An Aquifer in the Deep Subsurface - Microbiology and Sulfur Isotope Fractionation

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Dedicated to my parents, my family, and my friends. Thank you.

Contents

Abbreviations

CDT	Canyon Diablo Troilite
DAPI	4',6-Diamidino-2-phenylindole
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures)
dm	dry mass
FISH	Fluorescence in-situ Hybridization
$G^{0'}$	Change of Gibbs free energy of reaction at standard conditions, pH = 7
HPLC	High Performance Liquid Chromatography
MPN	Most-Probable-Number
PCR	Polymerase Chain Reaction
PDB	Belemnitella americana from the Cretaceous Peedee formation
R	gas constant (= $8.31451 \cdot 10^{-3}$ kJ • mol^{-1} • K^{-1})
SMOV	Standard Mean Ocean Water
s(p)p.	species
T	Absolute thermodynamic temperature (K)

Summary

Sulfate-reducing bacteria and sulfur isotope fractionation were examined near the open-pit lignite mine Garzweiler I, Germany. The general lithostratigraphic column in the Lower Rhene Embayment comprises a succession of marine sands of Tertiary age with intercalating lignite seams covered by Quaternary fluvial sediments. The lignite seams confine multiple aquifers. Evidence for recent reductive microbial processes in the deeper aquifers was furnished by a comprehensive microbiological characterization.

A complete anaerobic food chain was present in the investigated aquifer. MPN-counts revealed that sulfate-reducing and fermenting microorganisms were present up to a number of $1.5 \cdot 10^4$ cells/g dm. Groundwater from this aquifer harbored $7.3 \cdot 10^5$ cells/ml. *Bacteria*, 51.9% of total cells, were most dominant and another 25.7% of total cells could be affiliated to the domain *Archaea*. Among the *Bacteria*, the beta-subclass of the *Proteobacteria*, 21.0% of total cells, was most abundant and 2.5% of total cells were identified as *Desulfotomaculum* spp..

Members of the genus *Desulfotomaculum* were frequently encountered in samples from the Garzweiler aquifers. Newly isolated strains are proposed as *Desulfotomaculum garzweileriensis* and *Desulfotomaculum carboniphilus*. Obviously, *Desulfotomaculum* spp. with their ability to form spores and to perform homoacetogenesis during sulfate limitation are particularly adapted to aquifer conditions.

The characterization of the sulfur isotope fractionation during dissimilatory sulfate-reduction of aquifer isolates and another 31 sulfate-reducers revealed a fundamental difference between complete oxidizers and incomplete oxidizers. The type of electron donor is probably one of the regulating factors for sulfur isotope fractionation. Highest δ^{34}S-depletions (42.0‰) were determined for *Desulfonema magnum*. The fractionations observed for *Desulfotomaculum* spp. can explain the ^{34}S-depletions in the aquifers at the Garzweiler study site.

The sulfate-reduction rate was determined in one of the aquifers close to the lignite seam. The highest activity was observed at the interface between aquifer and lignite seam (8.4 mM sulfate • a^{-1}). Interestingly, this interface harbored also highest cell numbers of sulfate-reducing and fermentative microorganisms, suggesting that the sulfate-reduction in the aquifer is controlled by the availability of lignite-derived organic compounds of low molecular mass.

Nitrate-reducers were enriched on lignite as electron donor. During growth on lignite an increase in the H/C-ratio and a decrease in aromatic fraction for the residual lignite was observed, indicative of an anaerobic oxidation of lignite compounds.

Chapter 1.

General Introduction

1.1. Introduction

1.1.1. The deep subsurface

The first report on microbes in the subsurface goes back to the year 1677, when the Dutch microbiologist Antonie van Leeuwenhoek first observed microbial presence in a water well (130). In the years to come this phenomenon attracted scientists again and again. Today numerous deep drilling programs explore the geology and the biology of the subsurface (for review, see ref. 88). Considering the huge volumes of subsurface sediment and rock that may be colonized, the extent of subsurface biomass could potentially approach or exceed levels of colonization observed on the surface (47).

Several definitions for the term „deep subsurface" have been proposed (15, 46). In the following, it will be used as defined by Lovley & Chapelle for microbiological studies (82). This definition is independent of total depth; instead it depends entirely on the hydrologic framework of the system under study. Groundwater flow systems in the „deep subsurface" are characterized by relatively low recharge rates and have little connection to the surface (128).

Over the last decade several very different deep subsurface ecosystems, such as contaminated, marine, hot, granitic, and lithotrophic systems have been studied (for review, see refs. 46, 70, 71, 81, 82, 88, 103, 125). Because of increasing drinking water demands there is growing interest in the research of deep subsurface groundwater systems. Additionally, potential deep subsurface nuclear waste repository sites were investigated. Here, the understanding of the natural potential for microbial corrosion of nuclear waste containers and microbial radionucleotide transport is of importance (5, 65). In comparison to other countries, there has been little interest in microbiological research of deep subsurface ecosystems in Germany so far. Only three authors, Marxen (89), Hirsch *et al.* (52), and Kilb (68) have characterized shallow aquifers in the Fulda Valley, Segeberger Forst, and Insel Hengsen, respectively.

The site, investigated in the present study is situated near the open-pit lignite mine Garzweiler I, between Mönchengladbach and Cologne, Germany. The groundwater system has been geologically characterized (4, 25, 120, 121). The general lithostratigraphic column in the Lower Rhene Embayment comprises a succession of marine sands of Tertiary age with intercalating lignite seams covered by up to 100 m of Quaternary fluvial sediments (Fig. 1). The lignite seams confine a multiple aquifer system. Geochemical investigations revealed ferric iron-reduction, sulfate-reduction, and methano-genesis occuring in the aquifers. Sulfate-reduction proceeds throughout the deeper aquifers and at the interface between lignite seams and aquifers, as indicated by sulfur isotope data (120).

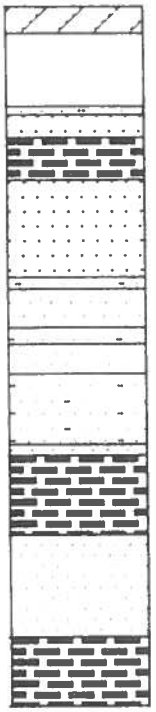

Fig. 1. Lithostratigraphic column of the study site (after ref. 121).

1.1.2. Stable isotopes and their use in the characterization of the subsurface

In a situation, where hydrochemical parameters undergo frequent temporal changes, hydrochemical measurements might represent only a snapshot and are, therefore, difficult to interpret. Based solely on hydrochemical parameters it is often impossible to characterize processes in detail, because the reaction equilibrium may be biologically catalyzed or kinetically inhibited. In this situation, stable isotopes can provide additional information and are, therefore, frequently used as part of a comprehensive geochemical approach. In many ecosystems the isotope records are available for long time periods and serve as a tracer for temporal variations of specific biological processes. Additionally, variations in the stable isotope composition may allow the identification of predominant processes and can help to elucidate the appropriate sources and sinks.

The isotope composition of a substance is commonly expressed in the δ-notation, which expresses the isotope composition of a sample (in ‰) relative to a standard. For sulfur isotopes the ratio is given by

$$\delta^{34}S = (R_{sample}/R_{standard} - 1) \cdot 1000 \quad (1)$$

where $R = {}^{34}S/{}^{32}S$ (carbon: ${}^{13}C/{}^{12}C$, oxygen: ${}^{18}O/{}^{16}O$). The reference standard for sulfur isotopes is the Canyon Diablo Troilite (CDT); for carbon it is Belemnitella americana from the Cretaceous Peedee formation (PDB), and for oxygen the standard is Standard Mean Ocean Water (SMOW) (54).

An unequal distribution (fractionation) of isotopes is frequently observed during physical, chemical, or biological reactions. Isotope fractionations are produced during a kinetic process or during an isotope exchange reaction. An isotope fractionation is expressed in terms of the ratios of the isotope composition in the product relative to the reactant. This ratio is termed the fractionation factor (α). During dissimilatory sulfate reduction for example, α is given by

$$\alpha = (R_{product}/R_{reactant}), \text{ where } R = {}^{34}S/{}^{32}S \quad (2)$$

Stable carbon, oxygen, sulfur, hydrogen, and nitrogen isotopes are of major interest in geochemistry (54). Stable carbon isotopes can be used, for example, to investigate the origin of inorganic carbon in the carbonate system (54) or to differentiate between different CO_2-fixation pathways in plants or bacteria (28, 40) and, thus, to reveal the biological precursors of fossil hydrocarbons. For example, the lignite in the Rhenish lignite mining area has a carbon isotope composition ($\delta^{13}C$) between –24‰ and –28‰ (PDB) indicating an origin from terrestrial plant (120). The isotope signature of the sedimentary organic carbon in the Garzweiler aquifers is characterized by $\delta^{13}C$-values around –26‰. The dissolved organic carbon displayed a $\delta^{13}C$-value of 28.9 ± 2.4‰. Together with additional hydrochemical data, this indicates that the dissolved organic matter in the groundwater is likely lignite derived and that the lignite originates from terrestrial C_3-plant detritus (120).

Oxygen isotopes, for example, can help to identify groundwater formation processes. For the Garzweiler aquifers it was shown that the groundwater is of meteoric origin (120). Additionally, an investigation of ${}^{18}O$ and ${}^{34}S$-isotopes of dissolved sulfate in rainfall and

groundwater revealed increased $\delta^{18}O$ and $\delta^{34}S$-values for the groundwater of the unsaturated zone above the uppermost lignite seam, indicating recent microbial sulfate-reducing activity in this zone (120).

Sulfur isotopes are relatively widely used in biogeochemistry, because they allow the differentiation between chemically and biologically mediated processes. Additionally, in recent years sulfur isotopes have been used to help elucidate mechanisms of biological sulfur transformation (26, 123). Generally, the sulfur cycle is controlled by oxidative, reductive, and disproportionation processes. There are contradictory results about the sulfur isotope fractionation during biological oxidation of sulfide (13). No fractionation (49) or a small inverse fractionation (38) were observed, however, available data is rare. The reduction of sulfate is coupled to a discrimination of the ^{34}S-isotope resulting in an enrichment of the ^{32}S isotope in the produced sulfide. This kinetic isotope effect produces the largest fractionations in the sulfur cycle (132). Modern sedimentary pyrites for example, are depleted in ^{34}S relative to sulfate by up to 50‰ (126) and some marine sedimentary sulfides even show depletions up to 70‰ (8, 9). Interestingly, these depletions were never observed in pure culture experiments. Studies on *Desulfovibrio desulfuricans* revealed a maximum fractionation of 46‰ (62). Additionally, the disproportionation of sulfite, thiosulfate, and sulfur is also coupled to an isotope fractionation. Maximum $\delta^{34}S$-depletions of sulfides with values of up to 37‰ were found during sulfite disproportionation (9, 49). Currently, the large ^{34}S-depletion of sedimentary sulfides is thought to result from initial fractionation by sulfate-reducing microorganisms followed by further fractionation during the disproportionation of re-oxidized sulfur compounds (9, 10, 60). This repeated cycling could explain the large ^{34}S-depletion of sulfides in natural sediments although it remains unclear to what extend the partly oxidized sulfur compounds like elemental sulfur, thiosulfate, and sulfite are formed under strictly anoxic conditions.

For the Garzweiler aquifers it has been shown, that with increasing depth the $\delta^{34}S$-values increased paralleled by increasing hydrogen sulfide and decreasing sulfate concentrations. Maximum depletions in ^{34}S of up to 50‰ have been observed, indicative for the activity of sulfate-reducing microorganisms (120).

1.1.3. Microbiological characterization of the subsurface

In recent years a variety of investigations have focused on microbially mediated processes in the subsurface (for reviews see refs. 46, 71, 81, 82, 103). The availability of uncontaminated samples is a major prerequisite for microbiological investigations of the deep subsurface. Several protocols, including different sampling procedures, various drilling methods, and the use of drilling fluids containing tracers to assure sample quality have been developed (48, 74, 92, 117).

Microbiological methods have been adapted to subsurface conditions for revealing structure and function of microbial communities in this ecosystem (88). The cultivation and isolation of subsurface microorganisms offers the possibility to investigate their physiological properties (refs. 27, 72, 97). Most-probable-number (MPN) approaches reveal the abundance of culturable physiological groups, but usually only a minority of abundant organisms is culturable to date (118). Nucleic acid-based methods provide more comprehensive information on the species composition (5, 35, 39, 104, 105), but due to the PCR- and cloning-biases, this approach provides little quantitative information. The fluorescence in-situ hybridization (FISH) has been rarely used in subsurface environments because of relatively high background autofluorescence. Membrane lipids specific for phylogenetic groups have been frequently isolated from subsurface environments (37, 67, 134), but this method is not quantitative and the interpretation of results strongly depends on the existing database, additionally, the formation of membrane lipids is strongly substrate-dependant (87). Integrated approaches, using cultivation techniques and molecular methods give a comprehensive overview of the microbial community structure (14, 58, 101, 113).

Geomicrobiological investigations frequently focused on the microbial activity in the deep subsurface. Several methods based on measurement of the consumption of electron donors and electron acceptors are available to determine the *in-situ* activity of microorganisms. The characterization of the carbon utilization capacity of a microbial

community by autoradiography using radiolabeled substrates allows a very sensitive measurement of *in-situ* metabolic rates (5, 64, 65, 106, 107). Radiolabeled electron acceptors, such as ^{35}S-sulfate, are sensitive tools in determining turnover rates on a community level (34, 57, 59, 73, 95). Biolog™-microplates may characterize the physiological potential of a community rather than the actual *in-situ* activity (23, 90). Messenger-RNA-based methods may be used to investigate the *in-situ* activity of specific phylogenetic groups, e.g. (110, 119).

The major limiting factor for microorganisms in the subsurface is the availability of nutrients. Additionally, in the deep subsurface, high temperatures may prevent microbial colonization (47) and in volcanic tuff in Yucca Mountain, Nevada, water availability is the major limiting factor for microorganisms (65). Another limiting factor, specific to the subsurface, may be the clogging of the sediment. The porosity of the sediment limits microbial activity (2, 24, 36); a well-documented example is the formation of intergranular secondary calcite cements due to microbial oxidation of organic matter (93).

Different subsurface geological settings select for unique physiological features within the resident microflora. Resting cells and spores confer the ability for long term survival (5, 66). Subsurface microorganisms are adapted to slow growth and low nutrient concentration (71). In pristine aquifers, microbial metabolism is often limited by the availability of electron donors. Slow oxidation of natural sedimentary organic matter leads to a sequential consumption of electron donors in the sequence oxygen – nitrate – ferric iron – sulfate – carbon dioxide (16, 82, 86). The *in-situ* hydrogen concentration is an indicator for the respective terminal electron accepting process (84).

Additionally, homoacetogenesis is also of importance in subsurface environments. When samples of sandstone and ground shale were combined, acetate was produced proportional to the addition of shale organic matter (73). The fermentation of organic matter is observed in organic rich subsurface sediments. Fatty acids produced within clay (93, 94) and fermentation products from lignite deposits (129) fueled sulfate-reduction in adjacent sandy sediments.

1.1.4. Lignite as a substrate for microorganisms

Coal is a physically and chemically heterogeneous material originating from living terrestrial organic matter (Fig. 2.) (51). During peatification, plant debris is partly mineralized by microorganisms. When sediments cover the peat over large periods of time, pressure and temperature slowly alter the chemical structure. Depending on geochemical conditions the coal subsequently undergoes a sequence of coalification phases and with increasing coal rank, the water content decreases whereas the carbon content and the aromaticity increases (50).

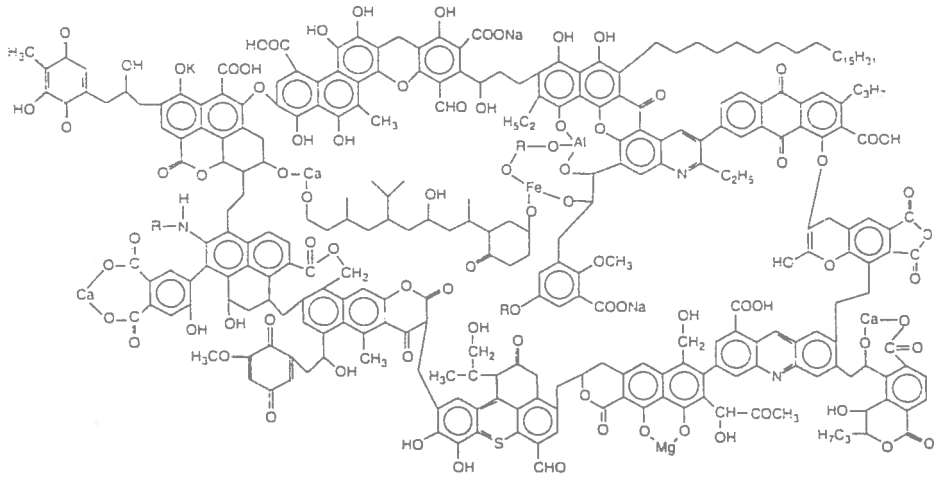

Fig. 2. Proposed structural unit of Rhenish lignite (after ref. 56).

The Rhenish lignite is characterized by relatively high moisture content and low sulfur content compared to lignite from other German deposits (56). The humic acids and the fulvic acids are water-soluble. They have an average molecular mass between 300 and 5000 Dalton. The hydrophobic fraction consists of resins, waxes, and aliphatic compounds (31, 53).

There is no generally accepted chemical structure for lignite available; a fundamental problem in the elucidation of lignite structure is the alteration of chemical structures during analysis. Consequently, current models depend on the employed methods. Microbial transformation and degradation of lignite and lignite-derived compounds are therefore monitored by relative changes in elemental composition, molecular mass, aromaticity, and functional groups (31).

The first report on microorganisms isolated from coal dates back to the year 1908 (108). In the following two decades, several investigations focused on microbiological aspects of coal storage and the use of coal as a fertilizer (41, 44, 76, 77, 78, 79). Subsequently, there was little further interest in this field until Fakoussa showed in 1981 that bacteria could utilize fractions of hard coal (30) using extracellular enzymes and solubilizing agents. Cohen & Gabriele observed that wood-rot fungi have an even higher potential for coal-solubilisation (19). In the following years, two bioconversion mechanisms were discovered. First, chelators and alkaline metabolic compounds mediate the solubilisation of lignite by fungi. Second, peroxidases, laccases, and hydrolases catalyze the depolymerisation of lignite fractions. The understanding of complex lignite-enzyme interactions is still developing and the yield of lignite bioconversion is too low to be economically interesting (31). There are attempts to produce biopolymers with polyhydroxyalkanoate-accumulating strains of *Pseudomonas oleovorans* and *Rhodococcus ruber*, growing on biosolubilised lignite fractions as sole carbon source (42).

A second application in coal microbiology is the biodesulfurisation of coal by sulfide oxidation. Strains of *Leptospirillum* sp. and *Rhodococcus* sp. play a key role in metal sulfide removal from lignite (6, 29, 69). Contradictory results about antibiotic effects of coal compounds are reported. However, several bacterial and fungal strains are obviously not inactivated by these compounds at natural concentrations (for review, see refs. 11, 31, 114, 115).

There are several reports on microbial communities oxidizing lignite-derived organic matter in pristine aquifers (17, 98, 129). Preparations of sterilized, anoxic, lignite-rich sediment slurries accumulated micromolar concentrations of formate, acetate, and propionate (129). Several lignite constituents (aromatic and aliphatic hydrocarbons) are known to be used as electron donor and carbon source under anoxic conditions, e.g. (45, 109, 116). Nevertheless, an oxidation of substantial amounts of natural lignite by a pure culture under anoxic conditions has not been shown yet.

Numerous investigations focused on the degradation of lignite model-compounds (1, 7, 32, 33, 43, 55, 61, 96, 99, 122, 131), however, the relative portion of each of these compounds in natural lignite is extremely low, allowing few conclusions on lignite degradation. Lignin, a substantial component of lignite, was found to be fairly resistant to

anoxic degradation (133), even though a partial transformation to CO_2 and methane was observed using ^{14}C-labeled lignin-derived oligomers (3, 20, 21, 22). Humic substances extracted from coal are oxidized under anoxic conditions by several microorganisms (83). Additionally, under anoxic conditions, humics can serve as electron acceptor for microbial respiration (18). Reduced humics can subsequently reduce Fe(III) oxides, thus shutteling electrons from humic reducers to Fe(III) oxides (85).

The heterogeneity of structures and bonds, partial insolubility and the high degree of polymerisation make lignite a difficult substrate for microorganisms.

1.2. Aims of the study

Evidence for sulfate-reduction and methanogenesis in the deep subsurface of the Garzweiler study site was found through geochemical methods. This finding raises further questions:

1.2.1. Structure, metabolic activity and sulfate-reduction rate of the microbial community

Generally, sulfate-reduction and methanogenesis in the subsurface are well-investigated processes (46, 82). Nevertheless, to reveal the microbiological potential of an ecosystem, specific physiological groups should be quantified (Chapter 2.). For a microbiological verification of the geochemical evidence, the responsible organisms should be cultured and characterized (Chapter 6.1.). Nucleic acid based methods may be used to characterize the non-culturable fraction of the microbial community (Chapter 3.). A determination of the sulfate-reduction rate to localize the sulfate-reducing activity completes the fundamental microbiological characterization of the deeper aquifers and the adjacent lignite seam (Chapter 2.).

1.2.2. Can lignite serve as substrate for microorganisms under anoxic conditions?

There have been speculations about the oxidation of lignite-derived compounds under anoxic conditions (17, 98, 129). However, no lignite-oxidizing pure culture is available to date (11). Obviously, it is difficult to obtain pure cultures growing on lignite as sole electron donor and carbon source. Nevertheless, it should be useful to establish enrichments on different types of lignite, to reveal the phylogeny of responsible organisms and to determine turnover rates (Chapter 6.2.).

1.2.3. Can the sulfur isotope signatures in nature be explained on the basis of enzymatic reactions during dissimilatory sulfate reduction?

A number of studies have focused on the sulfur isotope fractionation during dissimilatory sulfate-reduction by pure cultures. The influence of temperature, sulfate-reduction rate, substrate, species, and sulfate concentration were investigated (for review, see 13). However, previous studies have focused on the fractionation of only a few selected organisms that have been investigated at that time, mainly *Desulfovibrio* and two *Desulfotomaculum* species (12, 38, 62, 91). Today a broad variety of sulfate-reducers are available in pure culture, but only preliminary information on the abundance of these genera in their respective habitats is available (75, 80, 110, 111, 127).

Most of the strains investigated to date are incompletely oxidizing sulfate-reducers. These organisms oxidize the carbon source to acetate, which is then excreted. In contrast, the effect of complete-oxidizing metabolism on sulfur isotope fractionation during dissimilatory sulfate-reduction has yet to be investigated. Various attempts have been made to develop models for the sulfur isotope fractionation during dissimilatory sulfate reduction (13, 62, 112), but none of these models take the physiology of sulfate reducers into account. Furthermore, previous studies (62, 63) were conducted with substrates that may not be relevant in sulfate-reducing environments (102, 124). Aim of this part of the study is the characterization of a broad range of environmentally relevant sulfate-reducers, oxidizing natural substrates under a broad range of relevant environmental conditions (Chapter 4. & Chapter 6.3.). For the first time, this study reveals the effect of the electron donor on the sulfur isotope fractionation. A model explaining the fractionation on a physiologcal level is developed (Chapter 4.).

The reaction kinetics of microbes in biofilms is a subject of ongoing debate. For example, free-living sulfate-reducers have a different K_m-value for sulfate compared to those assembled in a biofilm (100). Thus, the formation of sulfate-reducing biofilms on sediment particles may affect the isotope fractionation and it is questionable whether fractionations observed for free-living cells can be applied to sediment conditions. This study reveals the affect of biofilm-formation on sulfur isotope fractionation (Chapter 5.).

1.3. Overview of the publications

[1] Aquifers at the Garzweiler study site were microbiologically characterized. In a combined approach hydrologic parameters, carbon and sulfur isotopes, and MPN-counts were determined, indicating the recent activity of sulfate-reducing bacteria in the aquifers. A complete anaerobic food chain (Ändern!?) (fermentative microorganisms, nitrate-reducers, ferric iron-reducers, sulfate-reducers, and methanogens) was present in the aquifers.
Cell numbers and activity of the sulfate-reducing bacteria as determined by MPN-counts and ^{35}S-radiotracer were highest at the interface between aquifer and lignite seam. The results are presented in the following manuscript:

Jan Detmers, Ulrike Schulte, Harald Strauss, and Jan Kuever: **Sulfate reduction at a lignite seam: A multidisciplinary approach**

H. Strauss and J. Kuever initiated the study. They contributed to this work with discussions about the methodology and the interpretation of results. U. Schulte did the work with the stable isotopes. J. Detmers carried out the microbiological experiments, interpretation of results and writing of the manuscript with help from co-authors. The manuscript is in press in „*Microbial Ecology*".

[2] In the second study, the microbial community in the groundwater of the aquifers at the Garzweiler study site was characterized by fluorescence in situ hybridization (FISH). This culture-independent method allows the identification of major phylogenic groups contributing to the microbial activity in the aquifers. FISH-data were combined with basic hydrochemical and isotope data for a comprehensive groundwater analysis of the study site. Results are compiled in the manuscript:

Jan Detmers, Harald Strauss, Ulrike Schulte, Axel Bergmann, Katrin Ravenschlag, and Jan Kuever: ***Desulfotomaculum*** **dominates the sulfate reducing community in a pristine aquifer**

J. Detmers initiated this study. A. Bergmann and U. Schulte determined hydrochemical and isotope parameters. FISH-determinations were performed by J. Detmers under supervision of K. Ravenschlag. J. Detmers interpreted the results and wrote the manuscript with editorial help of the co-authors. The manuscript has been submitted to „*Environmental Microbiology*".

[3] The third study is focused on the sulfur isotope fractionation by pure cultures during dissimilatory sulfate-reduction. A comprehensive overview is given of available strains oxidizing environmentally relevant substrates. A model is proposed to explain the effect of the electron donor on sulfur isotope fractionation. The results are summarized in the manuscript:

Jan Detmers, Volker Brüchert, Kirsten Habicht, and Jan Kuever: **Impact of electron flow on sulfur isotope fractionation during dissimilatory sulfate reduction**

J. Detmers and V. Brüchert, who did also the isotope analysis, developed the concept. K. Habicht contributed data about thermophilic strains. J. Kuever constructed the phylogenetic tree. J. Detmers carried out all microbiological work. V. Brüchert contributed extensively to the interpretation of data in the manuscript, that appeared in „*Applied and Environmental Microbiology*".

[4] The study on sulfur isotope fractionation in the presence of bentonit was carried out to evaluate how pure culture results can be applied to sediment conditions. The fractionation was determined at varying sediment conditions and oxygen isotope fractionation during dissimilatory sulfate-reduction was determined. Results are presented in the manuscript:

Michel E. Böttcher and Jan Detmers: **Sulfur isotope fractionation by *Desulfovibrio desulfuricans* in the presence of bentonite**

The idea was developed together with M. Böttcher. J. Detmers performed all microbiological experiments. M. Böttcher analyzed sulfur and oxygen isotopes. J. Detmers co-authored this manuscript, which is for submission in "*Geomicrobiology Journal*".

1.4. References

1. Afferden von, M., S. Schacht, J. Klein, and H.G. Trüper. 1990. Degradation of dibenzothiophene by *Brevibacterium* sp. Arch. Microbiol. 153:324 - 328.

2. Baveye, P., P. Vandevivere, B. Hoyle, P. DeLeo, and D. Sanchez de Lozada. 1998. Environmental impact and mechanisms of the biological clogging of saturated soils and aquifer materials. Crit. Rev. Envion. Sci. Tech. 28:123 - 191.

3. Benner, R. and R. Hodson. 1985. Thermophilic anaerobic biodegradation of [^{14}C]Lignin, [^{14}C]Cellulose, and [^{14}C]Ligninocellulose preparations. Appl. Environ. Microbiol. 50:971 - 976.

4. Bergmann, A. 1999. Hydrogeochemische Untersuchungen anoxischer Redoxprozesse in tiefen Porengrundwasserleitern der Niederrheinischen Bucht - im Umfeld des Tagebaus Garzweiler I, Institut für Geologie. Ruhr-Universität: Bochum. p. 167.

5. Boivin - Jahns, V., R. Ruimy, A. Bianchi, S. Daumas, and R. Christen. 1996. Bacterial diversity in a deep-subsurface clay environment. Appl. Environ. Microbiol. 62:3405 - 3412.

6. Bozdemir, T.O., T. Durusoy, E. Erincin, and Y. Yueruem. 1996. Biodesulfurization of turkish lignites. Fuel 75:1596 - 1600.

7. Cambell, J., D. Stewart, M. McClulloch, R. Lucke, and R. Bean. 1988. Biodegradation of coal-related model compounds, in ACS National Meeting, K. Vorres. American Society of Chemistry: Los Angeles. p. 514 - 521.

8. Canfield, D.E. and A. Teske. 1996. Late proterozoic rise in atmospheric oxygen concentration inferred from phylogenetic and sulfur-isotope studies. Nature 382:127-132.

9. Canfield, D.E. and B. Thamdrup. 1994. The production of ^{34}S-depleted sulfide during bacterial disproportionation of elemental sulfur. Science 266:1973 - 1975.

10. Canfield, D.E., B. Thamdrup, and S. Fleischer. 1998. Isotope fractionation and sulfur metabolism by pure and enrichment cultures of elemental sulfur-disproportionating bacteria. Limnol. Oceanogr. 43:253 - 264.

11. Catcheside, D. and J. Ralph. 1999. Biological processing of coal. Appl. Microbiol. Biotechnol. 52:16 - 24.

12. Chambers, L. and P. Trudinger. 1975. Fractionation of sulfur isotopes by continuous cultures of *Desulfovibrio desulfuricans*. Can. J. Microbiol. 21:1602 - 1607.

