and the transport of nitrate to the respective enzymes might become limiting. Then, the nitrate at the enzyme is no more in isotopic equilibrium with the nitrate outside the cell and the stable isotope enrichment factor has to become more positive. If the oxidation of the carbon source is rate limiting (toluene), the nitrate is always in isotope equilibrium with the nitrate in the medium and the stable isotope enrichment factor is fully measurable in the remaining nitrate.

The experiments could not show any backward fluxes of intermediates to residual nitrate during growth of *Thauera aromatica*. This is in contrast to bacterial sulfate reduction where such a reoxidation processes of sulfite to sulfate can be very pronounced (Brunner et al., 2005; Mangalo et al., 2007). Thus, the decisive fractionation of nitrogen has to occur during the first step of the denitrification pathway, when nitrate is reduced to nitrite.

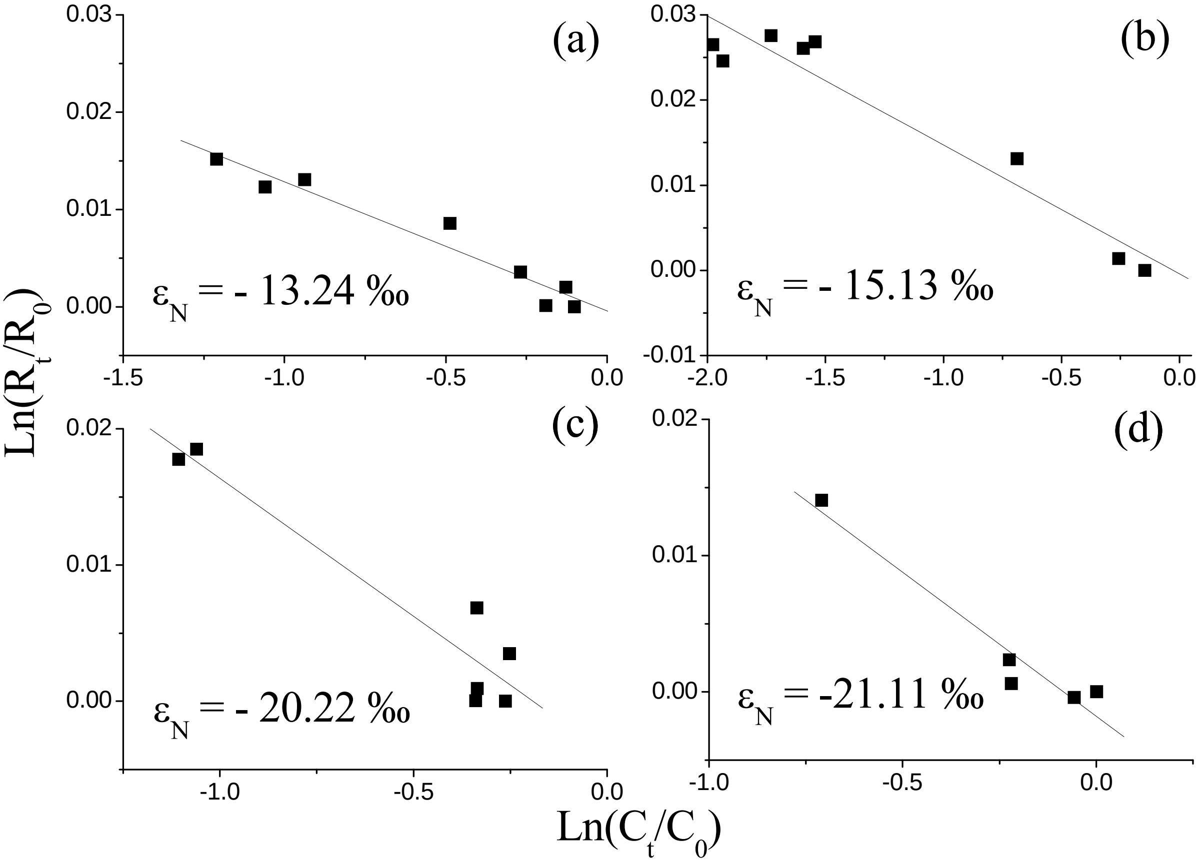


Figure 4.3: Determination of the nitrogen isotope enrichment factors by plotting the isotope data according to the Rayleigh equation. (a, b) growth with acetate as a carbon source with a standard deviation of ± 1.3 ‰, (c) growth with toluene as a carbon source with unlabeled water and (d) with toluene as a carbon source and a $\delta^{18}O_{water}$ ~ +700 ‰, with a standard deviation of ± 0.6 ‰, respectively.

4.4 CONCLUSION

The results of this study give strong indication that no significant backward fluxes of the metabolic intermediate nitrite to residual nitrate occurs during denitrification of *Thauera aromatica.* The oxygen isotopes of residual nitrate are therefore not regulated by an oxygen isotope exchange with water during denitrification unlike to bacterial sulfate reduction (Brunner et al., 2005; Knöller et al., 2006; Mangalo et al., 2007). However, the coupling of stable isotope fractionation and the electron flow from carbon source oxidation during denitrification showed some evidence that denitrification rates regulate isotope fractionation of nitrogen. Finally, this study also shows increasing $\delta^{15}N$ values combined with fairly constant $\delta^{18}O$ values formed during denitrification of *Thauera aromatica* found in manure or sewage (Smith et al., 1991; Clark and Fritz, 1997; Beller et al., 2004). Therefore, a combination of hydrodynamic, chemical and dual isotopic approaches (N, O) is needed to differentiate the mixing of respective nitrate sources and the microbial reduction of nitrate in complex aquatic systems.

The carbon source is just one aspect, to influence the denitrification rate and indirectly the stable isotope fractionation. However, to find out about parameters such as temperature, nitrate supply or strain specific attributes require further investigation on the stable isotope fractionation during denitrification.

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5. GENERAL CONCLUSIONS & OUTLOOK

A detailed knowledge about the biogeochemical turnover of elements contributes to the understanding of global and local changes and their consequences for environment and ecosystems.

Anaerobic microbial respiration processes constitute key processes in the global biogeochemical cycles of elements due to their omnipresence in nature. The characterization and quantification of these processes is of high importance to ensure the balance between the human and microbial world.

Such are for example bacterial sulfate reduction and denitrification fundamental processes for the anaerobe degradation of contaminants (Smith et al., 1991; Coates et al., 1996; Anderson and Lovley, 2000; Meckenstock et al., 2000; Widdel and Rabus, 2001; Rothermich et al., 2002; Meckenstock et al., 2004; Seidel et al., 2004; Kim and Pfaender, 2005), but intense activity can also have severe consequences on the bio-, geo- and atmosphere (Peng et al., 1994; Londry and Suflita, 1999; Casey et al., 2005; Greene et al., 2006).

By means of stable isotope analysis, it is possible to distinguish between the different biological, chemical and physical processes occurring in nature and quantify their impact on the sulfur and nitrogen turnover.