13. Chambers, L. and P. Trudinger. 1979. Microbiological fractionation of stable sulfur isotopes: A review and critique. Geomicrobiol. J. 1:249 - 293.

14. Chandler, D., S. Li, C. Spadoni, G. Drake, D. Balkwill, J. Fredrickson, and F. Brockman. 1997. A molecular comparison of culturable aerobic heterotrophic bacteria and 16S rDNA clones derived from a deep subsurface sediment. FEMS Microbiol. Ecol. 23:131 - 144.

15. Chapelle, F. 1993. Ground-water microbiology and geochemistry. 1. John Wiley and Sons, Inc., New York, Chichester, Brisbane, Toronto, Singapore.

16. Chapelle, F. 2000. The significance of microbial processes in hydrogeology and geochemistry. Hydrogeol. J. 8:41 - 46.

17. Chapelle, F., J. Zelibor Jr., D. Grimes, and L. Knobel. 1987. Bacteria in deep coastal plain sediments of Maryland: a possible source of CO_2 to groundwater. Wat. Resour. Res. 23:1625 - 1632.

18. Coates, J., D. Ellis, E. Blunt-Harris, C. Gaw, E. Roden, and D. Lovley. 1998. Recovery of humic-reducing bacteria from a diversity of environments. Appl. Environ. Microbiol. 64:1504 - 1509.

19. Cohen, M. and P. Gabriele. 1982. Degradation of coal by the fungi *Polyporus versicolor* and *Poria monticola*. Appl. Environ. Microbiol. 44:23 - 27.

20. Colberg, P.J. and L.Y. Young. 1982. Biodegradation of lignin-derived molecules under anaerobic conditions. Can. J. Microbiol. 28:886 - 889.

21. Colberg, P.J. and L.Y. Young. 1985. Anaerobic degradation of soluble fractions of [^{14}C-Lignin]Lignocellulose. Appl. Environ. Microbiol. 49:345 - 349.

22. Colberg, P.J. and L.Y. Young. 1985. Aromatic and volatile acid intermediates observed during anaerobic metabolism of lignin-derived oligomers. Appl. Environ. Microbiol. 49:350 - 358.

23. Colwell, F. and R. Lehman. 1997. Carbon source utilization profiles for microbial communities from hydrologically distinct zones in a basalt aquifer. Microb. Ecol. 33:240 - 251.

24. Colwell, F., T. Onstott, M. Delwiche, D. Chandler, J. Fredrickson, Q. Yao, J. McKinley, D. Boone, R. Griffiths, T. Phelps, D. Ringelberg, D. White, L. LaFreniere, D. Balkwill, R. Lehman, J. Konisky, and P. Long. 1997. Microorganisms from deep, high temperature sandstones: contraints on microbial colonization. FEMS Microbiol. Rev. 20:425 - 435.

25. Cremer, N. 1997. Hydrochemie des Grundwassers im Tagebauvorfeld Garzweiler. Diplomarbeit an der Ruhr-Universität Bochum .

26. Cypionka, H., A.M. Smock, and M.E. Böttcher. 1998. A combined pathway of sulfur compound disproportionation in *Desulfovibrio desulfuricans*. FEMS Microbiol. Let. 166:181 - 186.

27. Daumas, S., R. Cord-Ruwisch, and J.L. Garcia. 1988. *Desulfotomaculum geothermicum* sp. nov., a thermophilic, fatty acid-degrading, sulfate-reducing bacterium isolated with H_2 from geothermal ground water. Antonie van Leeuwenhoek 54:165 - 178.

28. Deines, P. 1980. The composition of reduced organic carbon, p. 329 - 406. In P. Fritz and J. Fontes (ed.), Handbook of environmental isotope geochemistry, Vol 1: The terrestrial environment. Elsevier, Amsterdam, Oxford.

29. Durusoy, T., T. Oezbas, A. Tanyolac, and Y. Yueruem. 1992. Biodesulfurization of some turkish lignites by Sulfolobus solfataricus. Energy & Fuels 6:804 - 808.

30. Fakoussa, R. 1981. Coal as substrate for microorganisms - investigations of the microbial decomposition of bituminous coals, Faculty of Higher Mathematics and Natural Sciences. Rheinische Friedrich-Wilhelms Universität: Bonn. p. 212.

31. Fakoussa, R. and M. Hofrichter. 1999. Biotechnology and microbiology of coal degradation. Appl. Microbiol. Biotechnol. 52:25 - 40.

32. Fedorak, P.M. and D. Grbic-Galic. 1991. Aerobic microbial cometabolism of benzothiophene and 3-methylbenzothiophene. Appl. Environ. Microbiol. 57:932 - 940.

33. Fedorak, P.M., J.D. Payzant, D.S. Montgomery, and D.W.S. Westlake. 1988. Microbial degradation of n-alkyl tetrahydrothiophenes found in petroleum. Appl. Environ. Microbiol. 54:1243 - 1248.

34. Fossing, H. and B.B. Jørgensen. 1989. Measurement of bacterial sulfate reduction in sediments: Evaluation of a single-step chromium reduction method. Biogeochemistry 8:205 - 222.

35. Franklin, R., D. Taylor, and A. Mills. 1999. The distribution of microbial communities in anaerobic and aerobic zones of a shallow coastel plain aquifer. Microb. Ecol. 38:377 - 386.

36. Fredrickson, J., J. McKinley, B. Bjornstad, P. Long, D. Ringelberg, D. White, L. Krumholz, J. Suflita, F. Colwell, R. Lehman, and T. Phelps. 1997. Pore-size constraints on the activity and survival of subsurface bacteria in a Late Cretaceous Shale-Sandstone sequence, Northwestern New Mexico. Geomicrobiol. J. 14:183 - 202.

37. Fredrickson, J., J. McKinley, S. Nierzwicki-Bauer, D. White, D. Ringelberg, S. Rawson, S. Li, F. Brockman, and B. Bjornstad. 1995. Microbial community structure and biogeochemistry of miocene subsurface sediments: implications for long-term microbial survival. Mol. Ecol. 4:619 - 624.

38. Fry, B., H. Gest, and J. Hayes. 1988. $^{34}S/^{32}S$ Fractionation in sulfur cycles catalysed by anaerobic bacteria. Appl. Environ. Microbiol. 54:250 - 256.

39. Fry, N., J. Fredrickson, S. Fishbain, M. Wagner, and D. Stahl. 1997. Population structure of microbial communities associated with two deep, anaerobic, alkaline aquifers. Appl. Environ. Microbiol. 63:1498 - 1504.

40. Fuchs, G. 1986. CO_2 fixation in acetogenic bacteria: variations on a theme. FEMS Microbiol Rev 39:181 - 213.

41. Fuchs, W. 1927. Über die Entwicklung von Bakterien auf Kohle. Brennstoff Chemie 20:324 - 326.

42. Füchtenbusch, B. and A. Steinbüchel. 1999. Biosynthesis of polyhydroxyalkanoates from low-rank coal liquefaction products by *Pseudomonas oleovorans* and *Rhodococcus ruber*. Appl. Microbiol. Biotechnol. 52:91 - 95.

43. Gallagher, J.R., E.S. Olson, and D.C. Stanley. 1993. Microbial desulfurization of dibenzothiophene: A sulfur-specific pathway. FEMS Microbiol. Let. 107:31 - 36.

44. Galle, E. 1910. Über die Selbstentzündung der Steinkohle. Zentralb. Bakteriol. Parasitenk. 28:461 - 473.

45. Galushko, A., D. Minz, B. Schink, and F. Widdel. 1999. Anaerobic degradation of naphthalene by a pure culture of a novel type of marine sulphate-reducing bacterium. Environ. Microbiol. 1:415 - 420.

46. Ghiorse, W. and J. Wilson. 1988. Microbial Ecology of terrestrial subsurface. Adv. Appl. Microbiol. 33:107 - 172.

47. Gold, T. 1992. The deep, hot biosphere. Proc. Nat. Acad. Sci. 89:6045 - 6049.

48. Griffin, W., T. Phelps, F. Colwell, and J. Fredrickson. 1997. Methods for obtaining deep subsurface microbiological samples by drilling, p. 23 - 44. In P. Amy and D. Haldeman (ed.), The microbiology of the terrestrial deep subsurface. Lewis, Boca Raton, New York.

49. Habicht, K., D. Canfield, and J. Rethmeier. 1998. Sulfur isotope fractionation during bacterial reduction and disproportionation of thiosulfate and sulfite. Geochim. Cosmochim. Acta 62:2585 - 2595.

50. Haenel, M. 1992. Recent process in coal structure research. Fuel 71:1211 - 1223.

51. Hatcher, P.G. and D.J. Clifford. 1997. The organic geochemistry of coal: from plant materials to coal. Org. Geochim. 27:251 - 274.

52. Hirsch, P. and E. Rades-Rohkohl. 1990. Microbial colonization of aquifer sediment exposed in a groundwater well in Northern Germany. Appl. Environ. Microbiol. 56:2963 - 2966.

53. Hodek, W. 1994. The chemical structure of coal in regard of microbiological degradation. Fuel Proc. Technol. 40:369 - 378.

54. Hoefs, J. 1997. Stable isotope geochemistry. 4th edition Springer, Berlin.

55. Hopper, W. and A. Mahadevan. 1997. Degradation of catechin by *Bradyrhizobium japonicum*. Biodegradation 8:159 - 165.

56. Hüttinger, K. and A. Michenfelder. 1987. Molecular structure of brown coal. Fuel 66:1164 - 1165.

57. Jakobsen, R. and D. Postma. 1994. In situ rates of sulfate reduction in an aquifer (Rømø, Denmark) and implications for the reactivity of organic matter. Geology 22:1103 - 1106.

58. Jimenez, L. 1990. Molecular analysis of deep-subsurface bacteria. Appl. Environ. Microbiol. 56:2108 - 2113.

59. Jørgensen, B.B. 1978. A comparison of methods for the quantification of bacterial sulfate reduction in coastal marine sediments. I. Measurement with radiotracer techniques. Geomicrobiol. J. 1:11-27.

60. Jørgensen, B.B. 1990. A thiosulfate shunt in the sulfur cycle of marine sediments. Science 249:152-154.

61. Kanagawa, T. and D.P. Kelly. 1987. Degradation of substituted thiophenes by bacteria isolated from activated sludge. Microb. Ecol. 13:47 - 57.

62. Kaplan, I.R. and S.C. Rittenberg. 1964. Microbiological fractionation of sulphur isotopes. J. Gen. Microbiol. 34:195 - 212.

63. Kemp, A. and H. Thode. 1968. The mechanism of bacterial reduction of sulfate and of sulfite from isotopic fractionation studies. Geochim. Cosmochim. Acta 32:71 - 91.

64. Kieft, T., J. Fredrickson, J. McKinley, B. Bjornstad, S. Rawson, T. Phelps, F. Brockman, and S. Pfiffner. 1995. Microbiological comparisons within and across contiguous lacrustine, paleosol, and fluvial subsurface sediments. Appl. Environ. Microbiol. 61:749 - 757.

65. Kieft, T., W. Kovacik Jr., D. Ringelberg, D. White, D. Haldeman, P. Amy, and L. Hersman. 1997. Factors limiting microbial growth and activity at a proposed high-level nuclear repository, Yucca Mountain, Nevada. Appl. Environ. Microbiol. 63:3128 - 3133.

66. Kieft, T., D. Ringelberg, and D. White. 1994. Changes in ester-linked phospholipid fatty acid profiles of subsurface bacteria during starvation and desiccation in a porous medium. Appl. Environ. Mcrobiol. 60:3292 - 3299.

67. Kieft, T., E. Wilch, K. O´Connor, D. Ringelberg, and D. White. 1997. Survival and phospholipid fatty acid profiles of surface and subsurface bacteria in natural sediment microcosms. Appl. Environ. Microbiol. 63:1531 - 1542.

68. Kilb, B. 1999. Analyse mikrobieller Lebensgemeinschaften in anaeroben Grundwassersystemen mittels molekulargenetischer Methoden, Institut für Wasserforschung. Gesamthochschule Essen: Essen. p. 169.

69. Klein, J. 1999. Biological processing of fossil fuels. Appl. Microbiol. Biotechnol. 52:2 - 15.

70. Krumholz, L. 1998. Microbial ecosystems in the earth's subsurface. ASM News 64:197 - 202.

71. Krumholz, L. 2000. Microbial communities in the deep subsurface. Hydrol. J. 8:4 - 10.

72. Krumholz, L., S. Harris, S. Tay, and J. Suflita. 1999. Characterization of two subsurface H_2-utilizing bacteria, *Desulfomicrubium hypogeium* sp. nov. and *Acetobacterium psammolithicum* sp. nov., and their ecological roles. Appl. Environ. Microbiol. 65:2300 - 2306.

73. Krumholz, L., J. McKinley, G. Ulrich, and J. Suflita. 1997. Confined subsurface microbial communities in cretaceous rock. Nature 386:64 - 66.

74. Lehmann, R.M., F.S. Colwell, D.B. Ringelberg, and D.C. White. 1995. Combined microbial community-level analyses for quality assurance of terrestrial subsurface cores. J. Microbiol. Methods 22:263 - 281.

75. Li, J., K. Purdy, S. Takii, and H. Hayashi. 1999. Seasonal changes in ribosomal RNA of sulfate-reducing bacteria and sulfate reducing activity in a fresh water lake sediment. FEMS Microbiol. Ecol. 28:31 - 39.

76. Lieske, R. 1928. Über das Vorkommen von Bakterien in der Steinkohle - Gesammelte Abhandlungen zur Kenntnis der Kohle. Brennstoff-Chemie 17:30 - 34.

77. Lieske, R. 1928. Über die in der Kohle lebenden Bacterien und einige ihrer Eigenschaften., p. 27 - 29. In F. Fischer (ed.), Kenntnis der Kohle. Verlag von Gebrüder Borntraeger, Berlin.

78. Lieske, R. 1932. Über das Vorkommen von Bakterien in Kohlenflözen. Biochem. Zentralschr. 250:339 - 351.

79. Lieske, R. and E. Hofmann. 1928. Untersuchungen über die Mikrobiologie der Kohlen und ihrer natürlichen Lagerstätten. Brennstoff-Chemie 9:282 - 285.

80. Llobet-Brossa, E., R. Rosselló-Mora, and R. Amann. 1998. Microbial community composition of Wadden Sea sediments as revealed by fluorescence in situ hybridization. Appl. Environ. Microbiol. 64:2691-2696.

81. Lovley, D. 1997. Microbial Fe(III) reduction in subsurface environments. FEMS Microbiol. Rev. 20:305 - 313.

82. Lovley, D. and F. Chapelle. 1995. Deep subsurface microbial processes. Rev. Geophys. 33:365 - 381.

83. Lovley, D., J. Fraga, J. Coates, and E. Blunt-Harris. 1999. Humics as an electron donor for anaerobic respiration. Environ. Microbiol. 1:89 - 98.

84. Lovley, D. and S. Godwin. 1988. Hydrogen concentrations as an indicator of the predominat terminal electron-accepting reactions in aquatic sediments. Geochim. Cosmochim. Acta 52:2993 - 3003.

85. Lovley, D.R., J.D. Coates, E.L. Blunt-Harris, E.J.P. Phillips, and J.C. Woodward. 1996. Humic substances as electron acceptors for microbial respiration. Nature 382:445 - 448.

86. Ludvigsen, L., H. Albrechtsen, G. Heron, P. Bjerg, and T. Christensen. 1998. Anaerobic microbial redox processes in a landfill leachate contaminated aquifer (Grindsted, Denmark). J. Contam. Hydrol. 33:273 - 291.

87. Ludvigsen, L., H. Albrechtsen, D. Ringelberg, F. Ekelund, and T. Christensen. 1999. Distribution and composition of microbial populations in a landfill leachate contaminated aquifer (Grindsted, Denmark). Microb. Ecol. 37:197 - 207.

88. Madsen, E. 2000. Nucleic-acid characterization of the identity and activity of subsurface microorganisms. Hydrogeol. J. 8:112 - 125.

89. Marxen, J. 1988. Investigations into the number of respiring bacteria in groundwater from sandy and gravelly deposits. Microb. Ecol. 16:65 - 72.

90. McCarthy, C. and L. Murray. 1996. Viability and metabolic features of bacteria indigenous to a contaminated deep aquifer. Microb. Ecol. 32:305 - 321.

91. McCready, R. 1975. Sulphur isotope fractionation by *Desulfovibrio* and *Desulfotomaculum* species. Geochim. Cosmochim. Acta 39:1395 - 1401.

92. McKinley, J.P. and F.S. Colwell. 1996. Application of perfluorocarbon tracers to microbial sampling in subsurface environments using mud-rotary and air-rotary drilling techniques. J. Microbiol. Methods 26:1 - 9.

93. McMahon, C., FH, W. Falls, and P. Bradley. 1992. Role of microbial processes in linking sandstone diagenesis with organic rich clays. J. Sediment. Petrol. 62:1 - 10.

94. McMahon, P. and F. Chapelle. 1991. Microbial production of organic acids in aquitard sediments and its role in aquifer chemistry. Nature 349:233 - 235.

95. Meier, J., A. Voigt, and H.-D. Babenzien. 2000. A comparison of ^{35}S-SO_4^{2-} radiotracer techniques to determine sulphate reduction rates in laminated sediments. J. Microbiol. Meth. 41:9 - 18.

96. Mormile, M.R. and R.M. Atlas. 1988. Mineralization of the dibenzothiophene biodegradation products 3-hydroxy-2-formyl benzothiophene and dibenzothiophene sulfone. Appl. Environ. Microbiol. 54:3183 - 3184.

97. Motamedi, M. and K. Pedersen. 1998. *Desulfovibrio aespoeensis* sp. nov., a mesophilic sulfate-reducing bacterium from deep groundwater at Äspö hard rock laboratory, Sweden. Int. J. Syst. Bacteriol. 48:311 - 315.

98. Murphy, E., J. Schramke, J. Fredrickson, H. Bledsoe, A. Francis, D. Sklarew, and J. Linehan. 1992. The influence of microbial activity and sedimentary organic carbon on the isotope geochemistry of the Middendorf aquifer. Wat. Resour. Res. 28:723 - 740.

99. Nelson, K.E., A.N. Pell, P. Schofiel, and S. Zinder. 1995. Isolation an characterization of an anaerobic ruminal bacterium capable of degrading hydrolyzable tannins. Appl. Environ. Microbiol. 61:3293 - 3298.

100. Okabe, S., P. Nielsen, W. Jones, and W. Chakacklis. 1995. Rate and stoichiometry of microbial sulfate reduction by *Desulfovibrio desulfuricans* in biofilms. Biofouling 9:63 - 83.

101. Orphan, V., L. Taylor, D. Hafenbradl, and E. Delong. 2000. Culture-dependent and culture-independent characterization of microbial assemblages associated with high-temperature petroleum reservoirs. Appl. Environ. Microbiol. 66:700-711.

102. Parkes, R.J., G.R. Gibson, I. Mueller-Harvey, W.J. Buckingham, and R.A. Herbert. 1989. Determination of the substrates for sulphate-reducing bacteria within marine and estuarine sediments with different rates of sulphate reduction. J. Gen. Microbiol. 135:175 - 187.

103. Pedersen, K. 1997. Microbial life in deep granitic rock. FEMS Microbiol. Rev. 20:399 - 414.

104. Pedersen, K., J. Arlinger, S. Ekendahl, and L. Hallbeck. 1996. 16S rRNA gene diversity of attached and unattached bacteria in boreholes along the acess tunnel to the Äspö hard rock laboratory, Sweden. FEMS Microbiol. Ecol. 19:249 - 262.

105. Pedersen, K., J. Arlinger, L. Hallbeck, and C. Petterson. 1996. Diversity and distribution of subterranean bacteria in groundwater at Oklo in Gabon, Africa, as determined by 16S rRNA gene sequencing. Mol. Ecol. 5:427 - 436.

106. Pedersen, K. and S. Ekendahl. 1990. Distribution and activity of bacteria in deep granitic groundwaters of Southeastern Sweden. Microb Ecol 20:37 - 52.

107. Phelps, T., S. Pfiffner, K. Sargent, and D. White. 1994. Factors influencing the abundance and metabolic capacities of microorganisms in Eastern Coastal Plain sediments. Microb. Ecol. 28:351 - 364.

108. Potter, M.C. 1908. Bakterien als Agenzien bei der Oxydation amorpher Kohle. Proc. Roy. Soc. London. p. 647 - 665.

109. Rabus, R., R. Nordhaus, W. Ludwig, and F. Widdel. 1993. Complete oxidation of toluene under strictly anoxic conditions by a new sulfate-reducing bacterium. Appl. Environ. Microbiol. 59:1444-1451.

110. Ravenschlag, K., K. Sahm, C. Knoblauch, B. Jørgensen, and R. Amann. 2000. Community structure, cellular rRNA content and actvity of sulfate-reducing bacteria in marine arctic sediments. Appl. Environ. Microbiol. 66:3592 - 3602.

111. Ravenschlag, K., K. Sahm, J. Pernthaler, and R. Amann. 1999. High bacterial diversity in permanently cold marine sediments. Appl. Envrion. Microbiol. 65:3982-3989.

112. Rees, C. 1973. A steady-state model for sulfur isotope fractionation in bacterial sulfate reduiction processes. Geochim. Cosmochim. Acta 37:1141 - 1162.

113. Reeves, R., J. Reeves, and D. Balkwill. 1995. Strategies for phylogenetic characterization of subsurface bacteria. J. Microbiol. Methods 21:235 - 251.

114. Rogoff, M.H. and I. Wender. 1963. Materials in coal inhibitory to the growth of microorganism. United States Department of Interior - Bureau of Mines, Report 6279.

115. Rogoff, M.H., I. Wender, and R.B. Anderson. 1962. Microbiology of coal. United States Department of Interior - Bureau of Mines, Report 8075.

116. Rueter, P., R. Rabus, H. Wilkes, F. Aeckersberg, F.A. Rainey, H.W. Jannasch, and F. Widdel. 1994. Anaerobic oxidation of hydrocarbon in crude oil by new types of sulphate-reducing bacteria. Nature 372:455-457.

117. Russell, B., T. Phelps, W. Griffin, and K. Sargent. 1992. Procedures for sampling deep subsurface microbial communities in unconsidated sediments. GWMR 4:96 - 104.

118. Rusterholtz, K. and L. Mallory. 1994. Density, activity, and diverstity of bacteria indigenous to a karstic aquifer. Mibrob. Ecol. 28:79 - 99.

119. Sahm, K., B. MacGregor, B. Jorgensen, and D. Stahl. 1999. Sulphate reduction and vertical distribution of sulphate-reducing bacteria quantified by rRNA slot-blot hybridization in a coastel marine sediment. Environ. Microbiol. 1:65 - 74.

120. Schulte, U. 1998. Isotopengeochemische Untersuchungen zur Charakterisierung biologisch gesteuerter Redoxprozesse in Aquiferen der Niederrheinischen Bucht - Dissertation, Fakultät für Geowissenschaften. Ruhr-Universität: Bochum. p. 169.

121. Schulte, U., H. Strauß, A. Bergmann, and P. Obermann. 1997. Isotopenverhältnisse der Schwefel- und Kohlenstoffspezies aus Sedimenten und tiefen Grundwässern der Niederrheinischen Bucht. Grundwasser 3:103 - 110.

122. Setti, L., M. Rossi, G. Lanzarini, and P.G. Pifferi. 1993. Barrier and carrier effects of n-dodecane on the anaerobic degradation of benzothiophene by *Desulfovibrio desulfuricans*. Biotechnol. Let. 15:527 - 530.

123. Smock, A.M., M.E. Boettcher, and H. Cypionka. 1998. Fractionation of sulfur isotopes during thiosulfate reduction by *Desulfovibrio desulfuricans*. Arch. Microbiol. 169:460 - 463.

124. Sørensen, J., D. Christensen, and B.B. Jørgensen. 1981. Volatile fatty acids and hydrogen as substrates for sulfate-reducing bacteria in anaerobic marine sediment. Appl. Environ. Microbiol. 42:5-11.

125. Stevens, T. 1997. Lithoautotrophy in the subsurface. FEMS Microbiol. Rev. 20:327 - 337.

126. Strauss, H. 1997. The isotopic composition of sedimentary sulfur through time. Palaeogeogr. Palaeoclim. Palaeoecol. 132:97 - 118.

127. Teske, A., N.B. Ramsing, K. Habicht, M. Fukui, J. Küver, B.B. Jørgensen, and Y. Cohen. 1998. Sulfate-reducing bacteria and their activities in cyanobacterial mats of Solar Lake (Sinai, Egypt). Appl. Environ. Microbiol. 64:2943-2951.

128. Toth, J. 1963. A theoretical analysis of groundwater flow in small drainage basins. J. Geophyss. Res. 68:4795 - 4812.

129. Ulrich, G., D. Martino, K. Burger, J. Routh, E. Grossman, J. Ammerman, and J. Suflita. 1998. Sulfur cycling in the terrestrial subsurface: commensal interactions, spatial scales, and microbial heterogeneity. Microb. Ecol. 36:141 - 151.

130. van Leeuwenhoek, A. 1677. Observations communicated to the publisher by Mr. Antonie van Leeuwenhoek in a letter from October 9th, 1676.

131. Wang, P. and S. Krawiec. 1994. Desulfurization of dibenzothiophene to 2-hydroxybiphenyl by some newly isolated bacterial strains. Arch. Microbiol. 161:266 - 271.

132. Widdel, F. 1988. Microbiology and ecology of sulfate- and sulfur-reducing bacteria, p. 469-585. In A.J.B. Zehnder (ed.), Biology of anaerobic microorganisms. J. Wiley, New York.

133. Zeikus, J., A. Wellstein, and T. Kirk. 1982. Molecular-basis for the biodegradative recalcitrance of lignin in anaerobic environments. FEMS Microbiol. Let. 15:193 - 197.

134. Zlatkin, I., M. Schneider, F. de Bruijn, and L. Forney. 1996. Diversity among bacteria isolated from the deep subsurface. J. Industr. Microbiol. 17:219 - 227.

Chapter 2.

Sulfate reduction at a lignite seam: A multidisciplinary approach

Microbial Ecology (in press)

Sulfate reduction at a lignite seam: A multidisciplinary approach

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Abstract

In a combined isotope geochemical and microbiological investigation a setting of multiple aquifers was characterized. A succession of biologically mediated redox processes was observed in the aquifers situated in marine sands of Tertiary age and overlying Quaternary gravel deposits. Intercalated lignite seams define the aquitards which separate the groundwater horizons. Bacterial oxidation of organic matter is evident from dissolved inorganic carbon characterized by average carbon isotope values between −18.4‰ and −15.7‰ (PDB). Strongly positive sulfur isotope values of up to +50‰ (CTD) for residual sulfate indicate sulfate reduction under closed system conditions with respect to sulfate availability. Both, hydrochemical and isotope data are, thus, consistent with the recent activity of sulfate-reducing bacteria (SRB).

Microbiological investigations revealed the presence of an anaerobic food chain in the aquifers. Most-probable-number (MPN) determinations for SRB and fermenting microorganisms reached highest values at the aquifer-lignite seam interface ($1.5 \cdot 10^3$ cells/g sediment dry mass). Five strains of SRB could be isolated from highest MPN dilutions. Spore-forming bacteria seem to dominate the SRB-population. Sulfate reduction rates were determined by the ^{35}S-radiotracer method. A detailed assessment indicates an increase in the reduction rate in proximity to the lignite seam, with a maximum turnover of 8.4 mM sulfate/a, suggesting that lignite-drived compounds represent the substrate for sulfate reduction.

Introduction

To date little is known about the microorganisms of deep subsurface biosystems (17, 31). The microbial diversity and physiological adaptations to this ecosystem are currently explored. The physiological activity of nutritionally diverse microorganisms inhabiting these environments is strongly affected by physical and chemical conditions (16, 31). Electron donors can either be organic compounds and hydrogen from natural sources (21, 34, 36, 42, 51), xenobiotic compounds (33, 47, 50), or molecular hydrogen produced by geochemical reactions (28, 40, 48). Differences in energy yield from the terminal oxidation of the electron donor by different electron acceptors is a determining factor for the redox zonation in a stratified aquifer. Frequently, the sequence of electron accepting reactions in nature corresponds to the theoretical values of energy yield (31). However, changes in flow conditions, availability of electron donors and acceptors, and nutrients may all effect these redox processes and can also result in the formation of micro niches within the aquifer (31, 33).

In contrast to our knowledge about contaminated sites, the number of studies conducted in natural subsurface environments that are relatively poor in potential electron donors are limited (31, 40). Additionally, for most studies, the source of energy for microbial communities in the subsurface remains unclear. It becomes evident that lignite-derived compounds are one of the naturally occurring organic compounds in aquifer systems, that can act as electron donor and carbon source for microbial processes. So far, lignite was hardly regarded as a possible electron donor under anoxic conditions. In this study we investigated this geochemical process, determined the abundance of microorganisms in this habitat, isolated predominant organisms, and determined the microbial activity in proximity to a lignite seam.

Material and Methods

Study site and sampling procedure

The study site is situated in the western part of Germany next to the open-pit lignite mine Garzweiler I. The general lithostratigraphic column in the Lower Rhine Embayment comprises a succession of up to 250 m of marine sands of Tertiary age covered by up to 100 m of Quaternary fluvial sediments. A clay horizon (Reuver Ton) and up to three lignite seams (Morken, Frimmersdorf, Garzweiler) are intercalated, resulting in up to 5 different aquifers. The uppermost aquifer is unconfined while all lower levels are confined. Sampling of sediments was performed using a subcoring method as described in detail elsewhere (11). Samples were transported to the laboratory at in situ temperature within 6 hours.