However, the knowledge on the principles of stable isotope fractionation on a micro scale is scarce. This is required to avoid misinterpretation of processes on a macro scale. This thesis includes three chapters, which all address the same issue:

- How can a dual isotope approach contribute to the principle understanding of stable isotope fractionation during anaerobe processes?
- Can this dual isotope approach supply biochemical knowledge of anaerobic respiration pathways?

This part of the thesis briefly summarizes the findings of the conducted experiments and gives some food for thoughts for successors to continue with the work.

Chapter 1:

In this part of the thesis, a direct link could be established between oxygen and sulfur isotope fractionation effects observed during bacterial sulfate reduction. Batch experiments with four different sulfate-reducing strains verified the hypothesis that isotope fractionation during bacterial sulfate reduction is governed by the cell internal fluxes of the individual reduction steps. It is suggested that a rate limitation of the last reduction step increases the overall isotope fractionation. The so far only theoretical model of Rees (1973) could be demonstrated with the following additional findings as follows:

- An oxygen isotope exchange between metabolic intermediates and ambient water could be observed.
- This isotope exchange is only visible, because recycling processes of the metabolic intermediates merge oxygen isotopes from ambient (in the experiments labeled) water to residual sulfate.
- These reoxidation processes get more prominent, if the residence time of the intermediate increases.
- The magnitude of the residence time is controlled by the enzymatic activity of the last reduction step, which in turn affects the sulfur isotope fractionation, as it includes a kinetic isotope fractionation.

From a biogeochemical point of view, this oxygen isotope exchange has only small influence on the interpretation of field data, as the environmental δ^{18}O values of water ($\delta^{18}O_{groundwater} \sim -10$ ‰) does not sufficiently label the residual sulfate to show any effect. The oxygen isotope exchange would therefore only be visuable in very old waters with higher δ^{18}O values or in processes observed over geologic time scales. Hence, the oxygen isotope analysis of residual sulfate still constitutes a useful tool to assess bacterial sulfate reduction (Strebel et al., 1990; Mayer et al., 1995; Canfield et al., 2000; Spence et al., 2001; Kirste, 2003; Mandernack et al., 2003; Einsiedl and Mayer, 2005; Novák et al., 2005). Nevertheless, further investigations on the isotope effects during bacterial sulfate reduction are needed to improve the quality of this isotopic tool. However, if high sulfur isotope enrichment factors are found during an investigation of a field site, also a high magnitude of oxygen isotope exchange can be expected (Böttcher et al., 1998; Aharon and Fu, 2000). The oxygen isotope effect could be also more prominent in study sites, where sulfate underlies high turnover rates, for example in the marine system or if sulfate reduction is attenuated over geologic timescales.

This study could only give indirect evidence for the formation of the intermediate sulfite and also the presence of other sulfuroxy-intermediates, could be followed by stable isotope analysis.

Chapter 2:

The last step of the sulfate reduction pathway was purposeful inhibited by the addition of nitrite in several concentrations in further batch experiments (Greene et al., 2003; Greene et al., 2006). With increasing nitrite concentrations, the sulfur isotope enrichment factors were shifted to larger values. This direct positive correlation between the nitrite concentration and sulfur isotope fractionation confirms the hypothesis of chapter 1 that isotope fractionation highly depends on the kinetic conversions in the sulfate reduction pathway. Furthermore, the oxygen isotope values in the residual sulfate increased as well, which constitutes a more pronounced reoxidation of the intermediate. It was not possible to influence the sulfur isotope fractionation in a way that the maximally observed isotope fractionations were retrieved.

Recent studies suggest that other additional fractionating mechanisms have to be present when high fractionations (above – 50 ‰) occur (Johnston et al., 2007). A detailed investigation of the isotope fractionation of every sulfate reducing step, by measurement of the fractionation potential of the individual enzymes may give more information, how the overall sulfur isotope fractionation comes about. Also a direct evidence of sulfite accumulation would further support the latter hypothesis.

This finding has also an interesting ecological aspect, as nitrite clearly affects the stable isotope composition of residual sulfate. Therefore, in systems with high microbial activity it would be possible, that the interaction of the different microbial groups, for example denitrification or ammonium oxidation influence the stable isotope fractionation of sulfate reducing bacteria, by production of nitrite. For future investigations, whether in laboratory or field studies, it might be interesting to find out more about further effects on the stable isotope fractionation during bacterial sulfate reduction due to the interaction of microbial communities.

Another aspect, which was not considered, is cell internal sulfate disproportionation. This could also have a severe effect on the stable isotope composition of both isotopes (S, O) in the residual sulfate (Böttcher et al., 2001).

Chapter 3:

Generally, same parameters have been also suggested to influence the isotope enrichment during bacterial denitrification, where special emphasis is placed on the rate of denitrification, causing a wide range of nitrogen isotope enrichment factors. Experiments were performed with a denitrifying strain, grown on two different carbon sources with the intent to cause different reduction rates. The direct comparison of the nitrate isotope fractionation confirmed the hypothesis of indirect rate-dependency of isotope fractionation during denitrification. However, unlike to the bacterial sulfate reduction, the oxygen isotope effect was absent with denitrification. This speaks for an unidirectional reduction pathway, where no reoxidation processes occur. Because of the complex sample procedure, the time was just sufficient to study one denitrifying strain. To broaden the knowledge on isotope fractionation during denitrification it is therefore crucial to perform similar experiments on other pure cultures. These should also include variations of other parameters, like it has already been done in many studies for bacterial sulfate reduction. The large variations in the sulfur isotope enrichment during bacterial sulfate reduction have been contributed to many different factors, such as temperature, electron donor and acceptor supply, the specific rates of sulfate reduction, and inherent differences among the sulfate-reducing species (Harrison and Thode, 1957; Kaplan and Rittenberg, 1963; Canfield, 2001; Detmers et al., 2001; Ohmoto et al., 2001; Habicht et al., 2002; Canfield et al., 2006).

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APPENDIX

SUPPORTING INFORMATION

CHAPTER 1:

Sulfide concentrations and mean values for the experiments Figures 2.2 and 2.3:

Desulfovibrio desulfuricans in ^{18}O-depleted media

Sulfide concentrations and mean values calculated from 3 parallels (n = 3) with standard deviation (± 1 σ)

Time (hours)	H_2S (mM) A	H_2S (mM) B	H_2S (mM) C	**H_2S (mM) Mean value**	± 1 σ
0	0.5	0.4	0.4	**0.4**	0.1
13	0.5	0.5	0.5	**0.5**	0.0
20	0.7	0.6	0.4	**0.6**	0.1
24	0.0	0.5	0.5	**0.3**	0.3
37	0.6	0.6	0.5	**0.6**	0.1
47	1.1	1.7	0.9	**1.2**	0.4
63	2.5	2.2	2.2	**2.3**	0.2
73	4.8	4.3	4.1	**4.4**	0.4
85	7.6	8.0	7.9	**7.8**	0.2
93	12.9	9.3	9.7	**10.6**	2.0
108	9.0	9.3	10.1	**9.5**	0.6