Chemical and isotope measurements

The main objective of the hydrogeological investigation was a characterization of the isotope compositions of carbon and sulfur components within the aquifer system with special emphasis on evidence for biologically driven processes. Basic hydrochemical data for groundwater in the study area were obtained using standard procedures as outlined elsewhere (5, 44). Hydrogen sulfide concentrations were quantified according to the method of Moncaster and Bottrell (38) and precipitated as silver sulfide. Sulfate concentrations in the groundwater were determined by ion chromatography. If necessary an enrichment by ion exchange (Poly-Prep, Bio Rad) was performed (20, 35). Subsequently, dissolved sulfate was precipitated as barium sulfate using a barium chloride solution. Sulfide and sulfate precipitates were prepared for mass spectrometry through conversion to SO_2 in vacuum as described by Yanagisawa and Sakai (53). Dissolved inorganic carbon (DIC) was quantified and liberated from the water samples as CO_2 through reaction with phosphoric acid (43). Mass spectrometric measurements of $\delta^{13}C$ and $\delta^{34}S$ were performed using a Finnigan MAT 251 or Delta S. Isotope values are presented in the standard delta notation as per mil difference to the international PDB ($\delta^{13}C$) or CDT ($\delta^{34}S$) standard, respectively. Reproducibility as determined through

replicate analyses was generally better than ± 0.2‰ for carbon and ± 0.3‰ for sulfur, respectively.

Laboratory measurements of sulfate-reduction

The sulfate reduction rates were determined in samples derived from core sections in proximal position to the lignite seam. Sediment subcores were taken from 10 different levels in the aquifer, at the interface between aquifer and lignite, and from the lignite seam. Anoxic slurries were prepared in a 1 l aspirator bottle under N_2 from sediment diluted 1:2 with N_2-purged sterilized aquifer water. During continuous stirring, aliquots of 50 ml slurries were transferred under N_2 to 100 ml serum bottles. These bottles were sealed with thick black butyl rubber stoppers and screw-capped. Portions of 100 µl $^{35}SO_4^{2-}$ containing approximately 500 kBq (Amersham) were injected into the bottles through the butyl stopper. The bottles were incubated at the aquifer in-situ temperature of 15 °C. In preparatory experiments an incubation time of 48 hours gave best results for this type of sediment. Duplicate incubations were stopped by addition of 20 ml 20% Zn-acetate solution and stored at 4 °C until distillation.

The $^{35}SO_4^{2-}$ turnover was determined using a single-step acid Cr(II)-distillation method to trap the total reduced sulfur species (14). The H_2S evolved from the total reduced inorganic sulfur (TRIS) was precipitated in 5% Zn-acetate. The resulting ZnS-suspension was mixed with scintillation fluid (Ultima Gold XR, Packard) and counted in a liquid scintillation counter. Sulfate reduction rates were calculated by a modified method according to Jørgensen (24). Another 50 ml portion of the sediment core was used to determine the porosity of the sediment and to gain pore water for further analysis. Pore water from the sediment cores was pressed under N_2 through 0.45 µm filters and preserved in 1% $ZnCl_2$-solution. Sulfate was measured by ion chromatography using a DIONEX BioLC system with AS 14 column.

Cultivation of microorganisms

The specific physiological groups of microorganisms were grown in cultivation media containing the following constituents (concentrations in g/l deionized water):

Ferric iron and manganese(IV)-reducing organisms: NaCl, 1.0; $CaCl_2 \cdot 2H_2O$, 0.1; $MgSO_4 \cdot 7H_2O$, 0.05; $MgCl_2 \cdot 6H_2O$, 0.4; NH_4Cl, 1.5; $KH_2PO_4 \cdot H_2O$, 0.6; Na-acetate, 1.64. Ferric iron- and manganese(IV)-minerals (ferrihydrite and vernadite) were prepared as described elsewhere (30, 46).

AFM-medium (anaerobic fermentative microorganisms): NaCl, 1.0; KCl, 0.5; $CaCl_2 \cdot 2H_2O$, 0.1; $MgSO_4 \cdot 7H_2O$, 0.05; $MgCl_2 \cdot 6H_2O$, 0.4; NH_4Cl, 1.5; $KH_2PO_4 \cdot H_2O$, 0.6; yeast extract (DIFCO), 0.25; tryptone (DIFCO), 0.25; peptone (DIFCO), 0.25; fructose, 0.25; Tween 80 (Fluka), 0.25; $Na_2MoO_4 \cdot 2H_2O$, 0.000036.

Sulfate-reducing organisms: NaCl, 1.0; $CaCl_2 \cdot 2H_2O$, 0.1; $MgCl_2 \cdot 6H_2O$, 0.4; NH_4Cl, 1.5; KCl, 0.5; $NaSO_4$, 4.0; $KH_2PO_4 \cdot H_2O$, 0.2. Substrates were added from sterile stock solutions as follows: benzoate (2 mM), palmitate (2 mM), butyrate (20 mM), acetate (20 mM), lactate (20 mM), and H_2/CO_2 (80:20, vol/vol).

Methanogenic organisms: $CaCl_2 \cdot 2H_2O$, 0.4; $MgCl_2 \cdot 6H_2O$, 1.0; NH_4Cl, 1.0; yeast extract (DIFCO), 0.05; tryptone (DIFCO), 0.05; cysteine•HCl, 0.5; $Na_2MoO_4 \cdot 7H_2O$, 0.000072; $KH_2PO_4 \cdot H_2O$, 0.4; Na-acetate, 1.64. The medium was incubated under an H_2/CO_2 atmosphere (80:20, vol/vol) at 1 bar overpressure. All media for cultivation of anaerobic microorganisms were bicarbonate buffered and amended with vitamins and trace mineral solutions described in detail elsewhere (52). To monitor the reduced state of the medium, resazurin was added to the cultivation media for sulfate-reducers and methanogens. The media were reduced by 1.5 mM sodium sulfide prepared as described previously (52). The pH was adjusted according to the sediment sample and the atmosphere overlying the medium was N_2/CO_2 (90:10, vol/vol). Enrichment for ferrous iron-oxidizing and sulfur-oxidizing microorganisms using $FeSO_4$ and thiosulfate as electron acceptor were prepared as described by Ulrich (51).

MPN procedure

Three subcores were equally pooled under anoxic conditions to reduce the effect of sediment heterogeneity. Cells attached to sediment particles were desorbed by vigorous shaking before inoculation. MPN dilution series were performed in triplicates up to a dilution of 10^{-8} according to (3) and incubated without agitation at 28 °C in the dark for 3 months. During that time, growth was monitored by significant increase in Fe^{2+} (32), Mn^{2+} (18), sulfide (12), methane (19), and optical density at 660 nm. MPN estimates and statistical significance were calculated as described elsewhere (3). Pure cultures were obtained using agar dilution series as described in detail by Widdel and Bak (52).

PCR amplification and sequencing of the 16S rRNA gene

To amplify the 16S rRNA encoding gene of isolates obtained, primers GM3F and GM4R were used in a 35-cycle PCR with an annealing temperature of 40 °C. PCR products were purified by using the QIAquick Spin PCR purification kit (Qiagen, Inc., Chatsworth, Calif.) as described by the manufacturer. The Taq Dyedeoxy Terminator Cycle Sequencing kit (Applied Biosystems, Foster Cit, Calif.) was used to directly sequence the PCR products according to the protocoll provided by the manufacturer. The sequencing primers have been described previously (8). The sequence reaction mixtures were electrophoresed on an Applied Biosystems 373S DNA sequencer. The nearly complete sequences for the 16S rRNA genes were compared to known sequences by using the BLAST search routine from GenBank (1). In addition, the sequences were loaded into the 16S rRNA sequence data base of the Technical University of Munich using the program package ARB (49). The tool ARB_ALIGN was used for sequence alignment. The alignment was visually inspected and corrected manually. The sequence identity was calculated using ARB. The sequences were deposited in GeneBank (Table 1).

Table 1. Strains isolated from highest (10^4) MPN-dilutions.

Isolate	Baselength of sequenced 16S rRNA gene	GenBank accension number	Closest relative	Sequence similarity (%)	Spore formation	Metabolism
GwA001	402	AF29568	*Aeromonas* sp.	100.0	-	Fermentation, Fe(III)-reduction
GwA050	400	AF295663	*Microbacterium* sp.	100.0	-	Fermentation
GwA159	810	AF295659	*Desulfotomaculum auripigmentum* (*Desulfosporosinus auripigmentum*)	97.4	+	Sulfate-reduction, Homoacetogenesis
GwA160	825	AF295660	*Desulfovibrio* sp. strain zt 31	99.4	-	Sulfate-reduction, Fermenation
GwA169	800	AF295661	*Acetonema longus*	91.7	+	Homoacetogenesis
GwA171	740	AF295662	*Desulfomaculum thermosapovorans*	96.0	+	Sulfate-reduction, Homoacetogenesis
GwA175	1428	AF295656	*Desulfotomaculum geothermicum*	93.5	+	Sulfate-reduction, Homoacetogenesis
GwA176	1324	AF295657	*Desulfomaculum thermosapovorans*	96.6	+	Sulfate-reduction, Homoacetogenesis

Results

Geochemistry

The main emphasis of this study was placed on the hydrochemistry of carbon and sulfur components in the aquifer system, their concentration and isotope composition, the microbial community, and possible implications for microbially mediated processes. The lithostratigraphic succession within the Lower Rhine Embayment comprises a series of Tertiary marine sands, Quaternary fluvial sands and up to three lignite seams (Fig. 1). A series of separate groundwater horizons can be distinguished. The lignite seams act as aquitards in this system, effectively inhibiting the vertical movement of groundwater and its dissolved constituents.

At the study site, average concentrations of dissolved inorganic carbon (DIC) range from 5.1 mM to 9.0 mM. The DIC is characterized by average $\delta^{13}C$-values between −18.4‰ and −15.7‰ (PDB), indicative for the mineralization of organic material. A depth gradient in concentration and isotope composition is apparent with low DIC-concentrations coupled to relatively high $\delta^{13}C$-values (less ^{13}C depleted) in the uppermost groundwater and higher DIC-concentrations and more negative $\delta^{13}C$-values in the deeper horizons. In the uppermost aquifer, dissolved sulfate displays an average concentration of 1.34 mM and decreases with depth to 0.02 mM (Fig. 1). This vertical gradient is paralleled by increasing hydrogen sulfide concentrations; in the deep water an average value of 0.76 mM dissolved sulfide is reached. The average sulfur isotope composition of dissolved sulfate ($\delta^{34}S_{sulfate}$) varies between +3‰ and +15‰ (CDT). Single values of up to +50‰ were observed in the lower aquifers. A negative correlation exists between sulfate concentration and isotope composition.

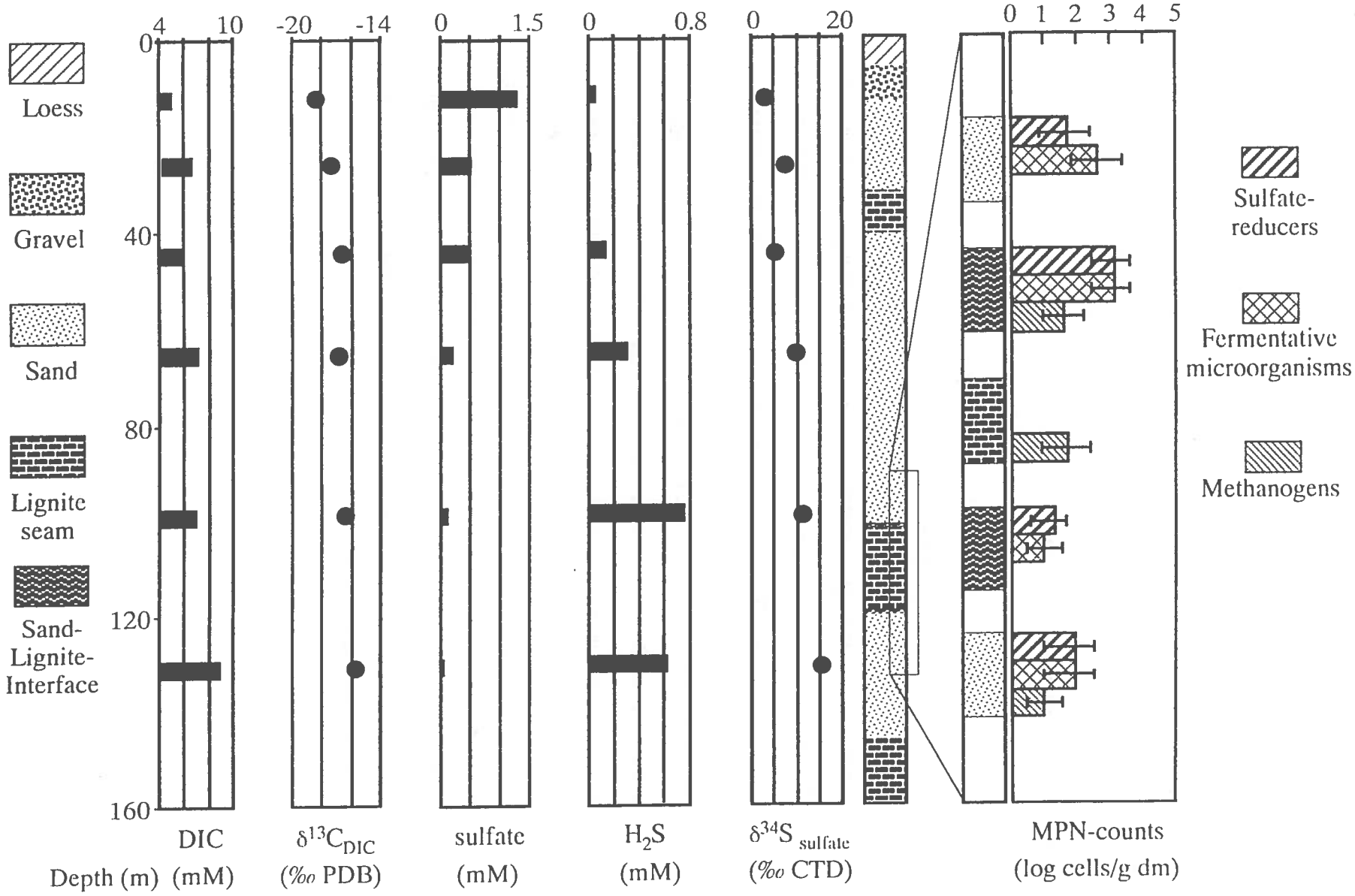

Fig.1. Sedimentology, geochemistry, and microbiology of the Garzweiler aquifers. Standard deviation was calculated according to (3).

Microbiology

A quantification of the microbial population in the sediment by direct counting of stained cells was impossible, because of low cell numbers and strong background fluorescence. A characterization of the microbial diversity by DNA-extraction and subsequent cloning failed due to a low DNA content, generally, the isolation of amplifyable DNA was difficult because of a high humics content of the sediment near the lignite seam. Thus, the most probable number (MPN) technique was used to quantify the microbial population. MPN series were conducted with samples from above, from the middle, and from below the second lignite seam. Results from MPN estimates using selective media for different physiological types of microorganisms are summarized in Figure 1. Within the lignite seam only methanogenic microorganisms were detected. Fermentative and sulfate-reducing bacteria were abundant in all samples above and below the lignite seam. Interestingly, highest cell numbers for these groups were detected at the interface between the aquifer and the lignite seam. MPN estimates for Fe^{3+} and Mn^{4+}-reducing bacteria as well as for Fe^{2+} and sulfur-oxidizing microorganisms gave negative results for the investigated levels.

We were able to isolate eight strains from the highest positive dilutions of the MPN series for fermentative and sulfate-reducing bacteria. Their 16S rDNA-genes were sequenced and the closest relative was determined by blast-search (Table 1). Strain GwA001 and strain GwA050 were isolated on AFM medium (anaerobic fermentative microorganisms). In addition to fermentation, strain GwA001 was able to reduce ferrihydrite with citrate as electron donor, indicating that ferric iron-reducing bacteria are also present in the system. The partial sequence of strain GwA001 (402 basepairs) was 100% identical to a ferric iron-reducer (*Aeromonas* sp.). Five sulfate-reducing bacteria (SRB) could be isolated from highest dilutions on SRB medium (Table 1). Interestingly, four of these isolates are spore-formers and can grow by homoacetogenesis as well as under sulfate-reducing conditions (strains GwA159, GwA160, GwA169, GwA171, GwA175, and GwA176).

The sulfate reduction rates were determined in the aquifer above the second lignite seam, at the upper interface between aquifer and lignite seam and directly in the

seam using the ^{35}S-radiotracer technique (Fig. 2). Above the lignite seam, the sulfate reduction rates were far below 1 μM sulfate/a. In proximity to the seam, rates increased by more than one order of magnitude. In the seam, one meter below the aquifer-lignite-interface, the sulfate-reducing activity reached maximum values (8.4 μM sulfate/a).

Discussion

Geochemical characterization

The aquifer system described in this study is characterized by several groundwater horizons, separated by lignite seams which act as aquitards, largely inhibiting the fluid exchange between the different aquifer levels. With increasing depth, conditions within the aquifers turned anoxic, $\delta^{34}S$ values increased with depth paralleled by decreasing sulfate concentrations and increasing hydrogen sulfide concentrations (Fig. 1). Bacterial sulfate reduction is associated with a strong isotope effect due to the preferential use of the ^{32}S isotope (9). Given the limited sulfate reservoir within an aquifer system, this results in an progressive increase in ^{34}S for the residual dissolved sulfate. Thus, sulfur isotope data obtained for the different aquifer levels provide clear evidence for recent microbial sulfate reduction.

Compared to sulfate reduction, ferric iron reduction in this system is interpreted as being of minor importance, because of the low concentration of bioreactive iron (5, 44, 45). These two reductive processes cannot be separated in hydrologically discrete zones; thus, ferric iron reduction might be limited to microniches where ferric iron and a suitable electron donor are available (5). Sulfate reduction is considered as the most important redox process in this part of the aquifer as indicated by geochemical and isotope results (44). Investigations were focused on the anaerobic redox processes within this aquifer. The microbiological investigation was therefore focused on the aquifer, where geochemical and isotope data indicated highest microbial activity (Fig. 1).

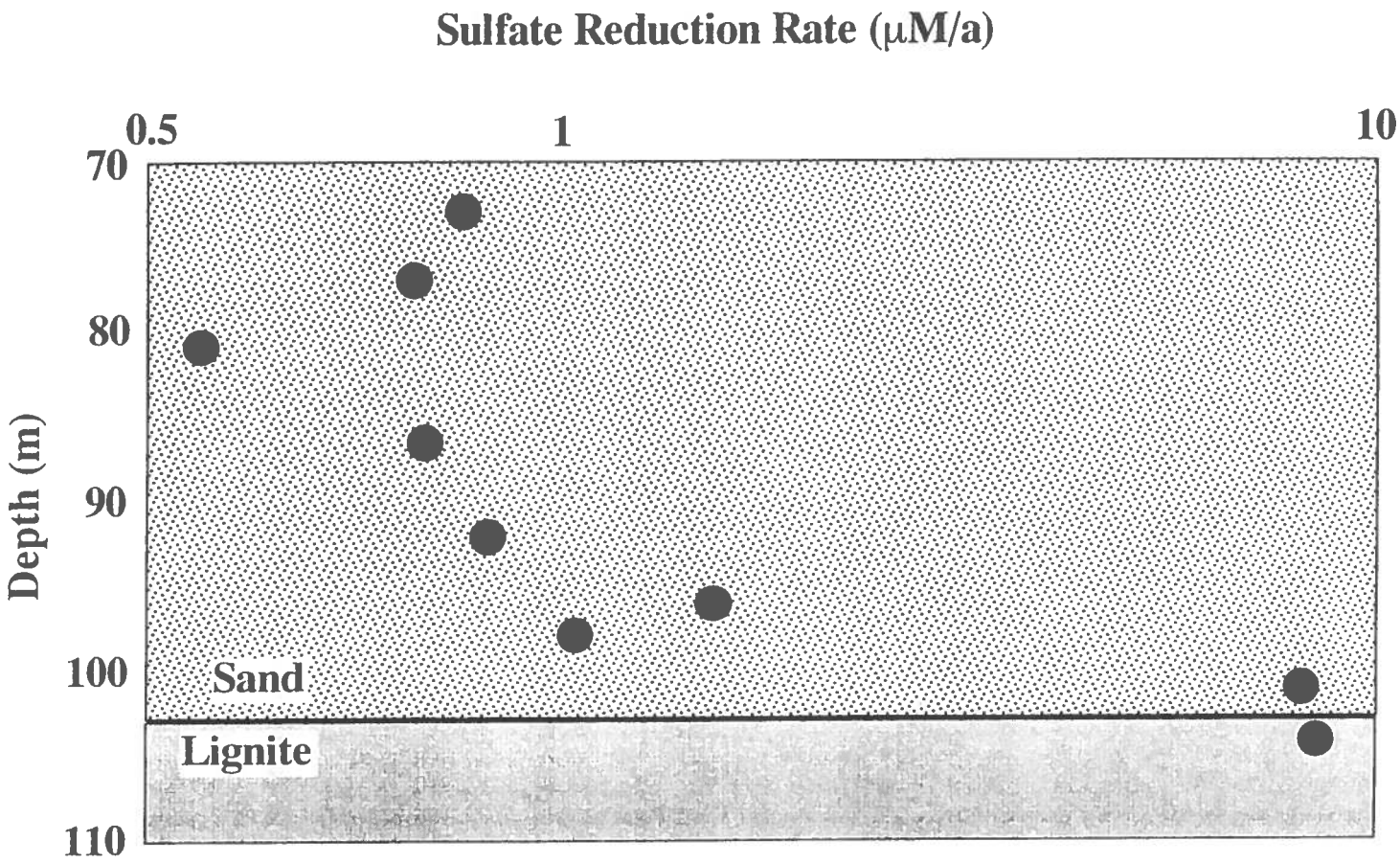

Fig.2. Increasing sulfate-reduction rates in approximation to the lignite seam.

The anaerobic food chain

The success of direct counting techniques and molecular approaches with the aim to reveal the microbial diversity are limited when cell numbers are low and the extraction of amplifyable DNA is impossible. Therefore the MPN estimation as a culture-dependent approach appeared most suitable. Generally, the numbers of microorganisms in groundwater samples detected by direct counting techniques ranged in the order of 10^3 to 10^5 cells (16, 25, 26, 41, 54). Due to the cultivation bias (2), direct counting techniques revealed much higher cell numbers than MPN determinations (16, 17, 21, 25, 26, 54). Therefore, our MPN results (Fig. 1) are likely to represent an underestimation of the *in-situ* microbial abundance. For comparison, anoxic coastal plain sediments harbored 10^3 to 10^6 viable bacteria compared to direct counts between 10^4 to 10^8 cells per gram of sediment (11). Similar discrepancies between these methods were obtained in other investigations including reports where MPN results were below the detection limit (25, 26).

The absence of Mn(IV)- and Fe(III)-reducing bacteria from the study site, as determined by MPN series, correlates well with the low concentrations of these electron acceptors (5). In these MPN series solely acetate was used as electron donor and only bacteria capable of growing by complete oxidation of substrates would, thus, be detected. Fermentative bacteria, capable of reducing Mn^{4+} or Fe^{3+} might be widespread and the isolation of ferric iron-reducing strain GwA001 (Table 1) can be regarded as an indication for this possibility. Therefore, the negative MPN results for this physiological group might be an underestimation of the metal-reducing capacity in the Garzweiler aquifer. The numbers of fermentative bacteria correlate well with the numbers of sulfate-reducing bacteria. Estimates for sulfate-reducing and fermentative bacteria obtained by our MPN analysis (Fig. 1) are in good agreement with results obtained previously for similar habitats (34, 47, 48, 51).

As previously shown, a large portion of the total microbial population in aquifers consists of Gram positive bacteria (13, 16, 54). Especially, spore-forming, sulfate-reducing *Desulfotomaculum* spp. and *Desulfosporosinus* spp. (formerly *Desulfotomaculum orientis*) seem to be well adapted to subsurface environments (Table 1). They were found to be dominant genera in several investigations (4, 13).

Boivin-Jahns affiliated 25% of the clones derived from a deep aquifer to the genus *Desulfotomaculum* (6). These sulfate-reducers and also the homoacetogenic strain GwA169 seem to be well adapted to subsurface environments because the formation of spores guarantees an efficient and long-term survival under varying redox conditions (15, 29). Sulfate-reducers can use a large variety of substrates for growth. In addition, these organisms can grow autotrophically on H_2 and CO_2, and even show homoacetogenic growth in the absence of sulfate as previously observed in subsurface environments (27, 29). In contrast to the geochemical formation of H_2 (48), the main source for H_2 at our study site might be the fermentation of lignite-derived organic matter, as indicated by $\delta^{13}C_{DIC}$-values and the finding of fermentative bacteria. The isolation of five strains capable of growing by homoacetogenesis (Table 1), present evidence for the occurrence of this process in our aquifer system. It should be kept in mind that close to the deeper lignite seams, sulfate abundance was nearly exhausted. The exclusive isolation of sulfate-reducers related to *Desulfotomaculum* spp., *Desulfosporosinus* spp. and *Desulfovibrio* spp. correlates well with low sulfate concentrations because all these organisms can grow by a fermentative metabolism (Table 1). However, the isolation of sulfate-reducing bacteria from a habitat does not necessarilly mean that they reduce sulfate in the natural habitat, they only have the potential for sulfate-reduction.

Quantification of sulfate reduction

The sulfate reduction rates determined by the ^{35}S-radiotracer method were in good agreement with other investigations in aquifer systems (Table 2). Different approaches to determine the sulfate turnover in aquifers gave very different results (23), therefore only rates determined by the ^{35}S radiotracer method were considered for comparison. Recently, Meier revealed that the slurry incubation method that we used in our study might underestimate the sulfate turnover on an average by one order of magnitude, possibly because of the disturbance of micro niches (37). The highest sulfate-reducing activity was determined at the interface between aquifer and lignite seam (Fig. 2). At this interface also highest MPN counts were observed (Fig. 1). In the aquifer above the lignite seam the sulfate-reducting activity was rather low but increased significantly in proximity to the lignite seam reaching a maximum turnover of 8.4 μM sulfate/a (Fig. 2). Obviously,

Table 2. Determinations of the sulfate-reduction by ^{35}S-radiotracer in different aquifers.

Aquifer	Incubation time	Sulfate (mM)	Rate	Unit	Reference
Grindsted, Denmark	Up to 20 days	< 2.5	3.7-657	µmol/kg dw*/yr	33
Yegua, Texas, USA	Up to 30 days	0.39	< 730	µmol/kg dw*/yr	34
Rømø, Denmark	18-40 h	0.3-0.5	20-4500	µM/yr	23
Rømø, Denmark	22-40 h	0.06-0.07	50-4500	µM/yr	23
Tuse Næs, Denmark	22-40 h	0.08-2.9	150-370	µM/yr	23
Garzweiler, Germany	48 h	0.45-0.92	0.30-8.36	µM/yr	this study

*dry weight

the sand/lignite interface has a pronounced effect on the bacterial population and their activity. Favourable conditions in close proximity to the lignite seam allow bacterial growth, because the suitable electron donor would be delivered either directly by the lignite or by the metabolism of fermentative bacteria. Additionally, the terminal electron acceptor sulfate would be transported by diffusion from the overlying sands. The microbial activity in sandy sediments is generally higher than in silt and clay rich sediments, because diffusion and exchange of water is not restricted (10, 11, 28, 34, 51). The only limitation in such habitats is the electron donor, which is often present only in low concentrations (Table 2). Interfaces like the spatially restricted area above the lignite seam represent ideal conditions for anaerobic bacteria, especially the sulfate-reducing bacteria as they benefit from both areas, the carbon rich and the electron acceptor rich one. Additional effects on the microbial growth might derive from nutrient and water availability.

Lignite as electron donor

The occurence of anaerobic reductive processes arises the questions for the electron donor in this system. Because the lignite seams act as aquitards, organic matter cannot be transported from the surface into the deep aquifer. Isotope data from DIC, the increasing sulfate reduction rates in proximity to the lignite seam, and the highest MPN enumerations at the aquifer/lignite interface justify the conclusion that lignite itself serves as an electron donor and carbon source. Several authors already proposed clay and also lignite as a source of organic carbon, inspired by an observed increase of DOC, DIC and microbial activity (7, 11, 34, 39, 51). Lignite is a rather complex substrate. Due to its chemical composition it was considered to be recalcitrant (22). Nevertheless, reports document the release of humic and fulvic acids, aromatic monomers and short chain fatty acids from lignite (11, 39, 51). A transformation or degradation of the polymer structure by fermenting bacteria could then deliver smaller molecules like organic acids, alcohols, aromatic monomers, and H_2 as electron donor and carbon source for other anaerobic processes.

In summary, the Garzweiler aquifer is a low activity ecosystem. This allows the observation of processes that are overlayed in high turnover systems. Lignite-derived compounds are presumably the electron donor and sulfate reduction is the predominant electron accepting process in this aquifer. Most sulfate-reducers isolated from this habitat are generalists being able to grow by sulfate reduction as well as by fermentation oxidizing a broad variety of electron donors, and beeing able to survive periods of starvation by spore-formation.

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References

1. Altschul, S., T. Madden, A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389 - 402.

2. Amann, R., W. Ludwig, and K. Schleifer. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol Rev 59:143 - 169.

3. American Public Health Association 1969. Standard methods for the examination of water, wastewater, including botton sediments and sludge. American Public Health Association 604 - 609.

4. Beeman, R. and J. Suflita. 1987. Microbial ecology of a shallow unconfined groundwater aquifer polluted by municipal landfill leachate. Microb Ecol 14:393 - 414.

5. Bergmann, A. 1999. Hydrogeochemische Untersuchungen anoxischer Redoxprozesse in tiefen Porengrundwasserleitern der Niederrheinischen Bucht - im Umfeld des Tagebaus Garzweiler I, *Institut für Geologie*. Ruhr-Universität: Bochum. p. 167.