Desulfovibrio desulfuricans in ^{18}O-enriched media

Sulfide concentrations and mean values calculated from 2 parallels (n = 2) with standard deviation (± 1 σ) during growth of

Time (hours)	H_2S (mM) B	H_2S (mM) C	**H_2S (mM) Mean value**	± 1 σ
0	1.1	1.6	**1.4**	0.4
53	2.3	2.8	**2.5**	0.4
79	2.6	2.3	**2.5**	0.2
87	4.2	2.8	**3.5**	1.0
110	11.4	8.4	**9.9**	2.1
127	9.8	10.1	**10.0**	0.2
135	11.4	11.4	**11.4**	0.0
151	10.6	10.9	**10.8**	0.1

Desulfonatronovibrio hydrogenovorans in ^{18}O-depleted media

Sulfide concentrations and mean values calculated from 3 parallels (n = 3) with standard deviation (± 1 σ)

Time (days)	H_2S (mM) A	H_2S (mM) B	H_2S (mM) C	**H_2S (mM) Mean value**	± 1 σ
0	3.3	3.4	3.6	**3.4**	0.2
7	2.7	3.1	3.0	**2.9**	0.2
15	11.5	11.5	11.7	**11.6**	0.1
18	17.9	19.4	19.0	**18.8**	0.8
20	14.8	18.0	18.3	**17.0**	1.9
22	16.1	16.7	16.2	**16.4**	0.3

Desulfonatronovibrio hydrogenovorans in ^{18}O enriched media

Sulfide concentrations and mean values calculated from 3 parallels (n = 3) with standard deviation (± 1 σ)

Time (days)	H_2S (mM) A	H_2S (mM) B	H_2S (mM) C	**H_2S (mM) Mean value**	± 1 σ
0	1.8	1.8	1.8	**1.8**	0.0
7	3.5	5.1	4.9	**4.5**	0.9
13	9.1	10.2	10.4	**9.9**	0.7
15	16.5	16.0	17.8	**16.7**	1.0
18	17.3	17.1	16.6	**17.0**	0.4
20	16.4	16.7	16.3	**16.5**	0.2

Desulfobacca acetoxidans in ^{18}O-depleted media

Sulfide concentrations and mean values calculated from 3 parallels (n = 3) with standard deviation (± 1 σ)

Time (days)	H_2S (mM) A	H_2S (mM) B	H_2S (mM) C	**H_2S (mM) Mean value**	± 1 σ
0	1.2	1.3	1.3	**1.3**	0.0
7	2.2	2.1	2.1	**2.1**	0.0
15	2.1	2.4	2.5	**2.4**	0.2
18	2.9	3.0	2.7	**2.9**	0.2
20	2.7	3.2	2.7	**2.9**	0.3
27	5.1	5.7	4.8	**5.2**	0.5
32	7.5	8.4	6.9	**7.6**	0.7
35	9.6	11.5	9.4	**10.2**	1.1
37	10.4	13.4	9.7	**11.2**	2.0
42	14.2	21.0	11.6	**13.3**	4.8
47	16.8	14.1	14.5	**16.6**	1.5
49	14.8	18.4	12.2	**13.5**	3.1
54	15.1	16.6	11.8	**15.1**	2.4

Desulfobacca acetoxidans in ^{18}O-enriched media

Sulfide concentrations and mean values calculated from 3 parallels (n = 3) with standard deviation (± 1 σ)

Time (days)	H_2S (mM) A	H_2S (mM) B	H_2S (mM) C	**H_2S (mM) Mean value**	± 1 σ
0	1.4	1.4	1.3	**1.4**	0.1
7	1.5	2.0	2.6	**2.0**	0.6
15	2.0	2.0	3.0	**2.3**	0.6
18	2.4	2.5	3.4	**2.8**	0.5
20	2.2	2.2	3.1	**2.5**	0.5
27	2.7	3.0	5.7	**3.8**	1.6
32	3.4	4.1	9.1	**5.6**	3.1
35	4.2	5.7	11.2	**7.0**	3.6
37	4.8	5.6	12.7	**7.7**	4.3
40	5.9	9.1	17.4	**10.8**	5.9
42	7.3	13.6	16.9	**12.6**	4.9
47	8.7	16.0	17.7	**14.1**	4.8
51	10.4	15.5	17.6	**14.5**	3.7

TRM1 in ^{18}O-depleted media

Sulfide concentrations and mean values calculated from 3 parallels (n = 3) with standard deviation (± 1 σ)

Time (days)	H_2S (mM) A	H_2S (mM) B	H_2S (mM) C	**H_2S (mM) Mean value**	± 1 σ
0	0.7	0.8	0.8	**0.7**	0.0
3	1.3	1.5	1.5	**1.4**	0.1
7	3.4	3.1	3.9	**3.5**	0.4
10	4.1	5.6	6.0	**5.2**	1.0
13	4.6	4.8	5.6	**5.0**	0.5
16	4.4	4.7	5.4	**4.8**	0.5
19	4.6	5.0	5.4	**5.0**	0.4
21	5.1	5.5	7.1	**5.9**	1.1
38	4.4	4.5	5.7	**4.9**	0.7
69	4.7	4.7	5.4	**4.9**	0.4

TRM1 in ^{18}O-enriched media

Sulfide concentrations and mean values calculated from 3 parallels (n = 3) with standard deviation (± 1 σ)

Time (days)	H_2S (mM) A	H_2S (mM) B	H_2S (mM) C	**H_2S (mM) Mean value**	± 1 σ
0	0.6	0.6	0.6	**0.6**	0.0
5	0.9	1.1	1.0	**1.0**	0.1
7	1.2	1.2	1.3	**1.2**	0.0
9	1.5	1.3	1.5	**1.4**	0.1
14	2.2	1.8	1.8	**1.9**	0.2
19	2.6	2.5	2.7	**2.6**	0.1
21	2.9	3.1	3.0	**3.0**	0.1
26	3.2	3.0	3.8	**3.3**	0.4
30	3.4	3.4	4.2	**3.7**	0.5
35	4.3	3.8	4.3	**4.1**	0.3
50	5.3	6.5	6.7	**6.2**	0.7

Oxygen isotope values and mean values for the experiments Figure 2.2 and Table 2.2:

Desulfovibrio desulfuricans in ^{18}O-depleted media

Oxygen isotope values and mean values calculated from 3 parallels (n = 3) with standard deviation (± 1 σ)

Time (hours)	δ^{18}O [‰] A	δ^{18}O [‰] B	δ^{18}O [‰] C	**δ^{18}O [‰] Mean value**	± 1 σ
13	n.a.	8.4	8.8	**8.6**	0.3
37	n.a.	9.8	n.a.	**9.8**	
47	11.2	10.4	n.a.	**10.8**	
63	12.5	n.a.	6.3	**9.4**	
73	n.a.	10.0	10.9	**10.4**	0.7
85	11.9	9.4	7.1	**9.5**	1.6
93	n.a.	9.8	n.a.	**9.8**	
108	n.a.	8.0	9.2	**8.6**	0.9
134	10.7	n.a.	10.7	**10.7**	