6. Boivin - Jahns, V., R. Ruimy, A. Bianchi, S. Daumas, and R. Christen. 1996. Bacterial diversity in a deep-subsurface clay environment. Appl Environ Microbiol 62:3405 - 3412.

7. Brown, C., J. Coates, and M. Schoonen. 1999. Localized sulfate-reducing zones in a coastal plain aquifer. Ground Water 37: 505-516.

8. Buchholz-Cleven, B.E.E., B. Rattunde, and K.L. Straub. 1997. Screening for genetic diversity of isolates of anaerobic Fe(II)-oxidizing bacteria using DGGE and whole-cell hybridization. System Appl Microbiol 20:301-309.

9. Chambers, L. and P. Trudinger. 1979. Microbiological fractionation of stable sulfur isotopes: A review and critique. Geomicrobiol J 1:249 - 293.

10. Chapelle, F. and P. McMahon. 1991. Geochemistry of dissolved inorganic carbon in a coastel plain aquifer. 1. Sulfate from confining beds as an oxidant in microbial CO_2 production. J Hydrol 127:85 - 108.

11. Chapelle, F., J. Zelibor jr., D. Grimes, and L. Knobel. 1987. Bacteria in deep coastel plain sediments of Maryland: a possible source of CO_2 to groundwater. Wat Resour Res 23:1625 - 1632.

12. Cline, J.D. 1969. Spectrophotometric determination of hydrogen sulfide in natural waters. Limnol Oceanogr 14:454-459.

13. Colwell, F., T. Onstott, M. Delwiche, D. Chandler, J. Fredrickson, Q. Yao, J. McKinley, D. Boone, R. Griffiths, T. Phelps, D. Ringelberg, D. White, L. LaFreniere, D. Balkwill, R. Lehman, J. Konisky, and P. Long. 1997. Microorganisms from deep, high temperature sandstones: contraints on microbial colonization. FEMS Microbiol Rev 20:425 - 435.

14. Fossing, H. and B.B. Jørgensen. 1989. Measurement of bacterial sulfate reduction in sediments: Evaluation of a single-step chromium reduction method. Biogeochemistry 8:205-222.

15. Fredrickson, J., J. McKinley, B. Bjornstad, P. Long, D. Ringelberg, D. White, L. Krumholz, J. Suflita, F. Colwell, R. Lehman, and T. Phelps. 1997. Pore-size constraints on the activity and survival of subsurface bacteria in a Late Cretaceous Shale-Sandstone sequence, Northwestern New Mexico. Geomicrobiol. J. 14:183 - 202.

16. Fry, N., J. Fredrickson, S. Fishbain, M. Wagner, and D. Stahl. 1997. Population structure of microbial communities associated with two deep, anaerobic, alkaline aquifers. Appl Environ Microbiol 63:1498 - 1504.

17. Ghiorse, W. and J. Wilson. 1988. Microbial Ecology of terrestrial subsurface. Adv Appl Microbiol 33:107 - 172.

18. Greene, A., B. Patel, and A. Sheehy. 1997. *Deferribacter thermophilus* gen. nov., sp. nov., a novel thermophilic manganese- and iron-reducing bacterium isolated from a petroleum reservoir. Intern J Syst Bacteriol 47:505 - 509.

19. Harder, J. 1997. Anaerobic methane oxidation by bacteria employing ^{14}C-methane uncontaminated with ^{14}C-carbon monoxide. Mar Geol 137:13 - 23.

20. Heinzer, C. 1997. Die Isotopenzusammensetzung von mikrobiell gebildetem Sulfat in kleinen Einzugsgebieten unter besonderer Berücksichtigung der Mineralisation organischer Schwefelverbindungen, *Institut für Geologie*. Ruhr-Universität Bochum p. 86.

21. Hirsch, P. and E. Rades-Rohkohl. 1990. Microbial colonization of aquifer sediment exposed in a groundwater well in Northern Germany. Appl Environ Microbiol 56:2963 - 2966.

22. Hüttinger, K. and A. Michenfelder. 1987. Molecular structure of brown coal. Fuel 66:1164 - 1165.

23. Jakobsen, R. and D. Postma. 1994. In situ rates of sulfate reduction in an aquifer (Rømø, Denmark) and implications for the reactivity of organic matter. Geology 22:1103 - 1106.

24. Jørgensen, B.B. 1978. A comparison of methods for the quantification of bacterial sulfate reduction in coastal marine sediments. I. Measurement with radiotracer techniques. Geomicrobiol J 1:11-27.

25. Kieft, T., J. Fredrckson, J. McKinley, B. Bjornstad, S. Rawson, T. Phelps, BrockmanFJ, and S. Pfiffner. 1995. Microbiological comparisons within and across contiguous lacrustine, paleosol, and fluvial subsurface sediments. Appl Environ Microbiol 61:749 - 757.

26. Kieft, T., W. Kovacik jr., D. Ringelberg, D. White, D. Haldeman, P. Amy, and L. Hersman. 1997. Factors limiting microbial growth and activity at a proposed high-level nuclear repository, Yucca Mountain, Nevada. Appl Environ Microbiol 63:3128 - 3133.

27. Kotelnikova, S. and K. Pedersen. 1997. Evidence for methanogenic archaea and homoacetogenic bacteria in deep granitic rock aquifers. FEMS Microbiol Rev 20:339 - 349.

28. Krumholz, L., J. McKinley, G. Ulrich, and J. Suflita. 1997. Confined subsurface microbial communities in cretaceous rock. Nature 386:64 - 66.

29. Kuever, J., F. Rainey, and H. Hippe. 1999. Description of *Desulfotomaculum* sp. Groll as *Desulfotomaculum gibsoniae*, sp. nov. Int J Syst Bacteriol 49:1801 - 1808.

30. Laha, S. and R.G. Luthy. 1990. Oxidation of Aniline and other Primary Aromatic Amines by Manganese Dioxide. Environ Sci Technol 24:363 - 373.

31. Lovley, D. and F. Chapelle. 1995. Deep subsurface microbial processes. Rev Geophys 33:365 - 381.

32. Lovley, D.R. and E.J.P. Phillips. 1986. Availability of ferric iron for microbial reduction in bottom sediments of the freshwater Tidal Potomac River. Appl Environ Microbiol 52:751 - 757.

33. Ludvigsen, L., H. Albrechtsen, G. Heron, P. Bjerg, and T. Christensen. 1998. Anaerobic microbial redox processes in a landfill leachate contaminated aquifer (Grindsted, Denmark). J Contam Hydrol 33:273 - 291.

34. Martino, D., E. Grossman, G. Ulrich, K. Burger, J. Schlichenmeyer, J. Suflita, and J. Ammerman. 1998. Microbial abundance and activity in a low-conductivity aquifer system in East-Central Texas. Microb Ecol 35:224 - 234.

35. Mayer, B. 1992. Untersuchungen zur Isotopengeochemie des Schwefels in Waldböden und neugebildetem Grundwasser unter Wald, *Fakultät für Geowissenschaften*. Ludwig-Maximilians-Universität, München. p. 179.

36. McKinley, J., T. Stevens, J. Fredrickson, and J. Zachara. 1997. Biogeochemistry of anaerobic lacustrine and paleosol sediments within aerobic unconfined aquifer. Geomicrobiol J 14:23 - 39.

37. Meier, J., A. Voigt, and H.-D. Babenzien. 2000. A comparison of $^{35}S\text{-}SO_4^{2-}$ radiotracer techniques to determine sulphate reduction rates in laminated sediments. J Microbiol Meth 41:9 - 18.

38. Moncaster, J. and S. Bottrell. 1991. Extraction of low -level sulphide from groundwater for sulphur isotope analysis. Chem Geol 94:79 - 82.

39. Murphy, E., J. Schramke, J. Fredrickson, H. Bledsoe, A. Francis, D. Sklarew, and J. Linehan. 1992. The influence of microbial activity and sedimentary organic carbon on the isotope geochemistry of the Middendorf aquifer. Wat Resour Res 28:723 - 740.

40. Pedersen, K. 1997. Microbial life in deep granitic rock. FEMS Microbiol Rev 20:399 - 414.

41. Pedersen, K. and S. Ekendahl. 1990. Distribution and activity of bacteria in deep granitic groundwaters of Southeastern Sweden. Microb Ecol 20:37 - 52.

42. Phelps, T., S. Pfiffner, K. Sargent, and D. White. 1994. Factors influencing the abundance and metabolic capacities of microorganisms in Eastern Coastal Plain sediments. Microb Ecol 28:351 - 364.

43. Sackett, W. and W. Moore. 1966. Isotopic variations of dissolved inorganic carbon. Chem Geol 1:323 - 328.

44. Schulte, U. 1998. Isotopengeochemische Untersuchungen zur Charakterisierung biologisch gesteuerter Redoxprozesse in Aquiferen der Niederrheinischen Bucht - Dissertation, *Fakultät für Geowissenschaften*. Ruhr-Universität Bochum. p. 169.

45. Schulte, U., H. Strauß, A. Bergmann, and P. Obermann. 1997. Isotopenverhältnisse der Schwefel- und Kohlenstoffspezies aus Sedimenten und tiefen Grundwässern der Niederrheinischen Bucht. Grundwasser 3:103 - 110.

46. Schwertmann, U. and R. Cornell. 1991. Iron oxides in the laboratory. VCH Verlagsgesellschaft, Weinheim.

47. Sonier, D., N. Duran, and G. Smith. 1994. Dechlorination of trichloroflouromethane (CF-11) by sulfate-reducing bacteria from an aquifer contaminated with halogenated aliphatic compounds. Appl Environ Microbiol 60:4567 - 4572.

48. Stevens, T.O. and J.P. McKinley. 1995. Lithoautotrophic microbial ecosystems in deep basalt aquifers. Science 270:450-454.

49. Strunk, O., O. Gross, B. Reichel, M. May, S. Hermann, N. Stuckmann, M. Nonhoff, M. Lenke, A. Ginhart, A. Vilbig, T. Ludwig, A. Bode, K.-H. Schleifer, and W. Ludwig. 1999. ARB: a software environment for sequence data. Department of Microbiology, Technische Universität München, Munich, Germany, http://www.mikro.biologie.tu-muenchen.de

50. Suflita, J., S. Gibson, and R. Beeman. 1988. Anaerobic biotransformations of pollutant chemicals in aquifers. J Industr Microbiol 3:179 - 194.

51. Ulrich, G., D. Martino, K. Burger, J. Routh, E. Grossman, J. Ammerman, and J. Suflita. 1998. Sulfur cycling in the terrestrial subsurface: commensal interactions, spatial scales, and microbial heterogeneity. Microb Ecol 36:141 - 151.

52. Widdel, F. and F. Bak. 1992. Gram-negative mesophilic sulfate reducing bacteria, p. 3352 - 3378. *In* A. Balows, H.G. Trüper, M. Dworkin, W. Harder, andK.H. Schleifer (ed.), The prokaryotes. Springer, Berlin, Heidelberg, New York.

53. Yanagisawa, F. and H. Sakai. 1983. Thermal decomposition of barium sulfate-vanadiumpentaoxide-silica glass mixtures for preparation of sulfur dioxide in sulfur isotope ratio measurements. Anal Chem 55:985 - 987.

54. Zlatkin, I., M. Schneider, F. de Bruijn, and L. Forney. 1996. Diversity among bacteria isolated from the deep subsurface. J Industr Microbiol 17:219 - 227.

Chapter 3.

Desulfotomaculum dominates sulfate reducing community in a pristine aquifer

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Desulfotomaculum dominates sulfate reducing community in a pristine aquifer

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Abstract

The hydrochemistry and the microbial diversity of a pristine aquifer system near Garzweiler, Germany were characterized. Hydrogeochemical and isotope data indicate a recent activity of sulfate-reducing bacteria in the Tertiary marine sands. The community structure in the aquifer was studied by fluorescence in situ hybridization (FISH). Up to 7.3×10^5 cells/ml were detected by DAPI-staining. *Bacteria* were most dominant, representing 51.9% of the total cell number. Another 25.7% of total cell counts could be affiliated with the domain *Archaea*. Within the domain *Bacteria*, the beta-subclass of the *Proteobacteria* were most abundant (21.0%). Using genus-specific probes for sulfate-reducing bacteria (SRB), 2.5% of the total cell number was identified as members of the genus *Desulfotomaculum*. This reflects the predominant role these microorganisms play in sulfate-reducing zones of aquifers.

Introduction

Cultivation based techniques for assessing the microbial diversity in natural environments are limited in respect of their ability to identify all contributing microorganisms. Only a small portion of the microbial population is revealed by the isolation of pure cultures, no matter whether they have been obtained from enrichment cultures or dilution series. Since molecular tools are available, a much higher diversity is detected, reflecting most of the natural microbial population in various environments including hot springs, soils, sewage sludge, intestines of higher organisms, marine habitats, freshwater systems, and contaminated groundwater systems (Chandler *et al.*, 1997; Hugenholtz *et al.*, 1998; Liesack and Stackebrandt, 1992; Orphan *et al.*, 2000; Ravenschlag *et al.*, 1999; Urukawa *et al.*, 1999; Zarda *et al.*, 1998). Compared to these habitats, the knowledge about structure and function of the microbial community in non-contaminated subsurface environments is rather limited (for review see Madsen, 2000). Until recently the microbial communities in subsurface environments were mostly described by cultivation based methods or by cloning of 16S rDNA-genes (Boivin - Jahns *et al.*, 1996; Chandler *et al.*, 1997; Martino *et al.*, 1998; Pedersen *et al.*, 1996a; Pedersen *et al.*, 1996b; Ulrich *et al.*, 1998). Nowadays FISH represents a powerful tool for the identification and quantification of different phylogenetic groups in environmental samples (Amann *et al.*, 1997; Amann *et al.*, 1995; Pedersen, 1997).

The investigated groundwater system, situated near Cologne, Western Germany, is geologically well characterized (Bergmann, 1999; Schulte, 1998; Schulte *et al.*, 1997). Marine sands of Tertiary age with intercalated lignite seams confine a multiple aquifer system. Before we started our investigation, we had information on the groundwater chemistry, pointing strongly towards a recent metabolic activity of microorganisms. A strongly positive isotope signature of dissolved sulfate and the occurrence of methane already indicated complete anoxic conditions and the presence of sulfate-reducing bacteria (SRB) and methanogenic microorganisms. Therefore, we used group- and genus-specific oligonucleotide probes to get an insight into the microbial diversity of this ecosystem.

Results

Groundwater

The hydrochemical and microbiological parameters of the investigated groundwater samples are summarized in Table 1. Water samples were retrieved from four groundwater monitoring wells at a depth of 120 – 125m and had a temperature between 11.3 and 13.0°C. Samples of well 2 and well 3 displayed circumneutral pH, wells 1 and 4 had a pH of 7.6. Except samples from well 2, all water samples were anoxic and contained only minor concentrations of nitrate. In contrast to water samples of well 1, all other samples showed comparatively low concentrations of sulfate, paralleled by strongly positive $\delta^{34}S$ values, indicative for a recent activity of SRB.

All samples contained dissolved organic carbon (DOC). Negative $\delta^{13}C$-values, indicative for microbial degradation of organic matter, were lowest in water samples of well 3. This groundwater contained the highest cell numbers (7.3×10^5 cells/ml) and additionally showed the highest percentage of FISH-detected cells with specific domain probes EUB338 and Arch915 (77.6% vs. 28.5% in groundwater well 4). This indicates that well 3 harbored the most active microbial population of all groundwater wells sampled. Groundwater samples from well 3 were therefore chosen for further characterization of the microbial diversity in this aquifer.

Table 1. Hydrochemical and microbiological properties of investigated water wells.

Well	Depth (m)	pH	Temperature (° C)	O_2 (mg/l)	NO_3^- (mg/l)	SO_4^{2-} (mg/l)	$\delta^{34}S_{sulfate}$ (‰)	DOC (mg/l)	$\delta^{13}C_{DIC}$ (‰)	total cell number (in 10^5 cells/ml)	Eub 338 (% DAPI)	Arc 915 (% DAPI)	Total FISH-detection (% DAPI)
1	120	7.6	13.0	0.0	0.04	17.1	+0.23	26.30	-15.79	0.5	10.1	0.0	10.1
2	125	6.9	13.2	1.1	0.10	2.8	+37.23	1.31	-16.55	0.9	0.0	0.0	0.0
3	123	7.0	11.8	0.0	0.04	5.3	+21.93	2.54	-17.72	7.3	51.9	25.7	77.6
4	121	7.6	11.3	0.0	0.04	7.1	+28.97	2.12	-15.94	1.3	9.6	18.9	27.5

Microbial diversity

FISH results are summarized in Fig. 1. 77.6% of the total cell counts as revealed by DAPI-staining could be affiliated with the domains *Bacteria* and *Archaea*. The *Bacteria* were shown to be most abundant in the groundwater (51.9% of total cells, Table 1). Among the cells detected with the bacterial probe EUB338 *Proteobacteria* were most abundant (29.0% of total cells). Within the *Proteobacteria*, members of the beta-subclass were dominating (21.0% of total cells) whereas the alpha-subclass played a minor role (1.0% of total cells). An important fraction of the beta-subclass of the *Proteobacteria* could be affiliated with the beta-1-subclass (8.2% of total cells). Members belonging to the gamma and epsilon-*Proteobacteria*, the *Cytophaga-Flavobacterium* cluster, *Planctomycetales*, and Gram-positives with high G+C DNA (Table 2) were below the detection limit for FISH. The major part of cells detected with the archaeal probe ARCH915 could be affiliated with the *Euryarchaeota* (21.5% of total cells).

Sulfate-reducing microorganisms

The general probe SRB385 gave signals for samples from groundwater well 3 (7.0% of total cells). This probe covers most bacteria of the delta-*Proteobacteria*, but targets also cells from other taxa (e. g. *Clostridia* and other Gram-positive bacteria) and might therefore cause an overestimation of this group. The use of more specific probes for certain genera of Gram-negative SRB (see Table 2) gave no signals. Members of the genera *Desulfobotulus*, *Desulfobulbus*, *Desulfomicrobium*, *Desulfomonile*, *Desulfovibrio*, and *Syntrophus*, which were all frequently isolated from freshwater habitats, were not found. The only positive result for SRB gave probe DTM229 targeting members of the genus *Desulfotomaculum*. Only *Desulfotomaculum acetoxidans*, *Desulfotomaculum alcaliphilum*, and *Desulfosporosinus orientis* (formerly *Desulfotomaculum orientis*-cluster) are not targeted by this probe (Hristova *et al.*, 2000).

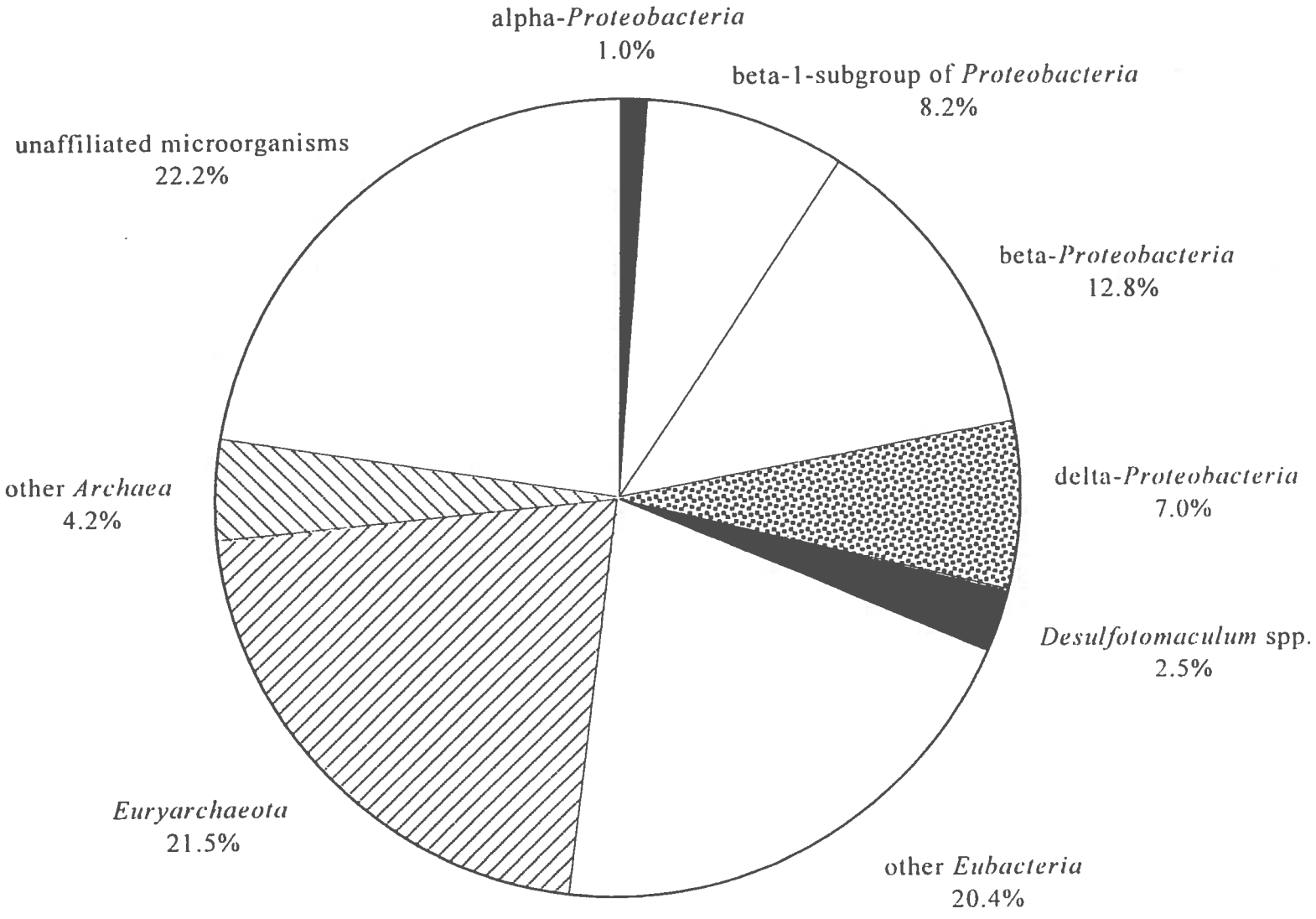

Fig. 1. Microbial diversity revealed by FISH in groundwater well 3.

Table 2. Oligonucleotide probes used in this study.

Probe	Specificity	Sequence (5'→ 3')	Target site [a] (rRNA position)	FISH (FA) [b]	Reference
EUB338	*Bacteria*	GCTGCCTCCCGTAGGAGT	16S (338-355)	10	Amman *et al.*,1990
NON338		ACTCCTACGGGAGGCAGC	16S (338-355)	10	Wallner *et al.*, 1993
ARCH915	*Archaea*	GTGCTCCCCCGCCAATTCCT	16S (915-935)	35	Stahl and Amann, 1991
CREN499R	*Crenarchaeota*	CCAGRCTTGCCCCCCGCT	16S (499-515)	0	Burggraf *et al.*, 1994
EURY498R	*Euryarchaeota*	CTTGCCCRGCCCTT	16S (498-510)	0	Burggraf *et al.*, 1994
ALF968	alpha-*Proteobacteria*	GGTAAGGTTCTGCGCGTT	16S (968-986)	35	Neef, 1997
BET42a	beta-*Proteobacteria*	GCCTTCCCACTTCGTTT	23S (1027-1043)	35	Manz *et al.*, 1992
BONE23a	beta-1-subgroup of *Proteobacteria*	GAATTCCATCCCCCTCT	16S (663-679)	35	Amann *et al.*, 1996
GAM42a	gamma-*Proteobacteria*	GCCTTCCCACATCGTTT	23S (1027-1043)	35	Manz *et al.*, 1992
CF319a	*Cytophaga-Flavobacterium* cluster	TGGTCCGTGTCTCAGTAC	16S (319-336)	35	Manz *et al.*, 1996
PLA886	*Planctomycetales*	GCCTTGCGACCATACTCCC	16S (886-904)	35	Neef *et al.*, 1998
HGC69a	Gram-positive with high G+C DNA content	TATAGTTACCACCGCCGT	23S (1901-1918)	20	Roller *et al.*, 1994
LGC354a LGC354b LGC354c	Gram-positive with low G+C DNA content	TGGAAGATTCCCTACTGC CGGAAGATTCCCTACTGC CCGAAGATTCCCTACTGC	16S (354-372)	35	Meier *et al.*, 1999
ARC94	*Arcobacter* spp.	TGCGCCACTTAGCTGACA	16S (94-111)	20	Snaidr *et al.*, 1997
ARC1430	*Arcobacter* spp.	TTAGCATCCCCGCTTCGA	16S (1430-1447)	20	Snaidr *et al.*, 1997
SRB385	SRB of the delta-*Proteobacteria* plus several gram-positive *Bacteria* (e.g., *Clostridium*)	CGGCGTCGCTGCGTCAGG	16S (385-402)	20	Amann *et al.*, 1990
DSR651	*Desulforhopalus*	CCCCCTCCAGTACTCAAG	16S (651-668)	35	Manz *et al.*, 1998
DSS658	*Desulfosarcina/ Desulfococcus/ Desulfofaba/ Desulfofrigus*	TCCACTTCCCTCTCCCAT	16S (658-685)	60	Manz *et al.*, 1998
DSV698	*Desulfovibrio*	GTTCCTCCAGATATCTACGG	16S (698-717)	35	Manz *et al.*, 1998
DSV214	*Desulfomicrobium*	CATCCTCGGACGAATGC	16S (214-230)	10	Manz *et al.*, 1998
DSV407	*Desulfovibrio*	CCGAAGGCCTTCTTCCCT	16S (407-424)	50	Manz *et al.*, 1998
DSV1292	*Desulfovibrio*	CAATCCGGACTGGGACGC	16S (1292-1309)	35	Manz *et al.*, 1998
DSD131	*Desulfovibrio*	CCCGATCGTCTGGGCAGG	16S (131-148)	20	Manz *et al.*, 1998
DSMA488	*Desulfovibrio/ Desulfomonile/ Syntrophus*	GCCGGTGCTTCCTTTGGCGG	16S (488-507)	60	Manz *et al.*, 1998
660	*Desulfobulbus*	GAATTCCACTTTCCCCTCTG	16S (660-679)	60	Devereux *et al.*, 1992
221	*Desulfobacterium*	TGCGCGGACTCATCTTCAAA	16S (221-240	35	Devereux *et al.*, 1992
DSB985	*Desulfobacter/ Desulfobacula*	CACAGGATGTCAAACCCAG	16S (985-1003)	20	Manz *et al.*, 1998
DRM432	*Desulfuromonas/ Pelobacter*	CTTCCCCTCTGACAGAGC	16S (432-449)	40	Ravenschlag *et al.*,2000
DTM229	*Desulfotomaculum*	TACCXAGGCGCAGGGTAA	16S (229-236)	15	Hristova *et al.*, 2000
DSBO224	*Desulfobotulus*	GGGACGCGGACTCATCCTC	16S (224-240)	60	Manz *et al.*, 1998

[a] Position in the 16S/23S rRNA of *E.coli*
[b] Formamide concentrations in the hybridization buffer

Discussion

Microbial Activity

In this communication we describe the community composition of the groundwater-associated microbial population. Hydrochemical data of the characterized groundwater monitoring wells indicated anaerobic processes in three of the four sampled wells (wells 2, 3, and 4). Positive $\delta^{34}S$-values paralleled by low concentrations of dissolved sulfate in wells 3 and 4 are pointing strongly towards recent bacterial sulfate-reduction (Schulte, 1998; Schulte *et al.*, 1997). The finding of highest cell numbers and highest percentage of FISH-detected cells in these samples supports this. Non-detectable cells may develop due to spore-formation, starvation, dormancy, or cell dead and are therefore considered to be an indicator for low *in-situ* activity (Amy, 1997). Only one out of four investigated groundwater samples harbored a microbial population with more than 75% hybridizable cells even though the other samples were retrieved from a similar depth (Table 1). Obviously, aquifer systems display a significant heterogeneity in respect of their microbial activity.

Microbial Diversity

FISH is a suitable method for the quantification of phylogenetic groups in environmental samples. Nevertheless, this method has limitations when cell walls are impermeable, target sites are poorly accessible, or when the cellular rRNA content is too low. By using FISH, 77.6% of the total cell number in samples from well 3 could be affiliated with specific domains giving an overview about structural and functional aspects of the microbial population in this aquifer.

Similar to other freshwater systems, a large portion of the abundant microorganisms belonged to the beta-*Proteobacteria* (Crump *et al.*, 1999; Glöckner *et al.*, 1999; Pedersen *et al.*, 1996b). Especially, the *Comamonas-Variovorax* group (beta-1 subclass) seems to be of importance. Microorganisms belonging to these taxa were frequently isolated from subsurface environments (Balkwill *et al.*, 1997; Chandler *et al.*, 1997; Jimenez, 1990; Pedersen *et al.*, 1996a; Pedersen *et al.*, 1996b; Zlatkin *et al.*, 1996). Obviously beta-*Proteobacteria* are generally well

adapted to freshwater conditions and might be important for the oxidation of dissolved organic carbon.

The abundance of *Euryarchaeota* can most probably be attributed to methanogens. This finding correlates well with the detection of biogenic methane as identified by typical $\delta^{13}C$ signatures (Schulte, 1998; Schulte *et al.*, 1997). The probe EURY498R would also target members of the genus *Archaeoglobus*, but the presence of these thermophilic sulfate-reducing archaea at this low temperature is very unlikely. Since sulfate concentrations are very low, carbon dioxide is of importance as electron acceptor in this aquifer system. In contrast to hydrogen-dependent methanogenesis, which would be outcompeted by sulfate-reduction, the acetate-dependent methanogenesis would probably not be affected, because here sulfate-reducing bacteria can be outcompeted by methanogens (Achtnich *et al.*, 1995). The coexistence of acetoclastic methanogenic archaea, homoacetogenic and sulfate-reducing bacteria has been demonstrated for deep granitic rock groundwater (Kotelnikova and Pedersen, 1997; Pedersen, 1997).