Desulfovibrio desulfuricans in ^{18}O-enriched media

Oxygen isotope values and mean values calculated from 2 parallels (n = 2) with standard deviation (± 1 σ)

Time (hours)	δ^{18}O [‰] A	δ^{18}O [‰] B	**δ^{18}O [‰] Mean value**	± 1 σ
0	n.a.	10.8	**10.8**	0.0
53	12.7	15.4	**14.1**	1.9
79	12.7	15.0	**13.8**	1.6
87	21.2	22.4	**21.8**	0.9
110	44.4	23.4	**33.9**	14.9
127	45.1	31.7	**38.4**	9.4
135	43.2	43.3	**43.2**	0.1
151	54.3	40.2	**47.2**	9.9

Desulfonatronovibrio hydrogenovorans in ^{18}O-depleted media

Oxygen isotope values and mean values calculated from 3 parallels (n = 3) with standard deviation (± 1 σ)

Time (days)	δ^{18}O [‰] A	δ^{18}O [‰] B	δ^{18}O [‰] C	**δ^{18}O [‰] Mean value**	± 1 σ
0	11.6	11.7	11.8	**11.6**	0.1
7	12.1	12.9	13.9	**12.1**	0.9
15	11.9	12.1	11.8	**11.9**	0.1
18	11.1	11.3	11.4	**11.1**	0.2
20	11.5	11.0	11.4	**11.5**	0.2
22	11.2	11.5	11.6	**11.2**	0.2

Desulfonatronovibrio hydrogenovorans in ^{18}O-enriched media

Oxygen isotope values and mean values calculated from 3 parallels (n = 3) with standard deviation (± 1 σ)

Time (days)	δ^{18}O [‰] A	δ^{18}O [‰] B	δ^{18}O [‰] C	**δ^{18}O [‰] Mean value**	± 1 σ
0	12.3	13.4	13.4	**13.0**	0.7
7	11.4	9.6	9.5	**10.2**	1.1
13	37.4	39.4	43.4	**40.1**	3.0
15	40.5	37.8	39.1	**39.1**	1.4
18	71.8	58.0	68.7	**66.2**	7.2
20	44.9	41.3	42.8	**43.0**	1.8

Desulfbacca acetoxidans in ^{18}O-depleted media

Oxygen isotope values and mean values calculated from 3 parallels (n = 3) with standard deviation (± 1 σ)

Time (days)	δ^{18}O [‰] A	δ^{18}O [‰] B	δ^{18}O [‰] C	**δ^{18}O [‰] Mean value**	± 1 σ
0	12.0	10.7	10.6	**11.1**	0.8
7	11.7	11.8	11.3	**11.6**	0.2
15	11.7	11.7	11.3	**11.6**	0.3
18	11.4	11.3	11.4	**11.4**	0.0
20	11.3	11.4	11.6	**11.4**	0.2
27	11.1	11.0	11.1	**11.1**	0.1
32	15.0	11.9	11.6	**12.8**	1.8
35	11.0	10.8	10.4	**10.7**	0.3
37	14.7	17.5	8.7	**13.6**	4.5
42	10.3	14.9	16.0	**13.7**	3.0

Desulfbacca acetoxidans in ^{18}O-enriched media

Oxygen isotope values and mean values calculated from 3 parallels (n = 3) with standard deviation (± 1 σ)

Time (days)	δ^{18}O [‰] A	δ^{18}O [‰] B	δ^{18}O [‰] C	**δ^{18}O [‰] Mean value**	± 1 σ
0	14.7	14.6	14.9	**8.8**	0.2
7	14.7	15.5	16.8	**12.2**	1.1
15	16.1	16.2	17.4	**15.9**	0.7
18	17.1	16.9	17.4	**17.5**	0.3
20	19.3	18.8	23.7	**20.4**	2.7
27	28.9	39.0	73.6	**39.1**	23.5
32	51.0	48.6	192.6	**71.2**	82.5
35	69.0	62.4	265.5	**93.4**	115.4
37	78.1	76.9	331.8	**112.2**	146.8
40	127.0	343.1	521.5	**215.1**	197.6
42	255.7	352.7	606.5	**261.8**	181.1
51	n.a.	595.9	n.a.	**595.9**	
54	n.a.	654.7	n.a.	**654.7**	

TRM1 in ^{18}O-depleted media

Oxygen isotope values and mean values calculated from 3 parallels (n = 3) with standard deviation (± 1 σ)

Time (days)	A [‰]	B [‰]	C [‰]	**δ^{18}O [‰]**	± 1 σ
3	n.a.	9.5	9.0	**9.5**	0.3
19	5.0	4.4	4.5	**4.6**	0.3
38	n.a.	3.2	2.6	**2.9**	0.5
69	3.9	3.5	3.4	**3.6**	0.2

TRM1 in ^{18}O-enriched media

Oxygen isotope values and mean values calculated from 3 parallels (n = 3) with standard deviation (± 1 σ)

Time (days)	δ^{18}O [‰] A	δ^{18}O [‰] B	δ^{18}O [‰] C	**δ^{18}O [‰] Mean value**	± 1 σ
5	31.4	30.7	30.2	**30.8**	0.6
7	46.2	45.8	47.6	**46.5**	0.9
9	128.7	110.5	128.8	**122.6**	10.5
14	208.9	200.2	217.1	**208.7**	8.4
19	239.0	228.9	246.4	**238.1**	8.8
26	338.0	312.4	354.5	**335.0**	21.2
35	364.6	391.6	337.0	**364.4**	27.3
50	560.7	636.4	559.0	**585.4**	44.2

Sulfur isotope values and mean values for the experiments Figure 2.3:

Desulfovibrio desulfuricans in ^{18}O-depleted media
Sulfur isotope values and mean values calculated from 3 parallels (n = 3) with standard deviation (± 1 σ)

Time (hours)	δ^{34}S [‰] A	δ^{34}S [‰] B	δ^{34}S [‰] C	**δ^{34}S[‰] Mean value**	± 1 σ
13	n.a	4.7	4.8	**4.7**	0.0
47	4.3	4.8	n.a	**4.6**	0.3
63	5.6	n.a	5.4	n.a	
73	n.a	7.4	n.a	**7.4**	
85	n.a	10.6	n.a	**10.6**	

Desulfovibrio desulfuricans in ^{18}O-enriched media
Sulfur isotope values and mean values calculated from 2 parallels (n = 2) with standard deviation (± 1 σ)

Time (hours)	δ^{34}S [‰] A	δ^{34}S [‰] B	**δ^{34}S[‰] Mean value**	± 1 σ
0	n.a	4.7	**4.7**	n.a
53	4.7	n.a	**4.7**	n.a
79	n.a	5.1	**5.1**	n.a
87	4.8	4.9	**4.9**	0.0
110	n.a	5.6	**5.6**	n.a
127	n.a	7.6	**7.6**	n.a
135	8.6	8.5	**8.5**	0.1
151	8.7	8.8	**8.8**	0.1

Desulfonatronovibrio hydrogenovorans in ^{18}O-depleted media
Sulfur isotope values and mean values calculated from 3 parallels (n = 3) with standard deviation (± 1 σ)

Time (days)	δ^{34}S [‰] A	δ^{34}S [‰] B	δ^{34}S [‰] C	**δ^{34}S[‰] Mean value**	± 1 σ
0	5.0	5.0	5.1	5.0	0.1
7	5.6	5.5	5.7	5.6	0.1
15	10.1	8.9	9.5	9.5	0.6
18	16.3	15.0	16.4	15.9	0.8
20	16.5	15.1	16.5	16.0	0.8
22	16.7	16.4	15.4	16.2	0.7

Desulfonatronovibrio hydrogenovorans in ^{18}O-enriched media

Sulfur isotope values and mean values calculated from 3 parallels (n = 3) with standard deviation (± 1 σ)

Time (days)	δ^{34}S [‰] A	δ^{34}S [‰] B	δ^{34}S [‰] C	**δ^{34}S[‰] Mean value**	± 1 σ
0	4.6	4.9	5.0	4.8	0.2
7	5.1	5.1	5.0	5.1	0.0
13	10.2	10.7	11.3	10.7	0.5
15	13.2	13.7	13.6	13.5	0.3
18	14.3	14.0	13.9	14.1	0.2
20	14.2	14.0	14.0	14.1	0.1

Desulfbacca acetoxidans in ^{18}O-depleted media

Sulfur isotope values and mean values calculated from 3 parallels (n = 3) with standard deviation (± 1 σ)

Time (days)	δ^{34}S [‰] A	δ^{34}S [‰] B	δ^{34}S [‰] C	**δ^{34}S[‰] Mean value**	± 1 σ
0	5.5	5.6	5.8	**5.6**	0.1
7	n.a	n.a	6.7	**6.7**	
15	6.8	6.9	7.4	**7.0**	0.3
18	7.6	7.7	8.1	**7.8**	0.3
20	8.0	8.4	n.a	**8.2**	0.3
27	11.1	11.9	10.6	**11.2**	0.7
32	19.3	17.9	16.7	**18.0**	1.3
35	19.2	21.6	23.8	**21.5**	2.3
37	22.3	24.6	n.a	**23.5**	1.6
42	n.a	62.6	n.a	**62.6**	

Desulfbacca acetoxidans in ^{18}O-enriched media

Sulfur isotope values and mean values calculated from 3 parallels (n = 3) with standard deviation (± 1 σ)

Time (hours)	δ^{34}S [‰] A	δ^{34}S [‰] B	δ^{34}S [‰] C	**δ^{34}S[‰] Mean value**	± 1 σ
0	5.4	5.1	5.3	**5.3**	0.1
7	6.8	6.7	7.4	**7.0**	0.4
15	7.0	6.4	6.6	**6.7**	0.3
18	6.1	6.1	7.8	**6.7**	1.0
20	6.5	6.4	7.5	**6.8**	0.6
27	6.7	7.5	10.6	**8.3**	2.0
32	8.6	8.4	17.7	**11.6**	5.3
35	9.6	9.5	21.7	**13.6**	7.0
37	11.0	n.a	30.1	**20.6**	13.5
42	18.0	32.2	n.a	**25.1**	10.1
47	n.a	32.5	n.a	**32.5**	
51	n.a	57.9	n.a	**57.9**	

TRM1 in ^{18}O-depleted media

Sulfur isotope values and mean values calculated from 3 parallels (n = 3) with standard deviation (± 1 σ)

Time (days)	δ^{34}S [‰] A	δ^{34}S [‰] B	δ^{34}S [‰] C	**δ^{34}S[‰] Mean value**	± 1 σ
3	n.a	10.3	10.6	10.5	0.2
19	20.6	19.3	21.7	20.5	1.2
38	n.a	20.0	20.0	20.0	0.0
69	24.1	22.2	23.4	22.8	0.9

TRM1 in ^{18}O-enriched media

Sulfur isotope values and mean values calculated from 3 parallels (n = 3) with standard deviation (± 1 σ)

Time (days)	δ^{34}S [‰] A	δ^{34}S [‰] B	δ^{34}S [‰] C	**δ^{34}S[‰] Mean value**	± 1 σ
5	n.a	n.a	6.8	6.8	
7	7.1	7.6	7.6	7.4	0.3
9	10.8	9.5	10.5	10.3	0.7
14	14.8	12.7	15.1	14.2	1.3
19	n.a	n.a	17.0	17.0	
26	19.8	n.a	23.0	21.4	2.2
35	22.8	28.0	19.7	23.5	4.2

Calculations for the isotope enrichment factor Figure 2.4:

Name	**^{18}O-depleted water**		**^{18}O-enriched water**	
	Ln(C_t/C_0)	**Ln(R_t/R_0)**	**Ln(C_t/C_0)**	**Ln(R_t/R_0)**
Desulfovibrio desulfuricans	-0.02583	0.00000	-0.02737	0
	-0.06294	-0.00018	-0.02276	3.68E-04
	-0.12104	0.00074	-0.07958	1.39E-04
	-0.24846	0.00264	-0.50418	0.00235
	-0.49512	0.00583	-0.50833	0.0034
	-	-	-0.57536	0.00383
	-	-	-0.50667	0.00401
Desulfonatronovibrio hydrogenovorans	-0.18693	0	-0.09376	0
	-0.15841	5.87E-04	-0.25231	2.59E-04
	-0.86394	0.00444	-0.68023	0.00585
	-2.79688	0.01077	-1.81401	0.0086
	-1.90717	0.01091	-1.89712	0.00916
	-1.70101	0.01104	-1.73161	0.00915
Desulfobacca acetoxidans	-0.06667	0	-0.13296	0.00151
	-0.12613	0.00108	-0.20887	0.00294
	-0.1549	0.0018	-0.32504	0.00623
	-0.15432	0.00249	-0.43386	0.00823
	-0.29975	0.00531	-0.48776	0.01509
	-0.47884	0.01129	-0.99021	0.01953
	-0.71233	0.01712	-1.22418	0.0267
	-0.81871	0.01865	-	-
TRM1	-0.42465	0.00567	-0.10425	0.00199
	-0.68518	0.0156	-0.13125	0.00265
	-0.66359	0.01507	-0.15315	0.00549
	-	-	-0.21567	0.00937
	-	-	-0.30246	0.01214
	-	-	-0.40347	0.01643
	-	-	-0.53273	0.01848

Calculated sulfate reduction rate and sulfur isotope enrichment factor for individual time steps Figure 2.5:

Group A	***Desulfobacca acetoxidans***		***TRM1***	
	SRR (sulfide/time)	ε_t	**SRR (sulfide/time)**	ε_t
	0.12	-3.91	0.22	-1.29
	0.03	-3.53	0.01	1.32
	0.17	-3.58	0	-9.52
	0.03	-14.23	0.08	-3.34
	0.32	-2.25	0.13	-14.62
	0.49	-2.64	0.1	-5.18
	0.86	-0.77	0.1	-1.78
	0.87	-0.45	0.16	3.16
	1.14	-0.81	0.14	-5.1
	0.1	-7.38	0.09	-0.89
	0.14	-0.04		
	0.18	-2.51		
	0.36	-2.91		
	0.5	-1.49		
	0.87	-2.88		
	1.08	-0.42		
	0.6	-1.25		