In the investigated aquifer system SRB are present and active as indicated by the isotope signature of the residual sulfate, depletion of sulfate and formation of sulfide in the groundwater. The absence of *Desulfovibrio* spp. and *Desulfomicrobium* spp. in the groundwater was surprising. The positive results with SRB385, but negative results using more specific probes for SRB might be explained by the presence of members of the *Geobacteraceae* or other bacteria. These microorganisms use sulfur or ferric iron as electron acceptor or are growing by fermentation.

The dominating SRB in our aquifer system are spore-forming members of the genus *Desulfotomaculum* (Fig. 1). These results perfectly reflect the isolates we have obtained from this aquifer system which were spore-forming SRB affiliated with *Desulfotomaculum* spp. and *Desulfosporosinus* spp. as indicated by comparative analysis of the 16S rRNA sequences (data not shown). All isolates are able to use a large variety of organic compounds as electron donor as it has been demonstrated for other members of this taxa (e. g. Kuever *et al.*, 1999). In contrast to *Desulfovibrio* and *Desulfomicrobium*, they are not restricted to a few organic compounds and hydrogen as electron donors. *Desulfotomaculum* spp. seem to be widespread in subsurface environments (Balkwill *et al.*, 1997; Boivin - Jahns *et al.*, 1996; Colwell *et al.*, 1997). Many

Desulfotomaculum spp. can cope with low levels or complete absence of sulfate, because they can grow by fermentation of organic compounds or by homoacetogenesis using hydrogen and carbon dioxide. Possibly, hydrogen, the favored electron donor for *Desulfovibrio* spp. and *Desulfomicrobium* spp. might not be present together with sulfate at sufficient levels in the aquifer or hydrogen is effectively removed by homoacetogenesis as it is the case in similar environments (Achtnich *et al.*, 1995; Kotelnikova and Pedersen, 1997; Pedersen, 1997). Additionally, the formation of spores allows survival under varying redox conditions. Generally Gram-positive bacteria play an important role in the subsurface (Zlatkin *et al.*, 1996). Members of the genera *Desulfosarcina* and *Desulfococcus* are widespread in marine environments, because of their universalistic physiological traits (Ravenschlag *et al.*, 2000; Sahm *et al.*, 1999) *Desulfotomaculum* spp. seem to be the counterpart in this niche in the subsurface.

Experimental procedures

Study site and sampling procedure

The study site is situated in the Lower Rhine Embayment, Germany next to the open-pit lignite mine Garzweiler I. The general lithostratigraphic column comprises a succession of up to 250 m of marine sands with a Tertiary age covered by up to 100 m of Quaternary fluvial sediments. A clay horizon (Reuver Ton) and up to three lignite seams (Morken, Frimmersdorf, Garzweiler) are intercalated, resulting in up to 5 different aquifers. The uppermost aquifer is unconfined while all lower levels are confined.

Sampling of groundwater monitoring wells was performed using a standard method as described in detail elsewhere (DVWK, 1992). Samples were taken after pumping for at least 40 minutes and when parameters such as temperature, pH, redox potential, oxygen and conductivity remained stable for at least 15 minutes due to recharge of aquifer water. For chemical analysis samples were stored under anoxic conditions at +4°C in the dark.

Chemical and isotope measurements

Basic hydrochemical information about the groundwater in the study area was obtained using standard procedures as outlined elsewhere (Bergmann, 1999; Schulte, 1998; Schulte *et al.*, 1997). The main objective of this study was a characterization of the isotope compositions of carbon and sulfur components within the aquifer system with special emphasis on evidence for biologically driven processes. Sulfate concentrations in the groundwater were determined by ion chromatography. If necessary an enrichment by ion exchange (Poly-Prep, Bio Rad) was performed. Subsequently, dissolved sulfate was precipitated as barium sulfate using a barium chloride solution. Sulfate precipitates were prepared for mass spectrometry through conversion to SO_2 in vacuum (Yanagisawa and Sakai, 1983). Dissolved inorganic carbon (DIC) was quantified and liberated as CO_2 from the water samples through reaction with phosphoric acid.

Mass spectrometric measurements of $\delta^{13}C$ and $\delta^{34}S$ were performed using a Finnigan MAT 251 or Delta S. Isotope values are presented in the standard delta notation as per mil difference to the international PDB ($\delta^{13}C$) or CDT ($\delta^{34}S$) standard, respectively. Reproducibility as determined through replicate analyses was generally better than ± 0.2‰ for carbon and ± 0.3‰ for sulfur, respectively.

Fluorescence in situ hybridization (FISH)

Samples for FISH were fixed with 4% (wt/v) paraformaldehyde solution on polycarbonate filters and stored as described in detail by Glöckner (Glöckner *et al.*, 1996). Cy3-labelled oligonucleotides were purchased from Interactiva (Ulm, Germany). Hybridization and microscopy counts of hybridized and 4',6'-diamidino-2-phenylindole (DAPI)-stained cells were performed as described previously (Snaidr *et al.*, 1997). Means were calculated from 10 to 20 randomly chosen fields on each filter section, corresponding to 800-1000 DAPI stained cells. Counting results were always corrected by subtracting signals observed with the probe NON338. Formamide concentrations and oligonucleotide probes used are given in Table 1. Probes BET42a, GAM42a, PLA886, CREN499R, EURY498R, and BONE23a were used with competitor oligonucleotides as described previously (Amann *et al.*, 1995; Burggraf *et al.*, 1994; Manz *et al.*, 1992; Neef *et al.*, 1998).

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References

Achtnich, C., Schuhmann, A., Wind, T., and Conrad, R. (1995). Role of interspecies H_2 transfer to sulfate and ferric iron-reducing bacteria in acetate consumption in anoxic paddy soil. *FEMS Microbiol Ecol* 16, 61-70.

Amann, R., Glöckner, F.-O., and Neef, A. (1997). Modern methods in subsurface microbiology: in situ identification of microorganisms with nucleic acid probes. *FEMS Microbiol Rev* 20, 191-200.

Amann, R., Ludwig, W., and Schleifer, K. (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol Rev* 59, 143 - 169.

Amann, R., Snaidr, J., Wagner, M., Ludwig, W., and Schleifer, K.-H. (1996). In situ visualization of high genetic diversity in a natural bacterial community. *J Bacteriol* 178, 3496-3500.

Amann, R. I., Binder, B. J., Olson, R. J., Chisholm, S. W., Devereux, R., and Stahl, D. A. (1990). Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl Envirol Microbiol* 56, 1919-1925.

Amy, P. (1997). Microbial dormancy and survival in the subsurface. *In* "The microbiology of the terrestrial deep subsurface" (P. Amy and D. Haldeman, eds.), pp. 185 - 203. Lewis, Boca Raton, New York.

Balkwill, D., Reeves, R., Drake, G., Reeves, J., Crocker, F., King, M., and Boone, D. (1997). Phylogenetic characterization of bacteria in the subsurface microbial culture collection. *FEMS Microbiol Rev* 20, 201-216.

Bergmann, A. (1999). Hydrogeochemische Untersuchungen anoxischer Redoxprozesse in tiefen Porengrundwasserleitern der Niederrheinischen Bucht im Umfeld des Tagebaus Garzweiler I, Ph.D. Thesis, Ruhr-Universität, Bochum.

Boivin - Jahns, V., Ruimy, R., Bianchi, A., Daumas, S., and Christen, R. (1996). Bacterial diversity in a deep-subsurface clay environment. *Appl Environ Microbiol* 62, 3405 - 3412.

Burggraf, S., Mayer, T., Amann, A., Schadhauser, S., Woese, C., and Stetter, K. (1994). Identifying members of the domain *Archaea* with rRNA-targeted oligonucleotide probes. *Appl Environ Microbiol* 60, 3112-3119.

Chandler, D., Li, S., Spadoni, C., Drake, G., Balkwill, D., Fredrickson, J., and Brockman, F. (1997). A molecular comparison of culturable aerobic heterotrophic bacteria and 16S rDNA clones derived from a deep subsurface sediment. *FEMS Microbiol Ecol* 23, 131 - 144.

Colwell, F., Onstott, T., Delwiche, M., Chandler, D., Fredrickson, J., Yao, Q., McKinley, J., Boone, D., Griffiths, R., Phelps, T., Ringelberg, D., White, D., LaFreniere, L., Balkwill, D., Lehman, R., Konisky, J., and Long, P. (1997). Microorganisms from deep, high temperature sandstones: contraints on microbial colonization. *FEMS Microbiol Rev* 20, 425 - 435.

Crump, B., Virginia Armbrust, V., and Baross, J. (1999). Phylogenetic analysis of particle-attached and free-living bacterial communities in the Columbia River, its estuary, and the adjacent coastal ocean. *Appl Environ Microbiol* 65, 3192 - 3204.

Devereux, R., Kane, M. D., Winfrey, J., and Stahl, D. A. (1992). Genus- and group-specific hybridization probes for determinative and environmental studies of sulfate-reducing bacteria. *System Appl Microbiol* 15, 601 - 609.

DVWK (1992). "Entnahme und Untersuchungsumfang von Grundwasserproben. DVWK-Regeln zur Wasserwirtschaft,". DVWK, Hamburg, Berlin.

Glöckner, F., Fuchs, B., and Amann, R. (1999). Bacterioplancton compositions of lakes and oceans: a first comparison based on fluorescence in situ hybridization. *Appl Envrion Microbiol* 65, 3721-3726.

Glöckner, F. O., Amann, R., Alfreider, A., Pernthaler, J., Psenner, R., Trebesius, K., and Schleifer, K.-H. (1996). An in situ hybridization protocol for detection and identification of planktonic bacteria. *Syst Appl Microbiol* 19, 403-406.

Hristova, L., Mau, M., Zheng, D., Aminov, R., Mackie, R., Gaskins, H., and Raskin, L. (2000). *Desulfotomaculum* genus and group-specific small subunit rRNA hybridization probes for envrionmental studies. *Envrion Microbiol* 2, 143 - 160.

Hugenholtz, P., Pitulle, C., Hershberger, K. L., and Pace, N. R. (1998). Novel division level bacterial diversity in a Yellowstone hot spring. *J Bacteriol* 180, 366-376.

Jimenez, L. (1990). Molecular analysis of deep-subsurface bacteria. *Appl Environ Microbiol* 56, 2108 - 2113.

Kotelnikova, S., and Pedersen, K. (1997). Evidence for methanogenic archaea and homoacetogenic bacteria in deep granitic rock aquifers. *FEMS Microbiol Rev* 20, 339 - 349.

Kuever, J., Rainey, F., and Hippe, H. (1999). Description of *Desulfotomaculum* sp. Groll as *Desulfotomaculum gibsoniae*, sp. nov. *Int J Syst Bacteriol* 49, 1801 - 1808.

Liesack, W., and Stackebrandt, E. (1992). Occurence of novel groups of the domain *Bacteria* as revealed by analysis of genetic material isolated from an Australian terrestrial environment. *J Bacteriol* 174, 5072-5078.

Madsen, E. (2000). Nucleic-acid characterization of the identity and activity of subsurface microorganisms. *Hydrogeol J* 8, 112 - 125.

Manz, W., Amann, R., Ludwig, W., Vancanneyt., M., and Schleifer, K.-H. (1996). Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum Cytophaga-Flavobacter-Bacteroides in the natural environment. *Microbiology* 142, 1097-1106.

Manz, W., Amann, R., Ludwig, W., Wagner, M., and Schleifer, K.-H. (1992). Phylogentic oligodeoxynucelotide probes for the major subclasses of proteobacteria: problems and solutions. *Syst Appl Microbiol* 15, 593-600.

Manz, W., Eisenbrecher, M., Neu, T. R., and Szewzyk, U. (1998). Abundance and spatial organization of Gram-negative sulfate-reducing bacteria in activated sludge investigated by in

situ probing with specific 16S rRNA targeted oligonucleotides. *FEMS Microbiol Ecol* 25, 43-61.

Martino, D., Grossman, E., Ulrich, G., Burger, K., Schlichenmeyer, J., Suflita, J., and Ammerman, J. (1998). Microbial abundance and activity in a low-conductivity aquifer system in East-Central Texas. *Microb Ecol* 35, 224 - 234.

Meier, H., Amann, R., Ludwig, W., and Schleifer, K.-H. (1999). Specific oligonucleotide probes for in situ detection of a major group of Gram-positive bacteria with low DNA G+C content. *Syst Appl Microbiol* 22, 186-196.

Neef, A. (1997). Anwendung der in situ-Einzelzell-Identifizierung von Bakterien zur Populations-Analyse in komplexen mikrobiellen Biozönosen, Diplom-Thesis, Technische Universität München, Munich.

Neef, A., Amann, R., Schlesner, H., and Schleifer, K.-H. (1998). Monitoring a widespread bacterial group: In situ detection of planctomycetes with 16S rRNA-targeted probes. *Microbiol* 144, 3257-3266.

Orphan, V., Taylor, L., Hafenbradl, D., and Delong, E. (2000). Culture-dependent and culture-independent characterization of microbial assemblages associated with high-temperature petroleum reservoirs. *Appl Environ Microbiol* 66, 700-711.

Pedersen, K. (1997). Microbial life in deep granitic rock. *FEMS Microbiol Rev* 20, 399 - 414.

Pedersen, K., Arlinger, J., Ekendahl, S., and Hallbeck, L. (1996a). 16S rRNA gene diversity of attached and unattached bacteria in boreholes along the acess tunnel to the Äspö hard rock laboratory, Sweden. *FEMS Microbiol Ecol* 19, 249 - 262.

Pedersen, K., Arlinger, J., Hallbeck, L., and Petterson, C. (1996b). Diversity and distribution of subterranean bacteria in groundwater at Oklo in Gabon, Africa, as determined by 16S rRNA gene sequencing. *Mol Ecol* 5, 427 - 436.

Ravenschlag, K., Sahm, K., Knoblauch, C., Jørgensen, B., and Amann, R. (2000). Community structure, cellular rRNA content and actvity of sulfate-reducing bacteria in marine arctic sediments. *Appl Environ Microbiol* 66, 3592 - 3602.

Ravenschlag, K., Sahm, K., Pernthaler, J., and Amann, R. (1999). High bacterial diversity in permanently cold marine sediments. *Appl Envrion Microbiol* 65, 3982-3989.

Roller, C., Wagner, M., Amann, R., Ludwig, W., and Schleifer, K.-H. (1994). In situ probing of gram-positive bacteria with high DNA G+C content using 23S rRNA-targeted oligonucleotides. *Microbiol* 140, 2849-2858.

Sahm, K., MacGregor, B., Jorgensen, B., and Stahl, D. (1999). Sulphate reduction and vertical distribution of sulphate-reducing bacteria quantified by rRNA slot-blot hybridization in a coastal marine sediment. *Environ Microbiol* 1, 65 - 74.

Schulte, U. (1998). Isotopengeochemische Untersuchungen zur Charakterisierung biologisch gesteuerter Redoxprozesse in Aquiferen der Niederrheinischen Bucht - Ph.D. Thesis, Ruhr-Universität, Bochum.

Schulte, U., Strauß, H., Bergmann, A., and Obermann, P. (1997). Isotopenverhältnisse der Schwefel- und Kohlenstoffspezies aus Sedimenten und tiefen Grundwässern der Niederrheinischen Bucht. *Grundwasser* 3, 103 - 110.

Schulte, U., Strauss, H., Detmers, J., and Kuever, J. (1999). Characterization of an aquifer system – stable isotopes and microbiology. *J Conf Abstr* 4, 578.

Snaidr, J., Amann, R., Huber, I., Ludwig, W., and Schleifer, K.-H. (1997). Phylogenetic analysis and in situ identification of bacteria in activated sludge. *Appl Environ Microbiol* 63, 2884-2896.

Stahl, D., and Amann, R. (1991). Development and application of nucleic acid probes in bacterial systematics. *In* "Nucleic acid techniques in bacterial systematics." (E. S. a. M. Goodfellow, ed.), pp. 205-248. John Wiley & Sons Ltd., Chichester, England.

Ulrich, G., Martino, D., Burger, K., Routh, J., Grossman, E., Ammerman, J., and Suflita, J. (1998). Sulfur cycling in the terrestrial subsurface: commensal interactions, spatial scales, and microbial heterogeneity. *Microb Ecol* 36, 141 - 151.

Urukawa, H., Kita-Tsukamoto, K., and Ohwada, K. (1999). Microbial diversity in marine sediments from Sagami Bay and Tokyo Bay, Japan, as determined by 16S rRNA gene analysis. *Microbiol* 145, 3305-3315.

Wallner, G., Amann, R., and Beisker, W. (1993). Optimizing fluorescent in situ hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. *Cytometry* 14, 136-143.

Yanagisawa, F., and Sakai, H. (1983). Thermal decomposition of barium sulfate-vanadiumpentaoxide-silica glass mixtures for preparation of sulfur dioxide in sulfur isotope ratio measurements. *Anal Chem* 55, 985 - 987.

Zarda, B., Mattison, G., Hess, A., Hahn, D., Höhener, P., and Zeyer, J. (1998). Analysis of bacterial and protozoan communities in an aquifer contaminated with monoaromatic hydrocarbons. *FEMS Microbiol Ecol* 27, 141-152.

Zlatkin, I., Schneider, M., de Bruijn, F., and Forney, L. (1996). Diversity among bacteria isolated from the deep subsurface. *J Industr Microbiol* 17, 219 - 227.

Chapter 4.

Diversity of sulfur isotope fractionation by sulfate-reducing procaryotes

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Diversity of Sulfur Isotope Fractionations by Sulfate-Reducing Prokaryotes†

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Batch culture experiments were performed with 32 different sulfate-reducing prokaryotes to explore the diversity in sulfur isotope fractionation during dissimilatory sulfate reduction by pure cultures. The selected strains reflect the phylogenetic and physiologic diversity of presently known sulfate reducers and cover a broad range of natural marine and freshwater habitats. Experimental conditions were designed to achieve optimum growth conditions with respect to electron donors, salinity, temperature, and pH. Under these optimized conditions, experimental fractionation factors ranged from 2.0 to 42.0‰. Salinity, incubation temperature, pH, and phylogeny had no systematic effect on the sulfur isotope fractionation. There was no correlation between isotope fractionation and sulfate reduction rate. The type of dissimilatory bisulfite reductase also had no effect on fractionation. Sulfate reducers that oxidized the carbon source completely to CO_2 showed greater fractionations than sulfate reducers that released acetate as the final product of carbon oxidation. Different metabolic pathways and variable regulation of sulfate transport across the cell membrane all potentially affect isotope fractionation. Previous models that explained fractionation only in terms of sulfate reduction rates appear to be oversimplified. The species-specific physiology of each sulfate reducer thus needs to be taken into account to understand the regulation of sulfur isotope fractionation during dissimilatory sulfate reduction.

The stable sulfur isotope ratio between ^{32}S and ^{34}S of solid and dissolved sulfur compounds is widely used as a marker for bacterial sulfate reduction and bacterial processes associated with the recycling of sulfide (5, 8, 18). The reduction of sulfate by sulfate reducers is coupled to a pronounced enrichment of ^{32}S in the produced sulfide. However, the extent of the isotope enrichment remains a matter of ongoing debate. Results from batch-culture, continuous-culture, and resting-cell experiments suggested that the isotope enrichment is inversely proportional to sulfate reduction rates (9, 22, 23). Furthermore, below a threshold concentration of sulfate, the discrimination against ^{34}S apparently decreases (21). Previous experimental studies of the isotope fractionation were conducted with only a few selected species that were known at that time, mainly *Desulfovibrio* spp. and two *Desulfotomaculum* spp. (9, 15, 22, 28). Moreover, since most of these species were isolated from freshwater environments, they are not necessarily of ecological importance in marine environments.

The different electron donors used in these early pure-culture studies included ethanol, lactate, acetate, pyruvate, glucose, yeast extract, and hydrogen (22, 23). Today, a number of sulfate reducers are known that can metabolize a wide range of substrates including long-chain fatty acids, alcohols, and even aromatic compounds that represent relevant substrates for natural environments (33, 45, 47). Hydrogen, propionate, butyrate, and acetate appear to be the most important electron donors for sulfate reducers in natural marine environments (31, 40), but propionate and butyrate have never been used as electron donors in sulfur isotope fractionation experiments. There is a need to expand the existing database of sulfur isotope fractionations by sulfate reducers with organisms that are important in natural environments and to conduct experiments with additional relevant electron donors. For this reason, we included microorganisms that cover the total temperature range of environments from which sulfate reducers have been isolated. Furthermore, we used a variety of likely natural substrates and conducted experiments at different salinities and pHs to cover as broad a range of natural conditions as possible.

MATERIALS AND METHODS

Cultures, growth conditions, and sampling. The investigated microorganisms (Table 1) were obtained from the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen [DSMZ], Braunschweig, Germany) or are recently isolated strains (*Desulfobacter* sp. ASv20; *Desulfovibrio* sp. strain X). The environmental sources for all organisms are listed in Table 1. In order to ensure reproducible growth conditions, all strains were transferred into fresh medium two times before an experiment was started. Cells were grown in strictly anoxic, carbonate-buffered mineral medium containing a single carbon source (see Table 2) and sodium sulfate in concentrations between 15 and 28 mM (46). Anoxic conditions were maintained by the addition of a 1 M sodium sulfide solution to a final concentration of 1 mM. The electron acceptor was not limiting. Strain-specific additives to the media (vitamins, trace metals, fatty acids) were prepared as described elsewhere in detail for each culture (DSMZ; http://www.dsmz.de/species/bacteria.htm). Growth experiments were performed in screw-cap glass bottles (56-ml volume) without headspace. To avoid cracking of the culture bottles from expanding hot media, the thermophilic strains were incubated in 125-ml butyl rubber-stoppered glass bottles containing 50 ml of growth media. The headspace was completely replaced by N_2-CO_2 (80/20 [vol/vol]). A similar incubation procedure was necessary for hydrogen-oxidizing *Desulfomicrobium autotrophicum*. For this

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TABLE 1. List of investigated strains

Microorganism	DSMZ[a]	Nucleotide[b]	Incubation (°C)	NaCl (g/liter)
Archaeoglobus fulgidus strain Z	4139	Y00275	80	18
"Desulfarculus baarsii"	2075	M34403	28	1
Desulfobacca acetoxidans	11109	AF002671	37	1
Desulfobacter sp. ASv20			20	20
Desulfobacterium autotrophicum	3382	M34409	28	20
Desulfobacula phenolica	3384	AJ237606	28	20
Desulfobacula toluolica	7467	X70953	28	20
"Desulfobotulus sapovorans"	2055	M34402	28	1
Desulfobulbus elongatus	2908	X95180	28	1
Desulfobulbus "marinus"	2058	M34411	28	20
Desulfocella halophila	11763	AF022936	28	40
Desulfococcus sp.	8541		28	20
Desulfofrigus oceanense	12341	AF099064	9	20
Desulfohalobium redbaense	5692	X99235	37	100
Desulfomicrobium baculatum	4028	M37311	28	1
Desulfonatronovibrio hydrogenovorans	9292	X99234	28	11
Desulfonatronum lacustre	10312	Y14594	28	1
Desulfonema magnum	2077	U45989	28	20
Desulfosarcina variabilis	2060	M34407	28	14
Desulfospira joergensenii	10085	X99637	28	20
Desulfotalea arctica	12342	AF099061	20	20
Desulfotalea psychrophila	12343	AF099062	9	20
Desulfotignum balticum	7044	AF233370	28	20
Desulfotomaculum geothermicum	3669	X80789	50	20
Desulfotomaculum gibsoniae	7213	Y11576	28	1
Desulfotomaculum thermocisternum	10259	U33455	60	21
Desulfovibrio halophilus	5663	U48243	28	70
"Desulfovibrio oxyclinae"	11498	U33316	28	70
Desulfovibrio profundus	11384	U90726	28	20
Desulfovibrio sp. strain X			28	20
Thermodesulfobacterium commune	2178	L10662	60	0
Thermodesulfovibrio yellowstonii	11347	L14619	60	0

[a] DSMZ strain number.
[b] Nucleotide sequence accession number.

culture, the headspace was replaced by H_2-CO_2 (80/20 [vol/vol]) at 10^5 Pa overpressure. The gas was replenished several times during the incubation. All strains were incubated in the dark without agitation. Bottles were shaken manually every second day for approximately 10 s to prevent biofilm formation.

For every experiment, a set of 10 bottles was inoculated with a culture grown to the mid-exponential growth phase. Measurements were made on individual culture bottles immediately after inoculation (T_0) and in the early exponential (T_1), mid-exponential (T_2), late exponential (T_3), and early stationary (T_4) phases. At each time point, a screw-cap bottle was opened to withdraw aliquots of the cultures to determine the concentrations of dissolved sulfate, dissolved sulfide, $\delta^{34}S_{sulfate}$, $\delta^{34}S_{sulfide}$, and cell numbers. The aliquots were withdrawn in less than 30 s to minimize loss or oxidation of hydrogen sulfide. For the serum vials, aliquots for the determination of cell numbers and sulfate/sulfide concentrations were withdrawn with a syringe through the septum. The remaining volume was used for sulfur isotope analysis.

Determination of sulfate and sulfide. A 50-μl aliquot of the culture was added to 300 μl of 20% zinc acetate to precipitate dissolved sulfide. This procedure guaranteed that loss or oxidation of dissolved sulfide was negligible. Sulfate was determined after further dilution by nonsuppressed anion chromatography and conductivity detection. The eluent was 1 mM isophthalic acid in 10% methanol adjusted to a pH of 4.7 with sodium tetraborate. The flow rate was 1 ml/min. Sulfide was determined spectrophotometrically by the methylene blue method (11).

csSRR. A 500-μl aliquot of each culture was used for cell counting using an Axioplan phase-contrast microscope (Carl Zeiss, Jena, Germany) and a modified Neubauer grid (0.0025 mm^2 by 0.02 mm). Cells were fixed in 2% glutaraldehyde and stained with 4',6'-diamidino-2-phenylindole (32). Cell-specific sulfate reduction rates (csSRRs, in moles $cell^{-1}$ day^{-1}) were calculated for the exponential phase using the change in concentration of sulfate and cell number (cn) between time points (T_1) and (T_2) according to the following equation:

$$\text{csSRR} = \frac{SO_4^{2-}{}_{(2)} - SO_4^{2-}{}_{(1)}}{\frac{(cn_{(1)} + cn_{(2)})}{2} \cdot (T_{(2)} - T_{(1)})}$$

We prefer to use this measure of metabolic activity because it can be directly compared to the change in isotopic composition of sulfate during cell growth. The equation is not valid for the lag and stationary phases.

Determination of stable sulfur isotopes. For the screw-cap bottles, 40 ml of the remaining culture was added to 10 ml of 20% zinc acetate to terminate microbial activity and to precipitate all dissolved sulfide. For the butyl rubber-stoppered serum vials, 10 ml of 20% zinc acetate was directly added though the septum. Dissolved sulfate and precipitated zinc sulfide were separated by filtration through 0.45-μm-pore-size Millipore filters. The filter was washed three times and the wash was added to the filtrate. Dissolved sulfate was precipitated as $BaSO_4$ with 1 M $BaCl_2$ at pH 4.0. For sulfur isotope determination, 300 to 400 μg of $BaSO_4$ was weighed into tin cups that contained a 10-fold excess of V_2O_5. The isotopic composition of $BaSO_4$ was determined by continuous-flow isotope-ratio-monitoring gas chromatography-mass spectrometry according to methods described elsewhere (16). The sulfur isotopic composition is expressed in the standard δ-notation given by $\delta^{34}S = (R_{sample}/R_{standard} - 1) \cdot 1{,}000$, where $R = {}^{34}S/{}^{32}S$. Values are expressed on a per mille (‰) basis using the VCDT scale (37). The mean and standard deviation for the international reference standard NBS 127 (20.0‰) was 20.0‰ ± 0.3‰ versus VCDT.

Determination of isotope fractionation factors. Microbial reduction of sulfate by the culture occurred in sealed serum vials without loss of product. These conditions are analogous to closed systems, allowing calculation of the isotope fractionation according to a Rayleigh fractionation model (27). Isotope fractionation factors (ε) were determined after non-linear regression to determine the function best reflecting the isotopic composition of dissolved sulfate ($\delta^{34}S$) at each time point (T_0 to T_4) on the basis of the isotopic composition of sulfate and the fraction of remaining sulfate (SO_4^{2-}), according to the following equation: $\delta^{34}S^{T1}{}_{SO_4} = -\varepsilon \ln {}_{(SO_4^{2-})} + \delta^{34}S^{T0}{}_{(SO_4)^{2-}}$. In dual experiments, the standard deviation of ε usually was smaller than 1‰.

Comparative analysis of 16S rRNA sequences. The sequences of the 16S rRNA genes were determined as described previously (29). Sequences that were not included in the 16S rRNA sequence database of the Technical University Munich in the program package ARB (41) were added from other databases. All sequences contained at least 1,200 bases. The tool ARB_ALIGN was used for sequence alignment. The alignment was checked visually and corrected manually. Tree topologies were evaluated by performing maximum parsimony, neighbor joining, and maximum likelihood analysis. Alignment positions at which less than 50% of the sequences of the entire data set shared the same residues were excluded from the calculations.