Group B	***Desulfovibrio desulfuricans***		***Desulfonatronovibrio hydrogenovorans***	
	SRR (sulfide/time)	ε_t	**SRR (sulfide/time)**	ε_t
	0	-0.24	0	-0.39
	0.03	0.86	0	-0.93
	0.06	-3.6	0.58	-0.78
	0.2	-2.55	0.32	-0.44
	0.3	-1.36	0.38	-0.33
	-0.01	0.08	0.9	-1.75
	0.16	0.64	3.44	-0.2
	0.21	-0.4		
	0.09	-2.52		
	0.09	-1.06		

CHAPTER 2:

Sulfide concentrations and mean values for the experiments Figures 3.2 and 3.3:

Experiment A: *Desulfovibrio desulfuricans*, no addition of nitrite, no ^{18}O-enriched water
Sulfide concentrations and mean values (n = 2) with standard deviation (± 1 σ)

Time (hours)	H_2S (mM) Bottle 1	H_2S (mM) Bottle 2	**H_2S (mM) Mean values**	± 1 σ
0	1.3	1.0	**1.1**	0.2
19	2.8	2.5	**2.6**	0.3
26	6.4	5.6	**6.0**	0.6
48	8.6	8.2	**8.4**	0.2
70	7.6	5.3	**6.5**	1.7
191	8.6	8.4	**8.5**	0.2

Experiment B: *Desulfovibrio desulfuricans*, no addition of nitrite, ^{18}O-enriched water
Sulfide concentrations and mean values (n = 2) with standard deviation (± 1 σ)

Time (hours)	H_2S (mM) Bottle 1	H_2S (mM) Bottle 2	**H_2S (mM) Mean values**	± 1 σ
0	1.6	1.5	**1.5**	0.0
19	3.0	3.1	**3.1**	0.0
26	6.4	7.0	**6.7**	0.5
48	6.6	7.5	**7.1**	0.7
70	7.7	9.9	**8.8**	1.5
191	6.4	7.9	**7.1**	1.1

Experiment C: *Desulfovibrio desulfuricans*, 100 µM nitrite, ^{18}O-enriched water
Sulfide concentrations and mean values (n = 2) with standard deviation (± 1 σ)

Time (hours)	H_2S (mM) Bottle 1	H_2S (mM) Bottle 2	**H_2S (mM) Mean values**	± 1 σ
0	1.5	1.4	**1.4**	0.1
19	2.2	1.8	**2.0**	0.3
42	7.3	6.0	**6.7**	0.9
48	6.6	7.3	**7.0**	0.5
70	8.2	8.0	**8.1**	0.1
191	7.3	8.3	**7.8**	0.7

Experiment D: *Desulfovibrio desulfuricans*, 200 µM nitrite, ^{18}O-enriched water
Sulfide concentrations and mean values (n = 2) with standard deviation (± 1 σ)

Time (hours)	H_2S (mM) Bottle 1	H_2S (mM) Bottle 2	**H_2S (mM) Mean values**	± 1 σ
0	1.3	1.5	**1.4**	0.1
26	1.9	2.1	**2.0**	0.2
46	6.1	6.4	**6.3**	0.2
70	8.7	8.7	**8.7**	0.0
95	8.1	6.8	**7.5**	1.0
191	6.7	7.9	**7.3**	0.9

Experiment E: *Desulfovibrio desulfuricans*, 500 µM nitrite, ^{18}O-enriched water
Sulfide concentrations and mean values (n = 2) with standard deviation (± 1 σ)

Time (hours)	H_2S (mM) Bottle 1	H_2S (mM) Bottle 2	**H_2S (mM) Mean values**	± 1 σ
0	1.0	1.0	**1.0**	0.0
70	1.3	1.2	**1.2**	0.1
162	3.8	2.7	**3.3**	0.8
170	4.5	3.4	**4.0**	0.8
215	4.3	3.5	**3.9**	0.5
381	4.4	4.8	**4.6**	0.3

Experiment F: *Desulfovibrio desulfuricans*, 1000 µM nitrite, ^{18}O-enriched water
Sulfide concentrations and mean values (n = 2) with standard deviation (± 1 σ)

Time (hours)	H_2S (mM) Bottle 1	H_2S (mM) Bottle 2	**H_2S (mM) Mean values**	± 1 σ
0	0.5	0.7	**0.6**	0.2
330	1.6	1.4	**1.5**	0.1
403	2.9	3.0	**3.0**	0.1
953	4.8	5.1	**4.9**	0.2

Oxygen isotope values and mean values for the experiments Figure 3.2:

Experiment A: *Desulfovibrio desulfuricans*, no addition of nitrite, no ^{18}O-enriched water
Oxygen isotope values and mean values (n = 2) with standard deviation (± 1 σ)

Time (hours)	δ^{18}O [‰] Bottle 1	δ^{18}O [‰] Bottle 2	**δ^{18}O [‰] Mean values**	± 1 σ
0	12.3	12.0	**12.2**	0.2
19	12.1	11.5	**11.8**	0.4
26	11.4	11.4	**11.4**	0.0
48	11.7	12.3	**12.0**	0.4
70	12.8	11.2	**12.0**	1.1
191	13.2	13.5	**13.4**	0.2

Experiment B: *Desulfovibrio desulfuricans*, no addition of nitrite, ^{18}O-enriched water
Oxygen isotope values and mean values (n = 2) with standard deviation (± 1 σ)

Time (hours)	δ^{18}O [‰] Bottle 1	δ^{18}O [‰] Bottle 2	**δ^{18}O [‰] Mean values**	± 1 σ
0	14.3	14.5	**14.4**	0.1
19	20.7	21.5	**21.1**	0.6
26	33.3	33.4	**33.4**	0.0
48	46.7	49.2	**47.9**	1.7
70	46.9	50.2	**48.5**	2.3
191	48.3	50.8	**49.6**	1.8

Experiment C: *Desulfovibrio desulfuricans*, 100 µM nitrite, ^{18}O-enriched water
Oxygen isotope values and mean values (n = 2) with standard deviation (± 1 σ)

Time (hours)	δ^{18}O [‰] Bottle 1	δ^{18}O [‰] Bottle 2	**δ^{18}O [‰] Mean values**	± 1 σ
0	15.3	15.1	**15.2**	0.1
19	19.6	19.5	**19.6**	0.1
42	40.2	40.7	**40.5**	0.3
48	57.4	55.5	**56.4**	1.3
70	59.8	73.4	**66.6**	9.6
191	62.0	74.5	**68.2**	8.8

Experiment D: *Desulfovibrio desulfuricans*, 200 µM nitrite, ^{18}O-enriched water
Oxygen isotope values and mean values (n = 2) with standard deviation (± 1 σ)

Time (hours)	δ^{18}O [‰] Bottle 1	δ^{18}O [‰] Bottle 2	**δ^{18}O [‰] Mean values**	± 1 σ
0	16.5	15.5	**16.0**	0.7
26	26.9	24.8	**25.8**	1.5
46	45.8	47.0	**46.4**	0.9
70	86.6	67.7	**77.1**	13.4
95	84.6	68.3	**76.4**	11.5
191	87.2	68.4	**77.8**	13.3

Experiment E: *Desulfovibrio desulfuricans*, 500 µM nitrite, ^{18}O-enriched water
Oxygen isotope values and mean values (n = 2) with standard deviation (± 1 σ)

Time (hours)	δ^{18}O [‰] Bottle 1	δ^{18}O [‰] Bottle 2	**δ^{18}O [‰] Mean values**	± 1 σ
0	16.2	15.4	**15.7**	0.5
70	19.0	18.0	**17.8**	0.7
162	43.3	32.6	**31.8**	7.6
170	44.1	38.1	**40.9**	4.3
215	49.8	39.5	**43.4**	7.3
381	124.2	122.3	**123.5**	1.4

Experiment F: *Desulfovibrio desulfuricans*, 100 µM nitrite, ^{18}O-enriched water
Oxygen isotope values and mean values (n = 2) with standard deviation (± 1 σ)

Time (hours)	δ^{18}O [‰] Bottle 1	δ^{18}O [‰] Bottle 2	**δ^{18}O [‰] Mean values**	± 1 σ
0	13.8	14.4	**14.1**	0.4
330	26.3	22.8	**24.6**	2.5
403	45.0	46.6	**45.8**	1.2
953	84.6	81.1	**82.9**	2.5

Sulfur isotope values and mean values for the experiments Figure 3.3:

Experiment A: *Desulfovibrio desulfuricans*, no addition of nitrite, no ^{18}O-enriched water
Sulfur isotope values and mean values (n = 2) with standard deviation (± 1 σ)

Time (hours)	$\delta^{34}S$ [‰] Bottle 1	$\delta^{34}S$ [‰] Bottle 2	**$\delta^{34}S$ [‰] Mean values**	± 1 σ
0	5.1	5.0	**5.1**	0.1
19	6.4	6.4	**6.4**	0.0
26	8.3	8.1	**8.2**	0.1
48	10.8	10.7	**10.7**	0.0
70	10.8	10.5	**10.7**	0.2
191	10.7	10.4	**10.6**	0.2

Experiment B: *Desulfovibrio desulfuricans*, no addition of nitrite, ^{18}O-enriched water
Sulfur isotope values and mean values (n = 2) with standard deviation (± 1 σ)

Time (hours)	$\delta^{34}S$ [‰] Bottle 1	$\delta^{34}S$ [‰] Bottle 2	**$\delta^{34}S$ [‰] Mean values**	± 1 σ
0	5.1	5.0	**5.0**	0.0
19	6.4	6.5	**6.5**	0.1
26	8.3	8.5	**8.4**	0.1
48	10.3	10.9	**10.6**	0.5
70	10.5	11.0	**10.7**	0.4
191	10.5	10.9	**10.7**	0.3

Experiment C: *Desulfovibrio desulfuricans*, 100 µM nitrite, no ^{18}O-enriched water
Sulfur isotope values and mean values (n = 2) with standard deviation (± 1 σ)

Time (hours)	$\delta^{34}S$ [‰] Bottle 1	$\delta^{34}S$ [‰] Bottle 2	**$\delta^{34}S$ [‰] Mean values**	± 1 σ
0	4.9	5.0	**5.0**	0.1
19	5.5	5.7	**5.6**	0.1
42	8.9	8.3	**8.6**	0.4
48	11.1	10.0	**10.6**	0.8
70	11.3	11.5	**11.4**	0.1
191	11.3	11.5	**11.4**	0.1

Experiment D: *Desulfovibrio desulfuricans*, 200 µM nitrite, no ^{18}O-enriched water
Sulfur isotope values and mean values (n = 2) with standard deviation (± 1 σ)

Time (hours)	δ^{34}S [‰] Bottle 1	δ^{34}S [‰] Bottle 2	**δ^{34}S [‰] Mean values**	± 1 σ
0	5.0	4.8	**4.9**	0.2
26	5.6	5.5	**5.5**	0.0
46	7.9	9.1	**8.5**	0.9
70	10.8	10.8	**10.8**	0.0
95	10.7	10.8	**10.8**	0.1
191	10.9	11.1	**11.0**	0.1

Experiment E: *Desulfovibrio desulfuricans*, 500 µM nitrite, no ^{18}O-enriched water
Sulfur isotope values and mean values (n = 2) with standard deviation (± 1 σ)

Time (hours)	δ^{34}S [‰] Bottle 1	δ^{34}S [‰] Bottle 2	**δ^{34}S [‰] Mean values**	± 1 σ
0	4.9	5.0	**4.9**	0.0
70	5.2	5.6	**5.4**	0.3
162	7.9	6.7	**7.3**	0.8
170	8.0	6.7	**7.4**	0.9
215	8.7	7.5	**8.1**	0.9
381	11.0	11.3	**11.2**	0.2

Experiment F: *Desulfovibrio desulfuricans*, 1000 µM nitrite, no ^{18}O-enriched water
Sulfur isotope values and mean values (n = 2) with standard deviation (± 1 σ)

Time (hours)	δ^{34}S [‰] Bottle 1	δ^{34}S [‰] Bottle 2	**δ^{34}S [‰] Mean values**	± 1 σ
0	4.9	4.9	**4.9**	0.0
330	5.4	5.2	**5.3**	0.1
403	6.6	7.0	**6.8**	0.3
953	10.6	10.5	**10.5**	0.0

Calculations for the isotope enrichment factor Figure 3.4:

Desulfovibrio desulfuricans	Ln(C_t/C_0)	Ln(R_t/R_0)
Experiment A: no addition of nitrite, no ^{18}O-enriched water	0	0
	-0.08613	0.00131
	-0.28711	0.00312
	-0.46202	0.00563
	-0.32277	0.00558
	-0.4684	0.00548
Experiment B: no addition of nitrite, ^{18}O-enriched water	0	0
	-0.10844	0.00144
	-0.3345	0.0033
	-0.36268	0.00551
	-0.49852	0.00565
	-0.36594	0.00562
Experiment C: 100 µM nitrite, ^{18}O-enriched water	0	0
	-0.05132	6.39E-04
	-0.33435	0.00359
	-0.35473	0.00555
	-0.43901	0.00643
	-0.41579	0.00638
Experiment D: 200 µM nitrite, ^{18}O-enriched water	-0.02225	0
	-0.0496	5.95E-04
	-0.30489	0.00353
	-0.48746	0.00584
	-0.39096	0.00581
	-0.3787	0.00603
Experiment E: 500 µM nitrite, ^{18}O-enriched water	-6.09E-04	0
	-0.01226	4.24E-04
	-0.12009	0.0023
	-0.16085	0.00242
	-0.15711	0.00314
	-0.19771	0.00617
Experiment F: 1000 µM nitrite, ^{18}O-enriched water	0	0
	-0.0253	4.31E-04
	-0.10461	0.0019
	-0.21991	0.00562