RESULTS

Variability of isotope fractionation. All of the 32 sulfate-reducing bacteria discriminated against ^{34}S during sulfate reduction. *Desulfonema magnum* showed the largest fractionation (ε = 42.0‰), and *Desulfovibrio halophilus* showed the smallest (ε = 2.0‰) (Table 2). Complete-oxidizing sulfate reducers fractionated sulfate between 15.0‰ (*Desulfosarcina variabilis*) and 42.0‰ (*Desulfonema magnum*), whereas the acetate-excreting incomplete oxidizers showed fractionations between 2.0‰ (*Desulfovibrio halophilus*) and 18.7‰ (*Desulfonatrunum lacustre*) (Table 2). The average isotope fractionation of the complete oxidizers (ε = 25‰) was more than 15‰ greater than that of the incomplete oxidizers (ε = 9.5‰). When the electron donor for *Desulfobacterium autotrophicum* was changed from butyrate to hydrogen, the fractionation decreased from 32.7 to 14.0‰. The oxidation of formate by *Desulfonatronovibrio hydrogenovorans* yielded a fractionation of 5.5‰.

Phylogeny. In order to cover the known phylogenetic diversity of sulfate reducers, we investigated Archaea (*Archeaoglobus fulgidus*), members of the deep-branching Thermodesulfovibrio (*T. yellowstonii*) and Nitrospira (*Thermodesulfobacterium commune*) subgroups, the low G+C subgroup (*Desulfoto-*

TABLE 2. Cell-specific sulfate reduction rates and fractionation factors of investigated sulfate-reducing prokaryotes

Microorganism[a]	Isolated from:	Electron donor (mM)	csSRR (fmol cell^{-1} day^{-1})	ε (‰)
Complete oxidizing				
Desulfonema magnum	Marine mud	Benzoate (3)	5.9	42.0
Desulfobacula phenolica	Marine mud	Benzoate (3)	125.0	36.7
Desulfobacterium autotrophicum	Marine mud	Butyrate (20)	64.8	32.7
Desulfobacula toluolica	Marine mud	Benzoate (3)	40.3	28.5
Desulfotomaculum gibsoniae	Freshwater mud	Butyrate (20)	13.0	27.8
Desulfospira joergensenii	Marine mud	Pyruvate (20)	0.9	25.7
"Desulfarculus baarsii"	Lake mud	Butyrate (20)	11.5	23.2
Desulfotignum balticum	Marine mud	Butyrate (20)	4.2	23.1
Desulfofrigus oceanense	Arctic sediment	Acetate (20)	7.6	22.0
Desulfobacter sp. ASv20	Arctic sediment	Acetate (20)	6.6	18.8
Desulfobacca acetoxidans	Anaerobic sludge	Acetate (20)	17.0	18.0
Desulfococcus sp.	Marine mud	Pyruvate (20)	41.8	16.1
Desulfosarcina variabilis	Marine mud	Benzoate (3)	11.1	15.0
Incomplete oxidizing				
*Desulfonatronum lacustre**	Alkaline lake mud	Ethanol (20)	16.2	18.7
Archaeoglobus fulgidus strain Z*	Submarine hot spring	Lactate (20)	63.8	17.0
Thermodesulfovibrio yellowstonii	Thermal vent water	Lactate (20)	24.0	17.0
"Desulfobotulus sapovorans"	Freshwater mud	Lactate (20)	26.0	16.5
*Desulfotomaculum thermocisternum**	Oil reservoir	Lactate (20)	310.0	15.0
Desulfomicrobium baculatum	Manganese ore	Lactate (20)	4.8	12.7
*Desulfotomaculum geothermicum**	Aquifer	Lactate (20)	8.1	12.5
Desulfohalobium redbaense	Saline sediment	Lactate (20)	434.0	10.6
Desulfocella halophila	Great salt lake	Pyruvate (20)	10.2	8.1
Desulfobulbus "marinus"	Marine mud	Propionate (20)	28.9	6.8
Desulfotalea arctica	Arctic sediment	Lactate (20)	4.2	6.1
Desulfobulbus elongatus	Digester	Propionate (20)	35.3	5.5
Desulfovibrio sp. strain X	Hydrothermal vent	Lactate (20)	36.0	5.4
Thermodesulfobacterium commune	Thermal spring	Lactate (20)	45.4	5.0
"Desulfovibrio oxyclinae"	Hypersaline mat	Lactate (20)	69.1	4.5
Desulfotalea psychrophila	Arctic sediment	Lactate (20)	6.3	4.3
Desulfovibrio profundus	Deep sea sediment	Lactate (20)	17.0	4.1
Desulfovibrio halophilus	Hypersaline microbial mat	Lactate (20)	33.1	2.0
Hydrogen and formate				
Desulfobacterium autotrophicum	Marine mud	H_2 (10^5 Pa)	34.8	14.0
Desulfonatronovibrio hydrogenovorans	Lake mud	Formate (20)	12.7	5.5

[a] Under experimental conditions, strains followed by an asterisk are considered incomplete oxidizers.

maculum spp.), and the δ subclass of the *Proteobacteria* (δ-*Proteobacteria*) (Fig. 1). Strains of all three orders of the δ-*Proteobacteria* with dissimilatory sulfate-reducing activity (*Desulfovibrionales, Desulfobacterales*, and *Synthrophobacterales*) were selected for isotopic characterization. However, there was no relationship between the fractionation and the phylogeny of the investigated strains (Fig. 1). For example, the distant species *Desulfarculus baarsii* and *Desulfotignum balticum* yielded similar fractionations of 23.2 and 23.1‰, whereas *Desulfocella halophila* (8.1‰), which is closely related to *Desulfarculus baarsii*, showed a very different fractionation. On the other hand, very closely related strains such as *Desulfotalea arctica* and *Desulfotalea psychrophila* fractionated similarly (4.6 and 6.5‰, respectively).

Strain-specific factors. The sulfur isotope fractionation was independent of the sulfate reduction rates when the specific optimum growth conditions for each organism were used (Fig. 2). These rates can be considered as the maximum possible sulfate reduction rates for each organism under batch culture conditions. Nevertheless, the rates varied by more than 2 orders of magnitude. The scatter in Fig. 2 indicates that no uniform relationship exists between isotope fractionation and sulfate reduction rate that would be valid for all sulfate reducers. Furthermore, the lowest and highest rates measured, 0.9 fmol cell^{-1} day^{-1} for *Desulfospira joergensenii* and 4,340 fmol cell^{-1} day^{-1} for *Desulfohalobium redbaense*, yielded only intermediate fractionations of 25.7 and 10.6‰, respectively (Table 2). Conversely, despite similar sulfate reduction rates of 64.8 and 69.1 fmol cell^{-1} day^{-1} for *Desulfobacterium autotrophicum* and *Desulfovibrio oxyclinae*, the fractionations were very different, with values of 32.7‰ for *Desulfobacterium autotrophicum* when growing on butyrate and 4.5‰ for *Desulfovibrio oxyclinae* when growing on lactate.

We determined the isotope fractionation of 26 mesophilic sulfate reducers which had not been characterized previously with respect to their fractionation behavior. In addition, we also investigated psychrophilic sulfate reducers (*Desulfofrigus oceanense, Desulfotalea psychrophila*) and psychrotolerant (*Desulfotalea arctica*), thermophilic (*Desulfotomaculum geothermicum, Desulfotomaculum thermocisternum, Thermodesulfobacterium commune, Thermodesulfovibrio yellowstonii*), and hyperthermophilic (*Archeaoglobus fulgidus*) organisms. All organisms were incubated at or very close to their temperature optimum. A comparison of the incubation temperature and fractionation behavior also indicated no correlation (Tables 1 and 2).

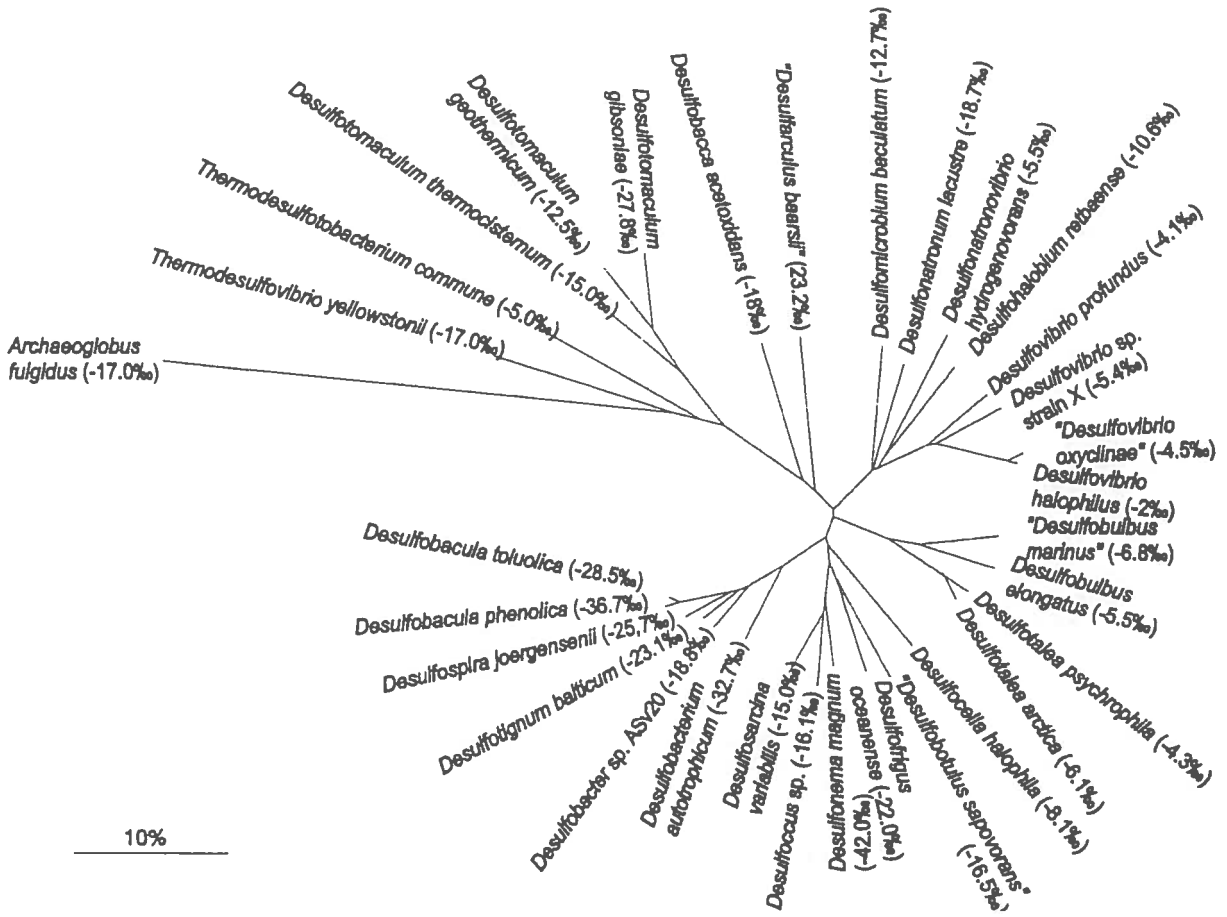

FIG. 1. Phylogenetic affiliation and sulfur isotope fractionation factors of investigated sulfate-reducing microorganisms. Neighbor-joining tree based on 1,308 positions of nearly full-length 16S rRNA sequences from 30 bacteria. *Archaeoglobus fulgidus* was taken to root the tree. Trees constructed with other tree reconstruction algorithms (maximum likelihood and parsimony) resulted in general in the same overall tree topology. The bar indicates 10% sequence divergence.

The strains were isolated from freshwater, brackish, marine, and hypersaline environments (Table 1), and their pH optima are between 6.7 (*Desulfotomaculum thermocisternum*) (30) and 9.6 (*Desulfonatronovibrio hydrogenovorans*) (48). All strains were grown under their optimal conditions with respect to salinity and pH. Comparison with the isotope fractionation also indicated no systematic relationship. Other strain-specific characteristics such as cell size, capability for spore formation, or oxygen sensitivity also did not affect the isotope fractionation.

DISCUSSION

Diversity of isotope fractionation. The overall range in sulfur isotope fractionation (ε = 2.0 to 42.0‰) for this diverse group of sulfate-reducing prokaryotes is very large and spans the full range of fractionations previously observed (4, 15, 22, 23, 28). In previous studies, very high fractionations (greater than 40‰) were obtained by growing cultures under physiologically stressed conditions, e.g., by determining fractionations with *Desulfovibrio desulfuricans* below the minimum temperature for growth (22). In contrast, our experimental conditions were optimized for each strain to permit a comparison of isotope fractionations.

There is no relationship between phylogenetic distance on the basis of 16S rRNA sequences and differences in isotope fractionation (Fig. 1). This is particularly apparent for the Archaeon *Archaeoglobus fulgidus*, whose isotope fractionation (ε = 17.0‰) was similar to that for a variety of incomplete-oxidizing sulfate reducers from the δ-*Proteobacteria* subgroup (Table 2). Thus, different isotope fractionation patterns do not reflect 16S rRNA-based phylogenetic relationships. Phylogenetic trees based on gene sequences that encode the dissimilatory bisulfite reductase (DSR) are not significantly different from 16S rRNA-based trees (44). Although the presently available data set is small, a comparison of DSR gene-based relative sequence dissimilarity with differences in isotope fractionation yields results similar to the 16S rRNA-based comparison.

Various attempts have been made to develop models for the sulfur isotope fractionation during dissimilatory sulfate reduction (10, 21, 22, 36), but none of these models take the physiological diversity of sulfate reducers into account. While it is

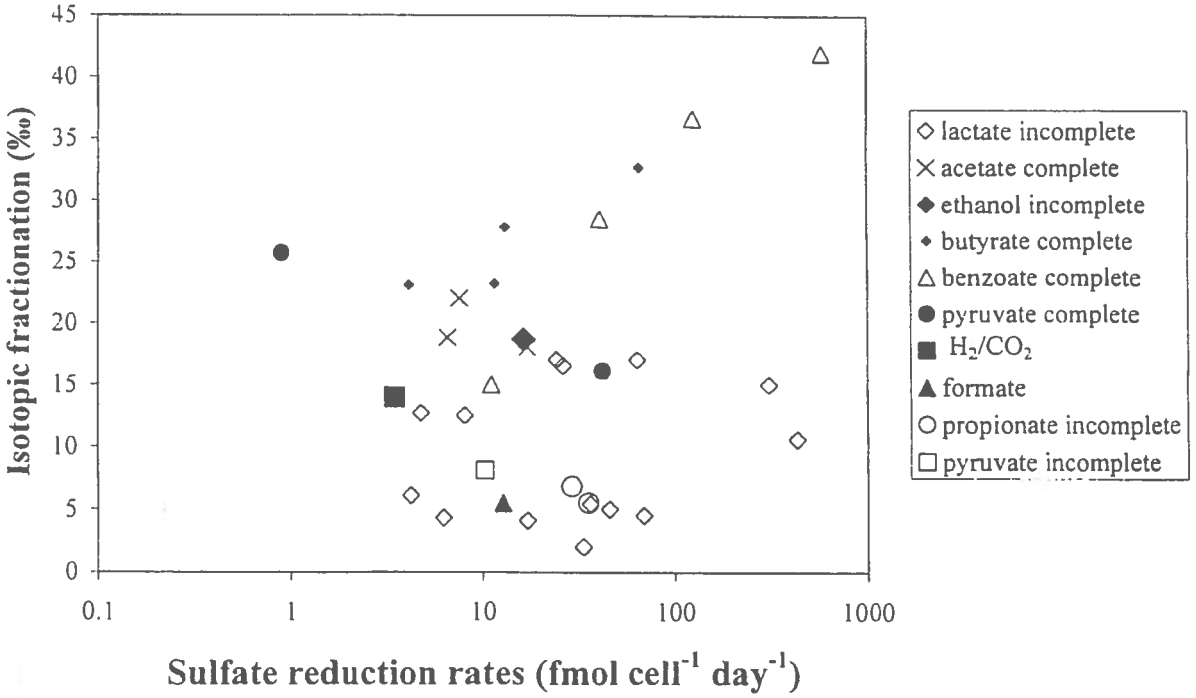

FIG. 2. Relationship between sulfate reduction rates in the mid-exponential growth phase (femtomoles of sulfate reduced per cell per day) and isotope fractionation. Each data point represents a different culture. The different substrates used are shown by the different symbols. Growth conditions were optimized for each culture so that the sulfate reduction rates were presumably close to the maximum potential rates for each organism.

clear that isotope fractionation most likely occurs when a sulfur-oxygen bond is broken, dissimilatory sulfate reduction proceeds in multiple steps (20). Isotope fractionation can occur at the adenosine phosphosulfate reductase (APSR) and the DSR (21, 22, 36), but too little is known about the structural differences between these enzymes among different sulfate reducers to assess their effect on isotope fractionation. It may be of interest, however, that the amino acid sequences important for gene function of the DSR are highly conserved (Bauer, personal communication). This may suggest a similarity in the structure of the reactive center and, possibly, similar isotope fractionation at these enzymes.

Sulfur isotope fractionation can also be modulated through a rate-limiting step that occurs either during sulfate uptake, at the APSR, or at the DSR (22, 23, 36). This rate-limiting step determines whether isotope fractionation can occur in successive reduction steps, but it may occur at different locations for the different sulfate reducers. Some sulfate reducers, in particular freshwater strains such as *Desulfobulbus propionicus*, have been shown to concentrate sulfate in their cells up to 2,500-fold (24). For these sulfate reducers, sulfate uptake is probably not the rate-limiting step. By contrast, marine species may not require a comparable preconcentration mechanism, and the regulation of sulfate uptake may take place by a different mechanism.

Complete versus incomplete electron donor oxidation are the only physiological characteristics that are consistently distinguished by sulfur isotope fractionation. In general, complete oxidizers fractionate more strongly (>15.0‰) than do incomplete oxidizers (<18.7‰). Only a small overlap exists between these two types. These results may be rationalized in terms of the energy conserved during electron donor oxidation for complete- and incomplete-oxidizing sulfate reducers (20, 47). In general, during incomplete oxidation of a substrate more energy is conserved per mole of sulfate reduced (Table 3). For example, the incomplete oxidation of lactate to acetate by sulfate yields more than three times as much energy as the complete oxidation of acetate to CO_2 (−160.1 versus −47.6 kJ mol^{-1} sulfate). All incomplete-lactate-oxidizing sulfate reducers fractionated between 2.0 and 17.0‰, whereas all examined acetate-oxidizing species fractionated between 18.0 and 22.0‰. The underlying causes for the correlation between a thermodynamic property such as energy yield and a kinetic property such as isotope fractionation remain unclear. A possible explanation could be that for a reaction yielding more free energy the redox potential difference ($\Delta E_0'$) is also higher. In this case, the reaction equilibria for the partial reactions during sulfate reduction are shifted toward the product side and the potential for isotope discrimination between APS at the APSR and bisulfite at the DSR is minimized. A test of this hypothesis requires determination of the redox potential of each enzyme participating in the electron transport chain during dissimilatory sulfate reduction.

Electron donor effects on isotope fractionation were already suggested by Kaplan and Rittenberg (22), who observed an increasing fractionation for *Desulfovibrio desulfuricans* in the sequence of lactate, acetate, and ethanol and significantly lower fractionations for autotrophic growth on H_2-CO_2. In these studies, the increase in fractionation always coincided with a decrease in sulfate reduction rates. Therefore, these two

TABLE 3. Free energy changes at standard state ($\Delta G^{0'}$) and corresponding range of isotope fractionations (ε) during dissimilatory sulfate reduction with various electron donors for complete and incomplete oxidation

Electron donor (type of oxidation)	Stoichiometry	$\Delta G^{0'}$ [kJ mol^{-1} $(SO_4)^{2-}$]^a	ε (‰)
Pyruvate (incomplete)	$4\ CH_3COCOO^- + 4\ H_2O + SO_4^{2-} \rightarrow 4\ CH_3COO^- + 4\ HCO_3^- + HS^- + 3\ H^+$	−340.9	8.1
Lactate (incomplete)	$2\ CH_3CHOHCOO^- + SO_4^{2-} \rightarrow 2\ CH_3COO^- + 2\ HCO_3^- + HS^- + H^+$	−160.1	2.0–17.0
Hydrogen	$4\ H_2 + SO_4^{2-} + H^+ \rightarrow 4\ H_2O + HS^-$	−152.2	14.0
Formate	$4\ HCOO^- + SO_4^{2-} + H^+ \rightarrow 4\ HCO_3^- + HS^-$	−146.9	5.5
Ethanol (incomplete)	$2\ CH_3CH_2OH + SO_4^{2-} \rightarrow 2\ CH_3COO^- + HS^- + 2\ H_2O + H^+$	−146.6	18.7
Pyruvate (complete)	$4\ CH_3COCOO^- + 4\ H_2O + 5\ SO_4^{2-} \rightarrow 12\ HCO_3^- + 5\ HS^- + 3\ H^+$	−106.3	16.1; 25.7
Propionate (incomplete)	$4\ CH_3CH_2COO^- + 3\ SO_4^{2-} \rightarrow 4\ CH_3COO^- + 4\ HCO_3^- + 3\ HS^- + H^+$	−50.2	5.5; 6.8
Benzoate (complete)	$C\text{-}H_5O_2^- + 3.75\ SO_4^{2-} + 4\ H_2O \rightarrow 7\ HCO_3^- + 3.75\ HS^- + 2.25\ H^+$	−49.7	15.0–42.0
Butyrate (complete)	$CH_3CH_2CH_2COO^- + 2.5\ SO_4^{2-} \rightarrow 4\ HCO_3^- + 2.5\ HS^- + 0.5\ H^+$	−49.2	23.1–32.7
Acetate (complete)	$CH_3COO^- + SO_4^{2-} \rightarrow 2HCO_3^- + HS^-$	−47.6	18.0–22.0

[a] Calculated from the free energy change of formation using data from Hanselmann (19) and Thauer et al. (43).

authors postulated that changes in substrate affected isotope fractionation only insofar as they affected sulfate reduction rates. A correlation between sulfate reduction rates and fractionation is also supported by continuous culture experiments (9). Our data do not support a single relationship between the type of substrate, the sulfur isotope fractionation, and sulfate reduction rates because isotope fractionation was independent of the sulfate reduction rate (Fig. 2). The dependence of isotope fractionation on electron donor oxidation appears to be more important. Possibly, a correlation between sulfate reduction rate and isotope fractionation could be found if various substrates were tested for each organism. However, our data require a relationship between isotope fractionation and sulfate reduction rate that is characteristic for each organism. At the present time, we have sufficient information on the sulfate transport mechanisms, as well as the electron donor and electron acceptor pathways, for only a few sulfate reducers (12, 13, 20, 46, 47). This prevents us from relating the observed isotope fractionations to the specific physiology of each species. Further studies are required to understand sulfur isotope fractionation during dissimilatory sulfate reduction at the biochemical level.

Abundance of the investigated microorganisms in natural environments. Most of the reported isotope fractionations by sulfate reducers published before this study were derived from experiments with *Desulfovibrio* spp. and *Desulfotomaculum* spp. (9, 15, 22, 23, 28). Although *Desulfovibrio* spp. were detected in marine sediments (1, 2, 38) and *Desulfotomaculum* spp. were encountered in aquifers (3, 14), these sulfate reducers are not abundant in many other environmental settings. Therefore, overall fractionations in sulfate-reducing environments may often be influenced by organisms other than *Desulfovibrio* spp. and *Desulfotomaculum* spp. Furthermore, all of the previously investigated strains were incomplete-oxidizing sulfate reducers. In some marine sediments, however, complete-oxidizing species represent more than 70% of the identifiable sulfate reducers (34). Some of the sulfate reducers investigated here are of quantitative importance in their natural habitats. For example, *Desulfococcus* spp. and *Desulfotalea* spp. were the most abundant sulfate reducers in marine arctic sediments (34, 35). In near-shore sediments of the Wadden Sea in northern Germany and in hypersaline mats, *Desulfonema* spp. accounted for an important fraction of the total bacterial biomass (26, 42). *Desulfobulbus* spp. were the most abundant sulfate reducers in a freshwater lake (25).

Biogeochemical implications for interpretation of the sulfur cycle from isotope abundances. The present study is the first one to demonstrate that sulfate-reducing prokaryotes can produce widely different sulfur isotope fractionations during sulfate reduction. However, not the phylogenetic differences between the organisms but the physiological differences appear to be decisive for the isotope fractionation. Natural environments commonly contain a mixture of sulfate-reducing prokaryotes (26, 34). Thus, the characteristic community in a particular marine habitat can affect the isotope fractionations during bacterial sulfate reduction. Which sulfate reducers are present in a particular environment and which specific substrates are utilized thus become relevant controlling parameters for the isotope fractionation. There is no general agreement about the dominant substrates for sulfate reducers in marine environments. Acetate is generally regarded as an important terminal substrate in marine environments, but hydrogen can be an important substrate in syntrophic bacterial communities (31). Since the composition of the organic matter varies from place to place it is likely that the anaerobic food chain and the microbial community of sulfate reducers in different habitats varies as well. There is now clear molecular genetic evidence for the presence of different sulfate-reducing communities in different marine habitats (26, 34, 35, 39). Consequently, the overall isotope fractionations by sulfate-reducing communities in different environments may vary because different sulfate reducers are present.

Isotopic differences between sulfate and sulfide in marine sediments and porewaters are generally much greater than the experimentally determined isotope fractionations for pure cultures (5, 6, 18). In the natural environment prokaryotes are generally limited by the availability of organic substrate (32). General substrate limitation may also increase the isotope fractionation. Furthermore, in the natural environment additional isotope effects exist in the oxidative part of the sedimentary sulfur cycle through disproportionation of thiosulfate, elemental sulfur, or sulfite (7, 17). Therefore, in addition to considering variations in the microbial community structure of sulfate reducers, interpretation of isotope signals preserved in sediments and porewaters also have to take into account isotope effects in the oxidative part of the sulfur cycle.