CHAPTER 3:

Nitrate concentrations and optical density for the experiments figures 4.2, 4.3, and 4.4:

Thauera aromatica with ^{18}O-unlabeled and labeled media grown on acetate
Optical density, measured at 380 nm and nitrate concentrations in mM

$\delta^{18}O_{water}$ ~ - 10 ‰			**$\delta^{18}O_{water}$ ~ + 700 ‰**		
Time (hours)	OD (380 nm)	NO_3^- (mM)	Time (hours)	OD	NO_3^- (mM)
0	0.14	9	0	0.13	8.6
5	0.15	8.8	5	0.18	7.7
9.5	0.14	8.3	9.5	0.30	5
15	0.24	7.6	15	0.43	2.1
17	0.33	6.1	17	0.45	2
20	0.50	3.5	19	0.41	1.8
24	0.44	3.9	24	0.41	1.4
45	0.45	3	47	0.40	1.4

Thauera aromatica with ^{18}O-unlabeled and labeled media grown on toluene
Optical density, measured at 380 nm and nitrate concentrations in mM

$\delta^{18}O_{water}$ ~ - 10 ‰			**$\delta^{18}O_{water}$ ~ + 700 ‰**		
Time (hours)	OD (380 nm)	NO_3^- (mM)	Time (hours)	OD	NO_3^- (mM)
5	0.16	7.1	10	0.15	8.4
10	0.17	7.1	15	0.16	7.2
20	0.18	7.8	20	0.19	7.1
24	0.26	7.1	30	0.35	4.4
39	0.40	3.3	n.a	n.a	n.a
44	0.46	3.5	n.a	n.a	n.a

Oxygen isotope values and mean values for the experiments Figure 4.3:

Thauera aromatica with ^{18}O-unlabeled and labeled media grown on toluene

Oxygen isotope mean values with standard deviation (± 1 σ)

$\delta^{18}O_{water}$ ~ - 10 ‰			**$\delta^{18}O_{water}$ ~ + 700 ‰**		
Time (hours)	δ^{18}O	± 1 σ	Time (hours)	δ^{18}O	± 1 σ
0	18.4	0.6	0	23	0.3
5	18.7	0.3	10	24.4	0.8
10	19	0	15	22.7	0
20	20.5	0	20	29.3	0.4
24	20.3	0.1	30	24.5	1.7
39	13.4	0			

Nitrogen isotope values and mean values for the experiments Figure 4.4:

Thauera aromatica with ^{18}O-unlabeled and labeled media grown on acetate
Nitrogen isotope mean values with standard deviation (± 1 σ)

$\delta^{18}O_{water}$ ~ - 10 ‰			**$\delta^{18}O_{water}$ ~ + 700 ‰**		
Time (hours)	$\delta^{15}N$	± 1 σ	Time (hours)	$\delta^{15}N$	± 1 σ
0	-2.8	0.2	0	-1.8	0.1
5	-0.9	0.2	5	-0.4	0.3
9.5	-2.7	0.1	9.5	11.4	0.3
15	0.7	0	15	25.4	0.2
17	5.8	0.4	17	24.6	2.5
20	9.5	0.7	19	26.1	3.2
24	10.3	0.5	24	25	1.5
45	12.4	1.1	47	23.1	2.6

Thauera aromatica with ^{18}O-unlabeled and labeled media grown on toluene
Nitrogen isotope mean values with standard deviation (± 1 σ)

$\delta^{18}O_{water}$ ~ - 10 ‰			**$\delta^{18}O_{water}$ ~ + 700 ‰**		
Time (hours)	$\delta^{15}N$	± 1 σ	Time (hours)	$\delta^{15}N$	± 1 σ
0	-2.5	0.1	0	-0.1	-
5	-2.5	0.3	10	-0.5	-
10	-1.6	0.1	15	0.5	0.5
20	0.9	1.5	20	2.2	0.4
24	4.3	0.6	30	14.1	0.3
39	15.3	0.2			
44	16.1	1.4			

Calculations for the isotope enrichment factor Figure 4.5:

Thauera aromatica with ^{18}O-unlabeled and labeled media grown on acetate

$\delta^{18}O_{water}$ ~ - 10 ‰		**$\delta^{18}O_{water}$ ~ + 700 ‰**	
Ln(C_t/C_o)	Ln(R_t/R_o)	Ln(C_t/C_o)	Ln(R_t/R_o)
-0.1008	0.0000	-0.1475	0.0000
-0.1279	0.0020	-0.2577	0.0014
-0.1903	0.0001	-0.6874	0.0131
-0.2682	0.0036	-1.5440	0.0268
-0.4865	0.0086	-1.5935	0.0261
-1.0603	0.0123	-1.7311	0.0275
-0.9378	0.0131	-1.9778	0.0265
-1.2108	0.0152	-1.9344	0.0246

Thauera aromatica with ^{18}O-unlabeled and labeled media grown on toluene

$\delta^{18}O_{water}$ ~ - 10 ‰		**$\delta^{18}O_{water}$ ~ + 700 ‰**	
Ln(C_t/C_o)	Ln(R_t/R_o)	Ln(C_t/C_o)	Ln(R_t/R_o)
-0.2620	0.0000	0.0011	0.0000
-0.3396	0.0000	-0.0563	-0.0004
-0.3357	0.0010	-0.2187	0.0006
-0.2524	0.0035	-0.2244	0.0024
-0.3362	0.0069	-0.7086	0.0141
-1.1068	0.0178		
-1.0603	0.0185		

CURRICULUM VITAE

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EDUCATION

10/1997	Study of Geology/Paleontology at the Ludwig-Maximilians-University Munich
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Geological mapping of Blatt 7136 Neustadt at the Danube (Southeastern part)

DIPLOMA THESIS

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PROMOTION

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SELECTED PUBLICATIONS

Mangalo M., Meckenstock R.U., Einsiedl F. (2006): Oxygen and sulfur isotope effects governed by bacterial sulfate reduction. Poster, EGU Tagung Wien

Mangalo M., Meckenstock R.U., Stichler W., Einsiedl F. (2006): What can the oxygen-18 exchange of sulfate and water tell us about stable isotope fractionation during bacterial sulfate reduction. Poster, ISMSM Münster

Mangalo M., Meckenstock R.U., Stichler W., Einsiedl F. (2007): Stable isotope fractionation during BSR is governed by reoxidation of intermediates. Vortrag, EGU Tagung Wien

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Mangalo, M., Einsiedl F., Meckenstock R.U., Stichler W. (2008): Influence of the enzyme dissimilatory sulfite reductase on stable isotope fractionation during sulfate reduction. *Geochim. et Cosmochim. Acta* **72**: 1513-1520

Mangalo, M., Einsiedl F., Meckenstock R.U., Stichler W. (submitted to *ES&T*): Stable isotope fractionation during denitrification by strain *Thauera aromatica*.