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REFERENCES

1. **Bale, S. J., K. Goodman, P. A. Rochelle, J. R. Marchesi, J. C. Fry, A. J. Weightman, and R. J. Parkes.** 1997. *Desulfovibrio profundis* sp. nov., a novel barophilic sulfate-reducing bacterium from deep sediment layers in the Japan Sea. Int. J. Syst. Bacteriol. 47:515–521.
2. **Barnes, S. P., S. D. Bradbrook, B. A. Cragg, J. R. Marchesi, A. J. Weightman, J. C. Fry, and R. J. Parkes.** 1998. Isolation of sulfate-reducing bacteria from deep sediment layers of the Pacific ocean. Geomicrobiol. J. 15:67–83.
3. **Boivin-Jahns, V., R. Ruimy, A. Bianchi, S. Daumas, and R. Christen.** 1996. Bacterial diversity in a deep-subsurface clay environment. Appl. Environ. Microbiol. 62:3405–3412.
4. **Böttcher, M., S. Sievert, and J. Kuever.** 1999. Fractionation of sulfur isotopes during dissimilatory reduction of sulfate by a thermophilic gram-negative bacterium at 60°C. Arch. Microbiol. 172:125–128.
5. **Brüchert, V., and L. M. Pratt.** 1999. Stable sulfur isotopic evidence for historical changes of sulfur cycling in estuarine sediments from northern Florida. Aquat. Geochem. 5:249–268.
6. **Brüchert, V., M. E. Pérez, and C. B. Lange.** 2000. Coupled primary production, benthic formainiferal assemblage, and sulfur diagenesis in organic-rich sediments of the Benguela upwelling system. Mar. Geol. 163:27–40.
7. **Canfield, D. E., B. Thamdrup, and S. Fleischer.** 1998. Isotope fractionation and sulfur metabolism by pure and enrichment cultures of elemental sulfur-disproportionating bacteria. Limnol. Oceanogr. 43:253–264.
8. **Canfield, D. E., and B. T. Thamdrup.** 1994. The production of ^{34}S-depleted sulfide during disproportionation of elemental sulfur. Science 266:1973–1975.
9. **Chambers, L. A., P. A. Trudinger, J. W. Smith, and M. S. Burns.** 1975. Fractionation of sulfur isotopes by continuous cultures of *Desulvovibrio desulfuricans*. Can. J. Microbiol. 21:1602–1607.
10. **Chambers, L. A., and P. A. Trudinger.** 1979. Microbiological fractionation of stable sulfur isotopes. Geomicrobiol. J. 1:249–293.
11. **Cline, J. D.** 1969. Spectrophotometric determination of hydrogen sulfide in natural waters. Limnol. Oceanogr. 14:454–459.
12. **Cypionka, H.** 1989. Characterization of sulfate transport in *Desulvovibrio desulfuricans*. Arch. Microbiol. 152:237–243.
13. **Cypionka, H.** 1994. Sulfate transport. Methods Enzymol. 243:3–14.
14. **Daumas, S., R. Cord-Ruwisch, and J. L. Garcia.** 1988. *Desulfotomaculum geothermicum* sp. nov., a thermophilic, fatty acid-degrading, sulfate-reducing bacterium isolated with H_2 from geothermal ground water. Antonie Leeuwenhoek 54:165–178.
15. **Fry, B., H. Gest, and J. M. Hayes.** 1988. $^{34}S/^{32}S$ fractionation in sulfur cycles catalyzed by anaerobic bacteria. Appl. Environ. Microbiol. 54:250–256.
16. **Giesemann, A., H. J. Jaeger, A. L. Norman, H. R. Krouse, and W. A. Brand.** 1994. On-line sulfur isotope determination using an elemental analyzer coupled to a mass spectrometer. Anal. Chem. 66:2816–2819.
17. **Habicht, K. N., D. E. Canfield, and J. Rethmeier.** 1998. Sulfur isotope fractionation during bacterial reduction and disproportionation of thiosulfate and sulfite. Geochim. Cosmochim. Acta 62:2585–2595.
18. **Habicht, K. S., and D. E. Canfield.** 1997. Sulfur isotope fractionation during bacterial sulfate reduction in organic-rich sediments. Geochim. Cosmochim. Acta 61:5351–5361.
19. **Hanselmann, K. W.** 1991. Microbial energetics applied to waste repositories. Experientia 47:645–687.
20. **Hansen, T. A.** 1994. Metabolism of sulfate-reducing bacteria. Antonie Leeuwenhoek 66:165–185.
21. **Harrison, A. G., and H. G. Thode.** 1958. Mechanism of the bacterial reduction of sulphate from isotope fractionation studies. Trans. Faraday Soc. 54: 84–92.
22. **Kaplan, I. R., and S. C. Rittenberg.** 1964. Microbiological fractionation of sulphur isotopes. J. Gen. Microbiol. 34:195–212.
23. **Kemp, A. L. W., and H. G. Thode.** 1968. The mechanism of the bacterial reduction of sulphate and sulphite from isotope fractionation studies. Geochim. Cosmochim. Acta 32:71–91.
24. **Kreke, B., and H. Cypionka.** 1992. Proton motive force in freshwater sulfate-reducing bacteria, and its role in sulfate accumulation in *Desulfobulbus propionicus*. Arch. Microbiol. 158:183–187.
25. **Li, J., K. Purdy, S. Takii, and H. Hayashi.** 1999. Seasonal changes in ribosomal RNA of sulfate-reducing bacteria and sulfate-reducing activity in a fresh water lake sediment. FEMS Microbiol. Ecol. 28:31–39.
26. **Llobet-Brossa, E., R. Rosselló-Mora, and R. Amann.** 1998. Microbial community composition of Wadden Sea sediments as revealed by fluorescence in situ hybridization. Appl. Environ. Microbiol. 64:2691–2696.
27. **Mariotti, A., J. C. Germon, P. Hubert, P. Kaiser, R. Letolle, A. Tardieux, and P. Tardieux.** 1981. Experimental determination of nitrogen kinetic isotope fractionation: some principles. Illustration for the denitrification and nitrification processes. Plant Soil. 62:413–430.
28. **McCready, R. G. L.** 1975. Sulphur isotope fractionation by *Desulvovibrio* and *Desulfotomaculum* species. Geochim. Cosmochim. Acta 39:1395–1401.
29. **Muyzer, G., A. Teske, C. O. Wirsen, and H. W. Jannasch.** 1995. Phylogenetic relationships of Thiomicrospira species and their identification in deep-sea hydrothermal vent samples by denaturating gradient gel electrophoresis of 16S rRNA fragments. Arch. Microbiol. 164:165–172.
30. **Nilsen, K. R., T. Torsvik, and T. Lien.** 1996. *Desulfotomaculum thermocisternum* sp. nov., a sulfate reducer isolated from a hot North Sea oil reservoir. Int. J. Syst. Bacteriol. 46:397–402.
31. **Parkes, R. J., G. R. Gibson, I. Mueller-Harvey, W. J. Buckingham, and R. A. Herbert.** 1989. Determination of the substrates for sulphate-reducing bacteria within marine and estuarine sediments with different rates of sulphate reduction. J. Gen. Microbiol. 135:175–187.
32. **Porter, K. G., and Y. S. Feig.** 1980. The use of DAPI for identifying and counting aquatic microflora. Limnol. Oceanogr. 25:943–948.
33. **Rabus, R., and F. Widdel.** 1995. Anaerobic degradation of ethylbenzene and other aromatic hydrocarbons by new denitrifying bacteria. Arch. Microbiol. 163:96–103.
34. **Ravenschlag, K., K. Sahm, C. Knoblauch, B. Jørgensen, and R. Amann.** 2000. Community structure, cellular rRNA content and activity of sulfate-reducing bacteria in marine arctic sediments. Appl. Environ. Microbiol. 66: 3592–3602.
35. **Ravenschlag, K., K. Sahm, J. Pernthaler, and R. Amann.** 1999. High bacterial diversity in permanently cold sediments. Appl. Environ. Microbiol. 65: 3982–3989.
36. **Rees, C. E.** 1973. A steady state model for sulphur isotope fractionation in bacterial reduction processes. Geochim. Cosmochim. Acta 37:1141–1162.
37. **Robinson, B. W.** 1995. Reference and intercomparison materials for stable isotopes of light elements. TECDOC-825. International Atomic Energy Agency, Vienna, Austria.
38. **Sahm, K., C. Knoblauch, and R. Amann.** 1999. Phylogenetic affiliation and quantification of psychrophilic sulfate-reducing isolates in marine arctic sediments. Appl. Environ. Microbiol. 65:3976–3981.
39. **Sahm, K., B. J. MacGregor, B. B. Jørgensen, and D. A. Stahl.** 1999. Sulphate reduction and vertical distribution of sulphate-reducing bacteria quantified by rRNA slot-blot hybridization in a coastal marine sediment. Environ. Microbiol. 1:65–74.
40. **Sørensen, J., D. Christensen, and B. B. Jørgensen.** 1981. Volatile fatty acids and hydrogen as substrates for sulfate-reducing bacteria in anaerobic marine sediment. Appl. Environ. Microbiol. 42:5–11.
41. **Strunk, O., O. Gross, B. Reichel, M. May, S. Hermann, N. Stuckmann, M. Nonhoff, M. Lenke, A. Ginhart, A. Vilbig, T. Ludwig, A. Bode, K.-H. Schleifer, and W. Ludwig.** 1999. ARB: a software environment for sequence data. Department of Microbiology, Technische Universität München, Munich, Germany.
42. **Teske, A., N. B. Ramsing, K. Habicht, M. Fukui, J. Küver, B. B. Jørgensen, and Y. Cohen.** 1998. Sulfate-reducing bacteria and their activities in cyanobacterial mats of Solar Lake (Sinai, Egypt). Appl. Environ. Microbiol. 64: 2943–2951.
43. **Thauer, R. K., K. Jungermann, and K. Decker.** 1977. Energy conservation in chemotrophic anaerobic bacteria. Bacteriol. Rev. 41:100–180.
44. **Wagner, M., A. J. Roger, J. L. Flax, G. A. Brusseau, and D. A. Stahl.** 1998. Phylogeny of dissimilatory sulfite reductase supports an early origin of sulfate respiration. J. Bacteriol. 180:2975–2982.
45. **Widdel, F.** 1980. Ph.D. thesis. University of Göttingen, Göttingen, Germany.
46. **Widdel, F., and F. Bak.** 1992. Gram-negative mesophilic sulfate reducing bacteria, p. 3352–3378. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), The prokaryotes, 2nd ed., vol. IV. Springer, New York, N.Y.
47. **Widdel, F., and T. A. Hansen.** 1992. The dissimilatory sulfate- and sulfur-reducing bacteria, p. 583–624. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), The prokaryotes, 2nd ed., vol. I. Springer, New York, N.Y.
48. **Zhilina, T., G. Zavarsin, F. Rainy, E. Pikuta, G. Osipov, and N. Kostrikina.** 1997. *Desulfonatronovibrio hydrogenovorans* gen. nov., sp nov., an alkaliphilic, sulfate-reducing bacterium. Int. J. Syst. Bacteriol. 47:144–149.

Chapter 5.

Sulfur isotope fractionation by *Desulfovibrio desulfuricans* in the presence of bentonite

Sulfur isotope fractionation by *Desulfovibrio desulfuricans* in the presence of bentonite

Michael E. Böttcher and Jan Detmers

Abstract

Stable sulfur isotope fractionation was investigated experimentally during reduction of sulfate by growing batch cultures of *Desulfovibrio desulfuricans* (DSM 642) in the presence or absence of natural bentonite, consisting essentially of smectite. With lactate as electron donor a cell-specific sulfate-reduction rate (csSRR) of $10^{-9.6 \pm 0.5}$ μmol cell^{-1} h^{-1} was observed. Sulfate-reduction by *Desulfovibrio desulfuricans* yielded a sulfur isotope discrimination of −7 ± 2‰ that was not significantly influenced by the presence of bentonite. The isotope partitioning observed here is consistent with previous results for the variation of sulfur isotope fractionation with csSRR for *Desulfovibrio desulfuricans* and *Desulfovibrio vulgaris* found in resting-cell and chemostat experiments. Tentative oxygen isotope measurements indicate an enrichment of ^{18}O in residual sulfate during the microbial sulfate-reduction process most likely due to an intra-cellular isotope exchange of sulfur intermediates with water.

1. Introduction

Up to 50% of organic matter oxidation on the continental shelf is related to dissimilatory reduction of dissolved sulfate (Jørgensen, 1982). The activity of sulfate-reducing bacteria (SRB) within the sediment is related to the availability of reactive organic substances (e.g., Berner, 1980; Westrich & Berner, 1988). It has been found, that clay minerals, especially smectite are capable of adsorbing large amounts of organic matter (e.g., Keil *et al.*, 1994; Mayer, 1994). This leads to a positive correlation between the mineral surface area, the mud contents of sediments, and organic matter contents in marine sediments (e.g., Schröder & van Es, 1980; deFlaun & Mayer, 1983; Böttcher *et al.*, 2000a). Additionally, a positive relationship seems to exist between the abundance of bacteria and the grain size distribution in sediments (e.g., Schröder & van Es, 1980; deFlaun & Mayer, 1983). These empirical findings indicate that solid-solution interfaces may play an important role in determining the abundance of bacteria in sediments. The interaction of bacteria on mineral surfaces is determined by factors, such as ionic strength, bacterial species and mineralogy (e.g., van Loodsrecht *et al.*, 1989; Yee *et al.*, 2000). Other factors include the availability of metal oxide compounds and fermentation products (Banfield & Nealson, 1997; Lovley, 1991). Although the effect of particles on growth and activity of SRBs is still a matter of continuous discussion (Okabe *et al.* 1995), it is known that most sulfate-reducing bacteria in sediments are particle-associated (Fukui & Take 1996), and some gliding sulfate-reducers even require a surface for growth (Widdel 1980).

Most microbial reactions in the biogeochemical cycle of sulfur are accompanied by fractionations of the stable sulfur isotopes ^{34}S and ^{32}S with different magnitudes (e.g., Chambers & Trudinger 1979). H_2S formed upon reduction of sulfate, for instance, is significantly enriched in the lighter isotope ^{32}S compared to dissolved sulfate (e.g., Kaplan & Rittenberg, 1964). During the reaction of dissolved metals and sulfide to solid metal sulfides, no significant isotope fractionation occurs (Böttcher *et al.* 1998b). Therefore, the isotope signature of sedimentary sulfides should reflect the isotope composition of

hydrogen sulfide produced during overall bacterial metabolism in natural environments. Sulfur isotope partitioning in natural systems has been shown to be a valuable fingerprint of microbial activity in natural sediments (e.g., Hartmann & Nielsen, 1969; Ohmoto *et al.* 1990; Nielsen *et al.* 1991; Böttcher *et al.* 1998a). Isotopic signals in nature, however, often reflect the superimposition of different reactions and may be influenced by environmental boundary conditions, as temperature or substrate availability and quality. Therefore, a proper interpretation of the sedimentary sulfur isotope record requires calibration with results obtained with pure cultures under defined experimental conditions (e.g., Chambers & Trudinger, 1979; Canfield & Thamdrup, 1994; Böttcher *et al.*, 1999). Previous studies with mesophilic sulfate-reducing bacteria showed a strong influence of environmental parameters, such as temperature, on sulfur isotope discrimination leading to highly variable isotope fractionation factors (Harrison & Thode, 1958; Kaplan & Rittenberg, 1964; Kemp & Thode, 1968; Chambers *et al.*, 1975; McCready, 1975; Smock *et al.*, 1998). Additionally, sulfur isotope effects upon interaction of dissolved sulfate with the mineral matrix of natural sediments have been described (Nriagu, 1974; Cortecci, 1978). Although sulfate-reduction in sediments generally takes place in the presence of minerals, none of the previous laboratory studies considered solid surfaces to approach natural conditions.

In the present study, the effect of the presence of mineral surfaces on sulfur isotope fractionation during microbial sulfate-reduction is investigated experimentally. Experiments were conducted with *Desulfovibrio desulfuricans* because members of the genus *Desulfovibrio* play an important role in many natural ecosystems (e.g.; Motamedi & Pedersen 1998; Deveraux *et al.* 1996; Bale *et al.* 1997; Caumette *et al.* 1991; van der Maarel *et al.* 1996 ; Ollivier *et al.* 1990; Trinkerl *et al.* 1991; Parkes *et al.* 1993). Additionally, the influence of a number of boundary conditions (e.g., temperature, pH, type of electron donor and concentrations) on sulfur isotope fractionation has been investigated experimentally for *Desulfovibrio desulfuricans* (Harrison & Thode, 1958; Kemp & Thode 1968; McCready, 1975; Chambers *et al.* 1975; Smock *et al.* 1998;

Cypionka *et al.* 1998). Besides fractionation of the sulfur isotopes, the fractionation of the stable oxygen isotopes ^{18}O and ^{16}O has also been found to be extremely useful for a characterization of biogeochemical reactions (e.g., Mizutani & Rafter, 1973; van Stempvoort & Krouse, 1994). Tentative measurements are presented in this study to demonstrate for the first time the effect of dissimilatory sulfate-reduction by a pure culture on the oxygen isotope composition of sulfate.

The present experimental study demonstrates that the availability of mineral surfaces does not significantly influence sulfur isotope fractionation, and the results obtained here under batch growth conditions agree with earlier experimental studies using chemostat or resting-cell systems.

2. Material and Methods

A freshwater sulfate-reducing strain, *Desulfovibrio desulfuricans* (DSM 642), was obtained from the German Collection of Microorganisms and Cell Cultures; Braunschweig, Germany. It was originally isolated from sandy sediment. The strain was grown at 28°C in PTFE sealed glass bottles in the dark using an unagitated bicarbonate-buffered medium containing about 20 mM lactate prepared as described by Widdel & Bak (1992). The 22 parallel experiments were inoculated from exponentially growing cultures (Table 1) in glass flasks with 4 different amounts of added bentonite powder, and stopped after distinct time intervals (up to 18 days) to obtain different degrees of substrate consumption. Biofilm-formation on bentonite particles was monitored microscopically (Axioplan, Zeiss, Jena). The initial cell number was 5×10^6 per ml. At the end of an experiment aliquots were taken anoxically for cell counting in a Neubauer-chamber. The rest of the solution was immediately mixed with Zn-acetate solution (20%) to precipitate dissolved hydrogen sulfide quantitatively as ZnS. All samples were stored at -20°C until further analysis.

ZnS was converted anoxically by the reaction with 6 M HCl into H_2S, which was then precipitated quantitatively as Ag_2S in 0.1 M $AgNO_3$ solution. The gravimetric determination of Ag_2S was used to determine the amounts of H_2S formed during the experiment. Dissolved sulfate was precipitated as $BaSO_4$ with excess $BaCl_2$. $BaSO_4$ was carefully washed with deionized water, dried, and prepared for isotope analysis.

Sulfur isotope ratios of $BaSO_4$ samples were measured by means of combustion isotope-ratio-monitoring mass spectrometry (C-irmMS) as described by Böttcher *et al.* (1998a). Samples were converted to SO_2 in an elemental analyzer (Carlo Erba EA 1108) which was directly connected to a mass spectrometer Finnigan MAT 252 via a Finnigan Conflo II split interface. Replicate measurements agreed within ±0.2‰. International isotope standards IAEA-S-1 and IAEA-S-2 were used to calibrate the mass spectrometer. The oxygen isotope composition of $BaSO_4$ was analyzed at the University of Tübingen by fluorination with BrF_5 (Clayton & Mayeda, 1963; Pickthorn & O'Neil, 1985). The results were corrected for systematic isotope fractionation resulting from the extraction of only 50% of the sulfate oxygen using NBS-127. Replicate measurements agreed to within ± 0.2‰. $^{18}O/^{16}O$ and $^{34}S/^{32}S$ ratios are reported in the δ-notation relative to the V-SMOW and V-CDT standards, respectively.

The bentonite powder was characterized previously for its mineralogical and surface properties, by powder X-ray diffraction, FT infrared spectroscopy, and BET and NH_4-exchange measurements (Table 1; Gehlken, 1998, personal communication). The mineral mixture consisted to about 85% of the mineral smectite, and illite. Quartz, feldspars and carbonates were only present as minor compounds. The bentonite powder had a high specific surface of 155 m^2/g (Table 1).

Table 1. Characterization of the natural bentonite sample *GIII020 Bn2* used in the experiments of the present study. Mineral composition was determined via powder X-ray diffraction and Fourier-transform infrared spectroscopy according to Flehmig & Kurze (1973). Specific surface and the cation exchange capacity were measured using BET and NH_4 exchange, respectively.

Mineral phases	relative portion
Dioctahedral smectite	~85%
Illite/mica	6-8%
Quartz	2%
Calcite	3%
Dolomite	1-2%
Feldspars	1%
Surface characterization	
Specific surface	155.4 ± 1.4 m^2g^{-1}
Cation exchange capacity	59.2 mval NH_4^+/100 g

3. Results and Discussion

3.1 Sulfate-reduction rates

In the present study the influence of clay minerals on dissimilatory sulfate-reduction and corresponding sulfur isotope fractionation by *Desulfovibrio desulfuricans* was investigated for the first time. Using lactate as electron donor, the batch experiments were incubated with different amounts of natural bentonite, which consisted to about 85% of smectite (Table 1). Smectite is the clay mineral in natural marine sediments which mainly controls the sorption properties for organic matter (Keil *et al.*, 1994; Mayer, 1994) and, as indicated by a positive relationship between organic matter and cell numbers, the

abundance of bacteria at a specific site. The latter influence may be via the direct attachment of bacteria on solid surfaces or indirect via the availability of substrates as fermentation products (Banfield & Nealson, 1997; Lovley, 1991). Considering a projected surface area of about 4 μm^2 per cell (e.g., Banfield & Nealson, 1997), even in the experiments with the lowest solid/solution ratio (#32; Table 2) and high cell numbers at the end of the experiments ($\approx 10^8$ cells ml^{-1}) the fraction of the smectite surface area which was covered by bacteria should not have exceeded more than 4%. Yee *et al.* (2000) showed experimentally, that the adsorption equilibrium of bacteria on selected minerals was established within 1 hour. We, therefore, assume that bacterial sorption on the mineral surface in our runs #32, #33, and #34 was fast compared to the duration of the experiments, and mineral surfaces were generally available in excess in experiments #32-34. However, it has to be considered that the interaction between bacteria and clay minerals can also geometrically be rather complex and lead to an overestimation of the available surface (Lünsdorf *et al.*, 2000).

During incomplete lactate oxidation, dissolved sulfate (28 mM) was reduced to a concentration of about 15 mM in the final experiments, corresponding to about 49% of the initial concentration (Table 2). At the same time, hydrogen sulfide was produced and the number of bacterial cells increased. From the variation of the hydrogen sulfide concentrations and the cell numbers with time (17 to 137 h) of experiments with no bentonite added (#31) and the highest amount of bentonite (#34), respective cell-specific sulfate-reduction rates (csSRR) were calculated according to:

$$csSRR\ (\mu mol\ SO_4\ cell^{-1}\ h^{-1}) = (S_i - S_{i-1}) \bullet ((C_i + C_{i-1})/2)^{-1} \bullet (t_i - t_{i-1})^{-1} \quad (1)$$

S, C and t refer to the amounts of hydrogen sulfide [µmol], the total cell number, and reaction time at time intervals i and i-1. Calculated csSRR values were $10^{-9.4\ \pm\ 0.3}$ and $10^{-9.6 \pm 0.5}$ µmol $cell^{-1}$ h^{-1}, respectively. These results do not differ significantly, indicating

that the cell-specific activity during sulfate-reduction of *Desulfovibrio desulfuricans* was not significantly affected by the presence of smectite minerals.

Table 2. Boundary conditions of setups for experiments #31, #32, #33, and #34.

	Experiment			
	#31	#32	#33	#34
Amount of bentonite (g/l)	0	0.118	0.463	0.918
Number of experiments	10	2	2	10
Initial SO_4 (mM): Grav; IC	27.8; 26.9	n.d.; 27.8	n.d.; 27.6	30.1; 30.0
Initial $\delta^{34}S(SO_4)$ (‰)	+2.54	+2.54	+0.87	+0.87
Initial $\delta^{18}O(SO_4)$ (‰)	+10.4	n.d.	n.d.	n.d.
Initial lactate (mM)	19.4	19.5	19.9	20.3
Run duration (h)	0 to 427	0 to 354	0 to 354	0 to 427
lactate consumption (%)	0 to 49	0 to 39	0 to 43	0 to 47
log csSRR (μmol cell^{-1} h^{-1})	-9.4 ± 0.3	n.d.	n.d.	-9.6 ± 0.5
ε (‰)	-6.4 ± 1	-7.6	-6.6	-7.5 ± 1

Grav: Gravimetry; IC: Ion chromatography. n.d.: not determined.

The csSRR were calculated based on counted cells and evolution of H_2S in the time interval 17 to 137 h.

3.2 Sulfur isotopes

During the sulfate-reduction process the isotope composition of the residual sulfate rose from its initial values continuously (Fig. 1.), as expected for a reaction in a system closed with respect to sulfate (e.g., Nakai & Jensen, 1964; Jørgensen, 1979). The isotope enrichment factors (in ‰; $\varepsilon = \{\alpha - 1\}$ 1000; α: fractionation factor) derived from the $\delta^{34}S$ values of sulfate were evaluated using a Rayleigh equation for a closed system according to Mariotti *et al.* (1981):

$$\delta^{34}S(SO_4^{2-}) = \delta^{34}S(SO_4^{2-0}) + \varepsilon \ln F \qquad (2)$$

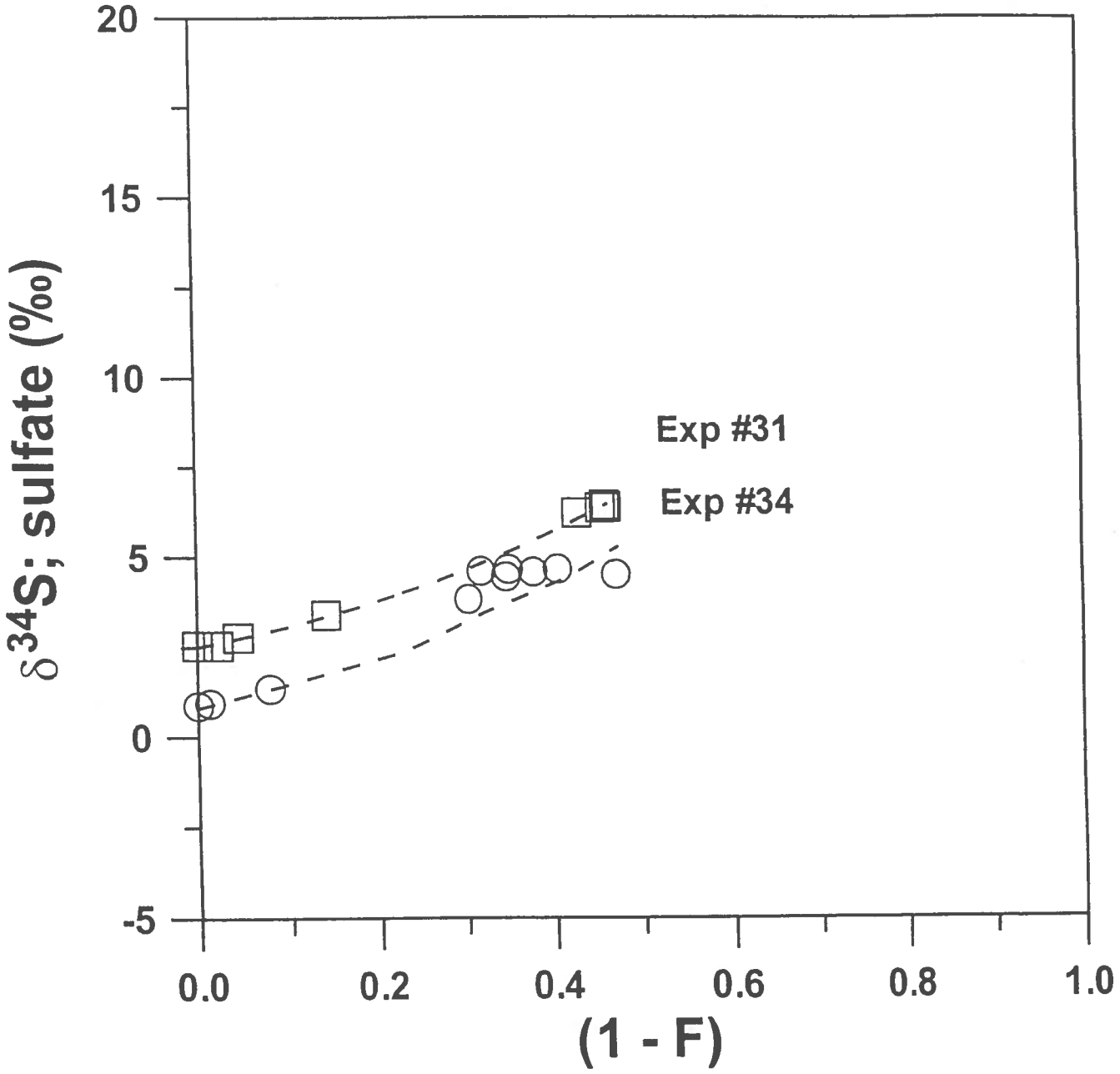

Fig. 1. Variation of the sulfur isotope composition of dissolved sulfate during microbial sulfate-reduction in the presence (experiment #31) and absence (experiment #34) of clay minerals. F: residual sulfate fraction.

F denotes the fraction and $\delta^{34}S(SO_4^{2-0})$ the initial isotope composition of the unreacted dissolved sulfate (Table 2). Evaluation of the sulfur isotope composition of dissolved sulfate of the four different sets of experiments according to equation (1) yields ε-values between -6.4 and -7.6‰ (average -7.0‰), without a significant influence of different amounts of bentonite in the experimental solutions (Table 2). The data are at the lower end of the range observed in previous experimental studies carried out with *Desulfovibrio desulfuricans* (Harrison & Thode, 1958; Kemp & Thode, 1968; Chambers *et al.*, 1975; McCready, 1975). Previous experimental studies showed that the dominant factor controlling the magnitude of sulfur isotope fractionation is the cell-specific sulfate-reduction rate (csSRR), leading to an increase in isotope fractionation with decreasing csSRR (Harrison & Thode, 1958; Kaplan & Rittenberg, 1964; Kemp & Thode, 1968; Chambers *et al.*, 1975). During experimental sulfate-reduction by members of the genus *Desulfovibrio* in the presence of excess sulfate, a maximum $^{34}S/^{32}S$ fractionation of 46‰ was observed (Kaplan & Rittenberg, 1964). The dependence of sulfur isotope fractionation from the csSRR was explained by a chain-reaction mechanism where several steps, with different individual fractionation factors, may become rate-limiting, depending on boundary conditions (Rees, 1973). Rees summarized the experimental results in the following mechanistic model:

$$\text{External } SO_4^{2-} \Leftrightarrow \text{Internal } SO_4^{2-} \Leftrightarrow \text{APS} \Leftrightarrow SO_3^{2-} \rightarrow HS^- \qquad (3)$$

Whereas only small isotope effects appear to be associated with the uptake and activation of sulfate, the reduction of APS (adenosine-5′-phosphosulfate) and especially sulfite results in substantial sulfur isotope fractionation. However, only at low csSRR a maximum isotope fractionation is found due to a superimposition of the different fractionation steps (Rees, 1973).

The experimental results of the present study are in agreement with the relationship between csSRR and sulfur isotope fractionation found previously for *Desulfovibrio desulfuricans* in chemostat and resting-cell experiments (Fig. 2.; Chambers *et al.*, 1975; Kemp & Thode, 1968). Similar results were also observed for *Desulfovibrio vulgaris* (Kaplan & Rittenberg, 1964; Kemp & Thode, 1968). The present experiments were carried out at relatively high csSRR and, therefore, low magnitudes of isotope discrimination were observed. Our results compare well with the measurements of McCready (1975) who investigated isotope fractionation by different growing cultures of sulfate-reducers, including *Desulfovibrio desulfuricans*, but did not estimate csSRR for his experiments. The close agreement between the two studies indicates that the bacteria reduced sulfate with similar cell-specific sulfate-reduction rates. The constancy observed in the present study for sulfur isotope fractionation in the absence or presence of different amounts smectite is in agreement with the negligible influence on the cell-specific sulfate-reduction rates (section 3.1).

3.3 Oxygen Isotopes

Besides sulfur isotope fractionation, the change in the $^{18}O/^{16}O$ ratio of residual sulfate was tentatively investigated for experiment #31. It was found that during sulfate-reduction by *Desulfovibrio desulfuricans* the oxygen isotope composition, $\delta^{18}O$, of dissolved sulfate increased by 1.6‰ from +10.4‰ in the initial solution to +12.0‰ after consumption of about 49% of the initial sulfate. The corresponding $\delta^{34}S$ values increased by 3.8‰ from +2.5‰ to +6.3‰. The results are consistent with experiments with mixed bacterial cultures (Mizutani & Rafter, 1973; Fritz *et al.*, 1989) and field observations (Zak *et al.*, 1980; Böttcher *et al.* 1998a,b; 2000b) indicating that reactions leading to oxygen isotope exchange between sulfate and water took place during the sulfate-reduction process.

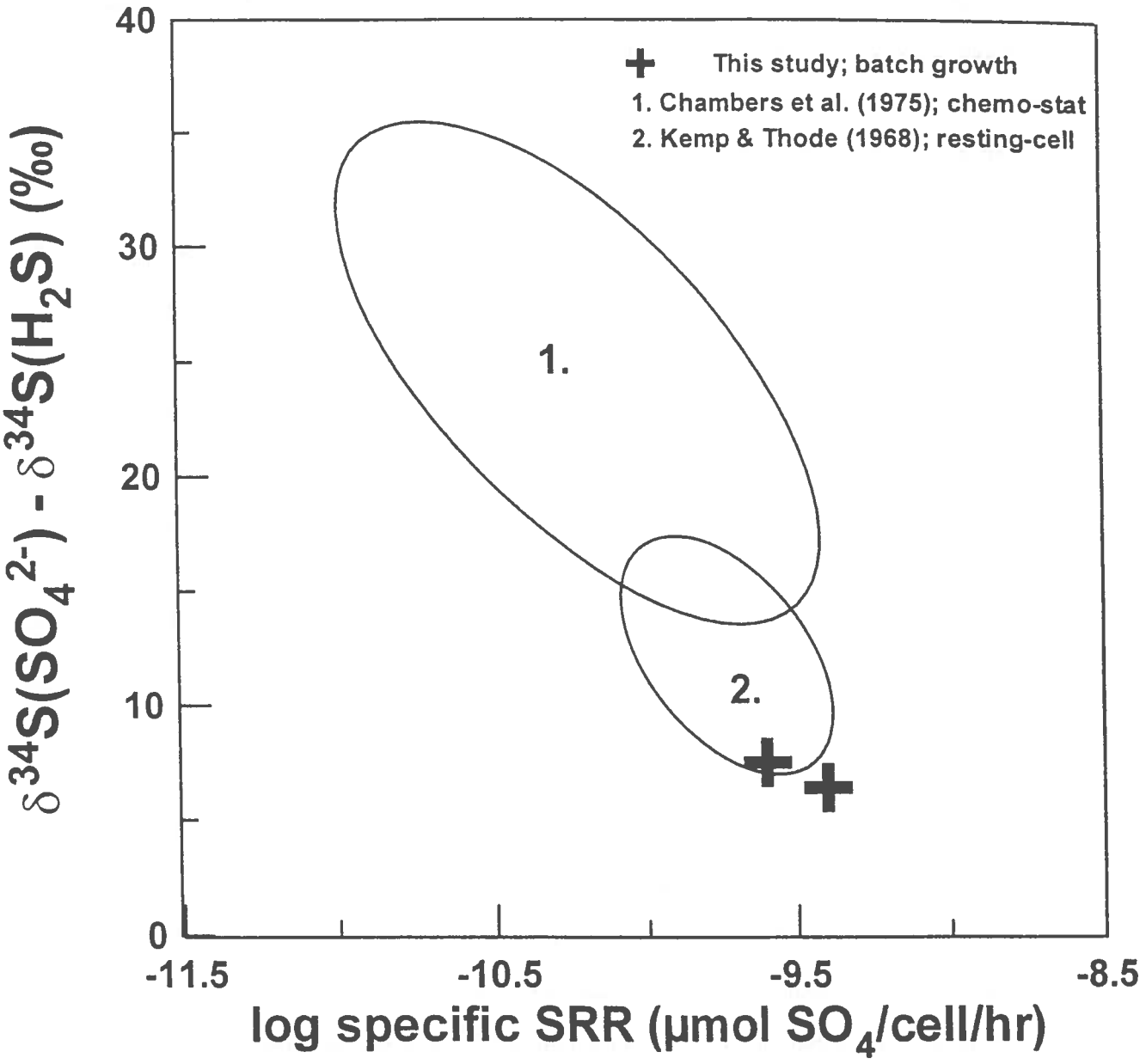

Fig. 2. Variation of sulfur isotope fractionation during microbial sulfate-reduction by *Desulfovibrio desulfuricans* as a function of the cell-specific sulfate-reduction rate. 1. Chemo-stat experiments with strain El Agheila Z (NCIBM No. 8380) (Chambers et al., 1975); 2. Resting-cell experiments with strain ATCC No. 7757 (Kemp & Thode, 1968). Rate units in Kemp & Thode (1968) were transfomed into cell-specific SRR according to Kaplan & Rittenberg (1964).

Non-biological oxygen isotope exchange between sulfate and water is extremely slow at neutral pH and low temperatures (e.g., Mizutani & Rafter, 1969) and can be neglected for the experimental conditions of the present study. Additionally, the bacterial sulfate-reduction experiments of Mizutani & Rafter (1973) & Fritz *et al.* (1989) with aqueous solutions of different oxygen isotope compositions clearly demonstrated that oxygen isotope exchange takes place between external sulfate and water during sulfate-reduction. Considering the reaction scheme proposed by Rees (1973) (equation 3), both APS (Fritz *et al.*, 1989) and sulfite (Mizutani & Rafter, 1973) were suspected to exchange oxygen isotopes with water leading to an increased equilibration between external sulfate and water with increasing reaction extent (Fritz *et al.*, 1989). Due to its very fast oxygen isotope exchange rate with water (van Stempvoort & Krouse, 1994), sulfite seems to be the most probable candidate to be responsible for the observed shift in ^{18}O of residual sulfate.

In figure 3 the co-variation of the sulfur and oxygen isotope composition of residual sulfate from experiment #31 is compared to results observed in anoxic sediments which are dominated by sulfate-reduction (Böttcher *et al.*, 2000b). Both the recent experiments with a pure culture and field measurements show a positive relationship between ^{34}S and ^{18}O-enrichment. The slopes between both parameters seem to be different most likely due to different isotope compositions of sulfate at equilibrium and different sulfate-reduction rates (Böttcher *et al.*, 1998b; Aharon & Fu, 2000).

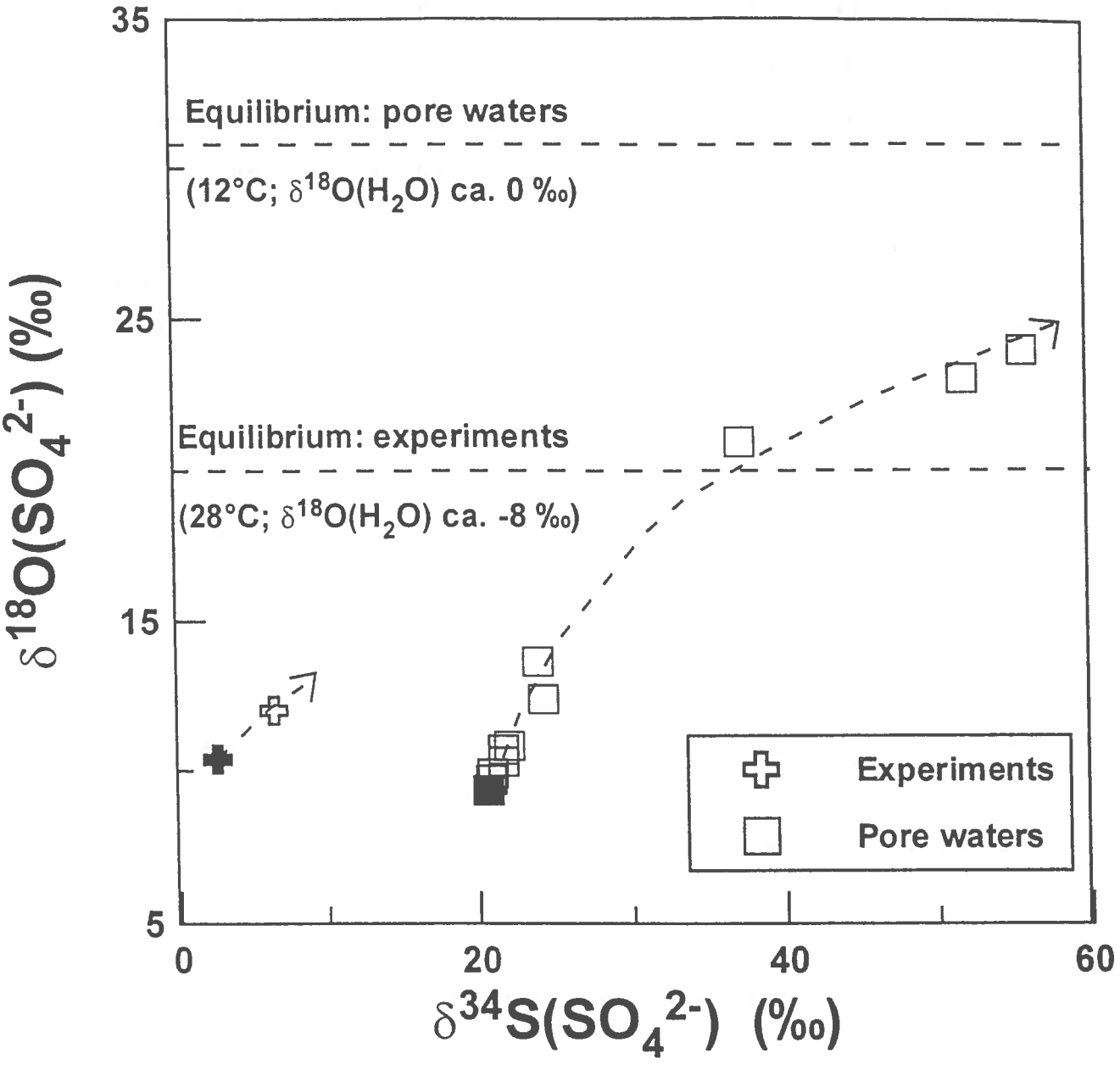

Fig. 3. Covariation of the sulfur and oxygen isotope compostion of residual sulfate in experiment #31 compared to the results for anoxic pore waters from upwelling-sediments off central Chile (Böttcher et al., 2000b). Oxygen isotope exchange equilibrium between dissolved sulfate and water was extrapolated from experimental results of Mizutani & Rafter (1969) considering estimated isotope compositions for waters at *in-situ* conditions. Closed symbols: Initial solutions.

3.4 Biogeochemical implications

Members of the genus *Desulfovibrio* have been shown to play an important role in many natural ecosystems under extremely different environmental conditions. They have been isolated, for instance, from coastal, estuarine and deep sea sediments, ground waters, and hypersaline lake sediments (Motamedi & Pedersen 1998; Deveraux *et al.* 1996; Bale *et al.* 1997; Caumette *et al.* 1991; van der Maarel *et al.* 1996; Ollivier *et al.* 1991; Trinkerl *et al.* 1990; Parkes *et al.* 1993). The RNA of *Desulfovibrionaceae* accounted for an important fraction of the total RNA in coastal marine sediment (Sahm *et al.* 1999) and in hypersaline microbial mats (Risatti *et al.* 1994). *Desulfovibrio* was present in high numbers in salt marsh sediments (Rooney-Varga *et al.* 1997, Hines *et al.* 1999) and made up more than 80% of the culturable fraction of sulfate-reducing bacteria in Lake Constance, a freshwater habitat (Bak & Pfennig 1991). A strain of *Desulfovibrio desulfuricans* accounted for up to 6×10^7 cells/cm^3 in a brackish sediment of the Baltic Sea (Lillebaek 1995). *Desulfovibrio desulfuricans* is known for the attachment to mineral surfaces and the formation of biofilms on particles along with the production of extracellular polymeric substances (Fukui & Takii 1994, Okabe *et al.* 1995).

It is, therefore, obvious that the results of the present study are of widespread importance for the interpretation of the sedimentary sulfur isotope record of natural environments. Our experimental results demonstrate that sulfur isotope partitioning observed in culture experiments without the presence of mineral surfaces can be transferred to natural sediments. Additionally, our results are consistent with an inverse relationship between cell-specific sulfate-reduction rate (csSRR) and sulfur isotope fractionation observed previously for members of the genus *Desulfovibrio*. In these studies a maximum isotope discrimination of -46‰ was observed (Kaplan & Rittenberg, 1964). The csSRR estimated recently for natural sediments (Sahm *et al.*, 1999; Böttcher *et al.*, 2000a), however, are lower than the lowest rates used in experimental studies on sulfur isotope fractionation (Kaplan & Rittenberg, 1964). Therefore, there is no a-priori

reason to assume that –46‰ is a natural maximum limit for sulfur isotope discrimination in sediments.

In the present study, oxygen isotope fractionation during sulfate-reduction was investigated for the first time for a pure culture of a sulfate-reducing bacterium, and our results are consistent with recent measurements in anoxic sediments. A more detailed experimental work is in progress to determine the relationship between csSRR and the rate of oxygen isotope exchange to provide a more fundamental biogeochemical base for the interpretation of oxygen isotope signatures in pore waters of the natural sediments.

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4. References

Aharon, P. & Fu, B. (2000). Microbial sulfate reduction rates and sulfur and oxygen isotope frcationation at oil and gas seeps in deepwater Gulf of Mexico. Geochim. Cosmochim. Acta 64, 233-246.

Bak, F. & Pfennig, N. (1991). Sulfate-reducing bacteria in littoral sediment of Lake Constance. FEMS Microbiol. Ecol. 85, 43 - 52.

Bale, S.J., K. Goodman, P.A. Rochelle, J.R. Marchesi, J.C. Fry, A.J. Weightman, and R.J. Parkes. (1997). *Desulfovibrio profundis* sp. nov., a novel barophilic sulfate-reducing bacterium from deep sediment layers in the Japan Sea. Int. J. System.Bacteriol. 47:515-521.

Banfield, J.F. & Nealson, K. H. (1997). Geomicrobiology: Interactions between microbes and minerals. Rev. Mineral. 35, 448 pp.

Berner, R.A. (1980). Sedimentary pyrite formation. Am. J. Sci., 268, 2-23.

Böttcher, M.E., Brumsack, H.-J. & de Lange, G.J. (1998a). Sulfate reduction and related stable isotope (^{34}S, ^{18}O) variations in interstitial waters from the eastern Mediterranean. In (eds. Robertson AHF, *et al.*) Proc. Oc. Dril. Prog., Scientific Results 160, 365-373.

Böttcher, M.E., Oelschläger, B., Höpner, T., Brumsack, H.-J. & Rullkötter, J. (1998b). Sulfate reduction related to the early diagenetic degradation of organic matter and "black spot" formation in tidal sandflats of the German Wadden Sea: Stable isotope (^{13}C, ^{34}S, ^{18}O) and other geochemical results. Org. Geochem. 29, 1517-1530.

Böttcher, M.E., Sievert, S.M. & Kuever, J. (1999). Fractionation of sulfur isotopes during dissimilatory reduction of sulfate by a thermophilic gram-negative bacterium at 60°C. Arch. Microbiol. 172, 125-128.

Böttcher, M.E., Llobet-Brossa, E., Hespenheide, B., Beardsley, C., Larsen, O., Schramm, A., Wieland, A., Böttcher, G., Berninger, U.-G. & Amann, R. (2000a).

Biogeochemistry, stable isotope geochemistry, and microbial community structure of a temperate intertidal mudflat: An integrated study. Cont. Shelf Res. 20, 1749-1769.

Böttcher, M.E., Thamdrup B. & Vennemann T.W. (2000b). Oxygen and sulfur isotope fractionation during anaerobic bacterial disproportionation of elemental sulfur. Geochim. Cosmochim. Acta, submitted.

Canfield D.E. & Thamdrup, B. (1994). The production of ^{34}S-depleted sulfide during bacterial disproportionation of elemental sulfur. Science 266, 1973-1975.

Caumette, P., Y. Cohen, and R. Matheron. (1991). Isolation and characterization of *Desulfovibrio halophilus* sp. nov., a halophilic sulfate-reducing bacterium isolated from Solar Lake (Sinai). System. Appl. Microbiol. 14:33 - 38.

Chambers, L. A. & Trudinger, P. A. (1979). Microbiological fractionation of stable sulfur isotopes: a review and critique. Geomicrobiol. J. 1, 249-293.

Chambers, L.A., Trudinger, P.A., Smith, J.W. & Burns, M.S. (1975). Fractionation of sulfur isotopes by continuous cultures of *Desulfovibrio desulfuricans*. Can. J. Microbiol. 21, 1602-1607.

Clayton, R.N. & Mayeda. T.K. (1963). The use of bromine pentafluoride in the extraction of oxygen from oxides and silicates for isotopic analysis. Geochim. Cosmochim. Acta 27, 43-52.

Cortecci, G. (1978). Fractionation of sulphate oxygen and sulphur isotopes by marine sediment retention. Bull. N.Z. Dept. Sci. Industr. Res. 20, 49-52.

Cypionka, H., Smock, A. & Böttcher, M.E. (1998). A combined pathway of sulphur compound disproportionation in *Desulfovibrio desulfuricans*. FEMS Microbiol. Lett. 166, 181-186.

deFlaun, M. F. & Mayer, M. (1983). Relationships between bacteria and grain surfaces in intertidal sediments. Limnol. Oceanogr. 28, 873-881.

Devereux, R., M.R. Winfrey, J. Winfrey, and D.A. Stahl. (1996). Depth profile of sulfate-reducing bacterial ribosomal RNA and mercury methylation in an estaurine sediment. FEMS Microbiol. Ecol. 20:23 - 31.

Flehmig, W. & Kurze, R. (1973). Die quantitative infrarotspektroskopische Phasenanalyse von Mineralgemengen. N. Jb. Mineral. Abh. 119, 101-112.

Fritz, P., Basharmal, G.M., Drimmie, R.J., Ibsen, J., & Qureshi, R.M., (1989). Oxygen isotope exchange between sulphate and water during bacterial reduction of sulphate. Chem. Geol. 79, 99-105

Fukui, M. & Takii, S. (1994). Kinetics of sulfate respiration by free-living and particle-associated sulfate-reducing bacteria. FEMS Microbiol. Ecol. 13, 241 - 248.

Fukui, M. & Take, S. (1996). Microdistribution of sulfate-reducing bacteria in sediments of a hypertrophic lake and their response to the addition of organic matter. Ecol. Res. 11(3), 257 - 267.

Harrison, A.G. & Thode, H.G. (1958). Mechanism of the bacterial reduction of sulfate from isotope fractionation studies. Faraday Soc. Trans. 54, 84-92.

Hartmann, M. & Nielsen, H. (1969). δ^{34}S-Werte in rezenten Meeressedimenten und ihre Deutung am Beispiel einiger Sedimentprofile aus der westlichen Ostsee. Geol. Runds. 58, 621-655.

Hines, M., Evans, R. *et al.* (1999). Molecular phylogenetic and biogeochemical studies of sulfate-reducing bacteria in the rhizophere of *Spartina alterniflora*. Appl. Environ. Microbiol. 65(5), 2209 - 2216.

Jørgensen, B.B. (1979). A theoretical model of the stable sulfur isotope distribution in marine sediments. Geochim. Cosmochim. Acta 43, 363-374.

Jørgensen, B.B. (1982). Mineralization of organic matter in the sea bed - the role of sulphate reduction. Nature 296, 643-645.

Kaplan, I.R. & Rittenberg, S.C. (1964). Microbiological fractionation of sulphur isotopes. J. Gen. Microbiol. 34, 195-212.

Keil, R.G., Martlucan, D.B., Prahl, F.G. & Hedges, J.I. (1994). Sorptive preservation of labile organic matter in marine sediments. Nature 370, 549-552.

Kemp, A.L.W. & Thode, H.G. (1968). The mechanism of the bacterial reduction of sulphate and of sulphite from isotope fractionation studies. Geochim. Cosmochim. Acta 32, 71-91.

Lillebaek, R. (1995). Application of antisera raised against sulfate-reducing bacteria for indirect immunofluorescent detection of immunoreactive bacteria in sediment from the german baltic sea. Appl. Environ. Microbiol. 61(9), 3436 - 3442.

Lovley, D.R. (1991). Dissimilatory Fe(III) and Mn(IV) reduction. Microb. Rev. 55, 259-287.

Lünsdorf, H., Erb, R.W., Abraham, W.-R. & Timmis, K.N. (2000). 'Clay hutches': a novel interaction between bacteria and clay minerals. Envrionm. Microbiol. 2, 161-168.

Mariotti, A., German, J.C., Hubert, P., Kaiser, P., Létolle, R., Tardieux, A. & Tardieux, P. (1981). Experimental determination of nitrogen kinetic isotope fractionation: some principles; illustration for the denitrification and nitrification. Plant Soil 62, 413-430.

Mayer, L.M. (1994). Relationships between mineral surfaces and organic carbon concentrations in soils and sediments. Chem. Geol. 114, 347-363.

McCready, R.G.L. (1975). Sulphur isotope fractionation by *Desulfovibrio* and *Desulfotomaculum* species. Geochim. Cosmochim. Acta. 39, 1395-1401.

Mizutani, Y. & Rafter, T.A. (1969). Oxygen isotopic composition of sulphates - part 3. Oxygen isotopic fractionation in the bisulphate ion - water system. New Zealand J. Sci. 12, 54-59.

Mizutani, Y. & Rafter, T.A. (1973). Isotopic behavior of sulfate oxygen in the bacterial reduction of sulfate. Geochem. J. 6, 183-191.

Motamedi, M. & Pedersen, K. (1998). *Desulfovibrio aespoeensis* sp. nov., a mesophilic sulfate-reducing bacterium from deep groundwater at Äspö hard rock laboratory, Sweden. Int. J. Syst. Bacteriol. 48, 31 - 315.

Nielsen, H. (1991). Lithospheric sources of sulfur. In (Krouse, H.R. & Grinenko, V.A. (eds.) Stable isotopes in the assesment of natural and anthropogenic sulphur in the environment. SCOPE, 65- 132.

Nriagu, J. (1974). Fractionation of sulphur isotopes by sediment adsorption of sulfate. Earth Planet. Sci. Lett. 22, 366-370.

Ohmoto, H., Kaiser, C.J. & Geer, K.A. (1990). Systematics of sulphur isotopes in recent marine sediments an ancient sediment-hosted basemetal deposits. In (Herbert, H.K. (ed.) Stable isotopes and fluid processes in mineralization. Proc. Conf. Stable Isotopes Fluid Proc. Mineralization, 70-120.

Okabe, S., P. Nielsen, W. Jones, and W. Chakacklis. (1995). Rate and stoichiometry of microbial sulfate reduction by *Desulfovibrio desulfuricans* in biofilms. Biofouling 9:63 - 83.

Ollivier, B., C. Hatchikian, G. Prensier, J. Guezennec, & Garcia, J. (1991) *Desulfohalobium retbaense* gen. nov., sp. nov., a halophilic sulfate-reducing bacterium from sediments of a hypersaline lake in Senegal. Int. J. Syst. Bacteriol. 41, 74 - 81.

Parkes, R., N. Dowling, D. White, R. Herbert, and G. Gibson. (1993). Characterization of sulfate-reducing bacterial populations within marine and estaurine sediments with different rates of sulfate reduction. FEMS Microbiol. Ecol. 102:235 - 250.

Pickthorn, W.J. & O'Neil, J.R. (1985). ^{18}O relations in alunite minerals: potential single-mineral thermometer. GSA Abstr. Progr. 17, 689.

Rees, C.E. (1973). A steady state model for sulphur isotope fractionation in bacterial reduction processes. Geochim. Cosmochim. Acta 37, 1141-1162.

Risatti, J., W. Capman, and D. Stahl. (1994). Community structure of a microbial mat: the phylogenetic dimension. Proc. Nat. Sci. USA 91:10173 - 10177.

Rooney-Varga, J.N., R. Devereux, R.S. Evans, and M.E. Hines. (1997). Seasonal changes in the relative abundance of uncultivated sulfate-reducing bacterie in a salt marsh sediment and in the rhizosphere of *Spartina alterniflora.* Appl. Environ. Microbiol. 63(10), 3895-3901.

Sahm, K., MacGregor, B.J., Jørgensen, B.B. & Stahl, D.A. (1999). Sulphate reduction and vertical distribution of sulphate-reducing bacteria quantified by rRNA slot-blot hybridization in a coastal marine sediment. Environm. Microbiol. 1, 65-74

Schröder, H.G.J. & van Es, F.B. (1980). Distribution of bacteria in intertidal sediments of the Ems-Dollard estuary. Neth. J. Sea Res. 14, 268-287

Smock, A., Böttcher, M.E. & Cypionka, H. (1998). Fractionation of sulfur isotopes during thiosulfate reduction by *Desulfovibrio desulfuricans*. Arch Microbiol 169, 460-463

Trinkerl, M., A. Breunig & Schauder R. (1990). *Desulfovibrio termitidis* sp. nov., a carbohydrate-degrading sulfate-reducing bacterium from the hindgut of a termite. System. Appl. Microbiol. 13, 372 – 377.

van der Maarel, M.J.E.C., M. Jansen, R. Haanstra, W.G. Meijer, and T.A. Hansen. (1996). Demethylation of Demethylsulfoniopropionate to 3-S-Methylmercapto-propionate by marine sulfate-reducing bacteria. Appl. Environ. Microbiol. (9), 3978 - 3984.

van Loodsrecht, M.C.C., Lyklema, J. , Norde, W. & Zehnder, A.J.B. (1989). Bacterial Adhesion: A physicochemical approach. Microbial Ecology 17, 1-15.

van Stempvoort, D.R. & Krouse, H.R. (1994). Controls of $\delta^{18}O$ in sulfate: Review of experimental data and application to specific environments. ACS Symp Ser. 550, 466-480.

Westrich, J.T. & Berner, R.A. (1988). The effect of temperature on rates of sulphate reduction in marine sediments. Geomicrobiol. J. 6, 99-117.

Widdel, F. (1980). Anaerober Abbau von Fettsäuren und Benzoesäure durch neu isolierte Arten Sulfat-reduzierender Bakterien. Ph.D. thesis, University of Göttingen, Göttingen.

Widdel, F. & Bak, F. (1992). Gram-negative mesophilic sulfate-reducing bacteria. In Balows, A., Trüper, H.G., Dworkin, M., Harder, W., Schleifer, K.H. (eds.) The Prokaryotes, 3352-3402, Springer, Berlin, Germany.

Yee N., Fein, J.B. & Daughney, C.J. (2000). Experimental study of the pH, ionic strength, and reversibility behavior of bacteria-mineral adsorption. Geochim. Cosmochim. Acta 64, 609-617.

Zak, I., Sakai, H. & Kaplan I.R. (1980). Factors controlling the $^{18}O/^{16}O$ and $^{34}S/^{32}S$ isotope ratios of ocean sulfate, evaporites and interstitial sulfates from deep sea sediments. In Isotope Marine Chemistry (eds. Goldberg *et al.*), 339-373.

Chapter 6.

Manuscripts in preparation

6.1. *Desulfotomaculum garzweileriensis*, sp. nov., and *Desulfotomaculum carboniphilus*, sp. nov., two novel spore-forming sulfate-reducing bacteria from a deep subsurface aquifer.

J. Detmers, V.Brüchert, and J. Kuever

Two novel, completely-oxidizing sulfate-reducing bacteria GwA 159 and GwA 171 have been isolated from a deep subsurface aquifer near Garzweiler, Germany. As revealed by analysis of the 16S rDNA-gene, both strains can be affiliated to the genus *Desulfotomaculum* within the delta-subclass of the *Proteobacteria*. We suggest, that both strains are new members of this genus.

The sulfur isotope fractionation during dissimilatory sulfate-reduction was characterized. Interestingly, *Desulfotomaculum garzweileriensis* showed a fractionation of 40.0‰, thus, explaining the strongly positive $\delta^{34}S_{sulfate}$-values, previously observed at the Garzweiler study site.

6.2. Characterization of a nitrate-reducing enrichment growing on lignite

J. Detmers and A. Weber

Different types of lignite were crushed, mortared, and sieved (< 1mm) under anoxic conditions. Nitrate-reducing microorganisms were enriched and subcultured on the lignite as sole electron donor. During growth on Rhenish lignite, highest turnover rates were achieved. The enrichment reduced 0.72 mM nitrate per g of lignite. During incubation the H/C-ratio of the lignite increased wheras the aromatic fraction decreased significantly which is indicative for an anoxic oxidation of the lignite.

The enrichment was characterized using 16S rRNA-directed probes. 76.6% of total DAPI-stained cells were identified as members of the beta-subclass of the *Proteobacteria* and another 6.6% of total cells could be affiliated to other *Eubacteria*. For the first time it was shown, that anaerobic microorganisms can use natural lignite or its solubilisation products as sole energy source.

6.3. Isotope fractionation of strain TD3 during oxidation of crude oil

J. Detmers, A. Behrends, and V. Brüchert

Thermophilic sulfate-reducing strain TD3 was grown on North Sea crude oil, decane, hexadecane, and butyrate. Growth on crude oil and butyrate was fast compared to decane and hexadecane.

The sulfur isotope fractionation during dissimilatory sulfate-reduction was determined. The fractionation during growth on butyrate (25.8‰) was comparable to previously characterized sulfate-reducers. The fractionation during oxidation of decane (27.3‰) and hexadecane (29.5‰) increased at slighly lower sulfate-reduction rates. Highest fractionations during crude oil oxidation (34.4‰) showed, that the sulfur isotope fractionation is controlled by the carbon oxidation, as it has been proposed previously.

Conclusions

The sulfate-reduction in the investigated aquifers depends on lignite-derived compounds.

At the aquifers of the Garzweiler study site, cell numbers of sulfate-reducers reached highest values at the lignite seam–aquifer interface. Additionally, maximum sulfate-reducing activity was found there, too. Highest cell numbers of fermenting microorganisms at the lignite–aquifer interface may indicate that a degradation of lignite-derived organic matter proceeds in two steps. Additionally, it was shown that lignite or its water-soluble compounds are oxidized under nitrate-reducing conditions. Thus, fermenting microorganisms may attack lignite compounds and the fermentation products are subsequently oxidized by sulfate-reducers.

***Desulfotomaculum* spp. are well adapted to aquifer habitats.**

Members of this genus were frequently isolated from the aquifers at the Garzweiler study site. Using 16S rRNA-directed probes for known sulfate-reducing genera, only *Desulfotomaculum* spp. were detected. Additionally, the sulfur isotope fractionation determined for the strains isolated from the aquifers can explain the ^{34}S-depletions in the sediment.

At sulfate-deficiency *Desulfotomaculum* spp. can turn to homoacetogenic metabolism or survive periods of starvation by spore-formation.

The sulfur isotope fractionation is controlled by the electron-flow to the sulfate-reducing enzymes.

It was shown, that the sulfur isotope fractionation during sulfate-reduction is not per se rate-dependent: When sulfate availability is not limiting, sulfate-reducers fractionate differently when they grow on different substrates at the same cellular sulfate-reduction rate.

The electron-flow to the sulfate-reducing enzymes is decisive for the fractionation when sulfate is not limiting. Thus, the electron-flow controls both: fractionation and rate of sulfate-reduction.