universität wien

# DIPLOMARBEIT

## Stable isotope probing-based ecophysiological analysis of sulphate-reducing microorganisms in an acidic fen

angestrebter akademischer Grad

Magister der Naturwissenschaften (Mag. rer.nat.)


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# 1 Introduction

## 1.1 The sulphur cycle

Besides carbon (C), nitrogen (N) and phosphorus (P), sulphur (S) is one of the main elements found in biomass. It is an essential constituent of many enzymes, vitamins and hormones. Sulphur is present in many different oxidation states, ranging from −2 ($H_2S$) to +6 ($SO_4^{2-}$), being constantly transformed chemically and biologically. Most of earths sulphur is present in rocks and sediments like pyrite ($FeS_2$) or gypsum ($CaSO_4$). As geological turnover times are long and these sulphur-species are biologically inaccessible, most of the biologically used sulphur can be found in the form of sulphate ions and hydrogen sulphide, playing essential roles in the biological sulphur cycle as illustrated in Fig.1 (Hollemann and Wiberg 1985).

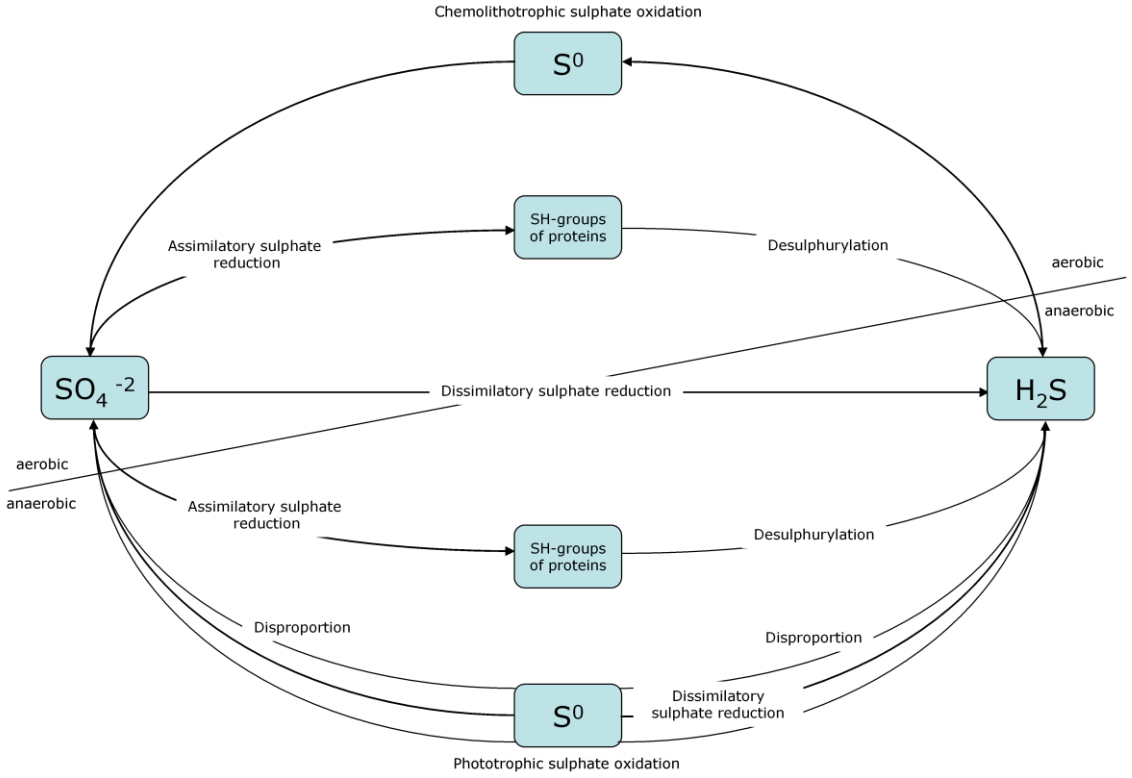



Fig.1 Biological sulphur cycle (modified from Madigan 2003).

Several plants, fungi and prokaryotes can use sulphate to build up the essential sulphur containing amino acids cystein and methionine through assimilatory sulphate reduction. Many organisms then use these amino acids for incorporation in their biomass. Beside the anabolic utilisation of these amino acids, many microorganisms remineralise them

to sulphide via desulphurylation, which is then converted back to sulphate by sulphur-oxidizing bacteria. Two distinct prokaryotic groups have the ability to oxidize sulphide. One group are anaerobic living, phototrophic sulphur bacteria, the others are aerobic chemolithotrophic bacteria (Madigan *et al.* 2003). Furthermore, elemental sulphur can be oxidised by some prokaryotes, like *Desulfocapsa sulfoexigens*, resulting in sulphate and closing the sulphur cycle (Frederiksen and Finster 2004).

Alternatively, sulphate, sulphite and thiosulphate can serve as an energy source for sulphate-reducing prokaryotes (SRP), not being converted to biomass (Karkhoff-Schweizer *et al.* 1995). Dissimilatory sulphate reduction is restricted to anoxic/microoxic habitats, playing an essential role in the turnover of sulphur and carbon in these environments (Brune *et al*. 2000). It is one of earth's history oldest biogeochemical processes which evolved 2.5 to 2.7 billion years ago (Canfield 1998) possibly emerging from sulphur metabolism which was verified in 3.5 billion years old sediments (Philippot *et al.* 2007).

Compared to denitrification in anoxic habitats, sulphate reduction is an energetically unfavourable process. Sulphate removal would therefore be expected to occur sequentially after depletion of nitrate, unless there are other potential electron acceptors such as iron and manganese. However, trough heterogeneous distribution of substrates, creating microenvironments, anaerobic sulphate reduction could be detected in almost any anaerobic environment (Whitmire and Hamilton 2005).

## 1.2 Physiology and phylogeny of SRP

Apart from being essential for sulphur turnover, research showed that SRP are mainly responsible for anaerobic carbon cycling. They are divided into two main groups, those that degrade organic compounds incompletely to acetate and those that degrade organic compounds to carbon dioxide. SRP that can degrade carbon compounds to carbon dioxide commonly can use acetate as a growth substrate. Two pathways of acetate oxidation are utilised, a modified citric acid cycle as shown in *Desulfobacter postgatei* (Brandisheep *et al.* 1983) and the acetyl-CoA pathway, as employed for example by *Desulfobacca acetoxidans*. Different species of sulphate reducers can utilise many kinds of organics, ranging from one carbon compounds (Nanninga and Gottschal 1987; Tanimoto and Bak 1994; Parshina *et al.* 2005), aromatic hydrocarbons (Rabus *et al.* 1993), long- and short-chain alkanes (Aeckersberg *et al.* 1998; Kniemeyer *et al.* 2007), to various sugars (Sass *et al.* 2002) (Fig. 2).

In terms of phylogeny, SRP are distributed throughout several prokaryotic lineages. Comparative analysis of 16S rRNA and *dsrAB* sequences of known SRP showed that they can be grouped into five bacterial and two archaeal phyla (Fig. 3a+b) (Castro *et al.* 2000; Mori *et al.* 2003; Wagner *et al.* 2005). Most of them belong to the class of *Del-*

*taproteobacteria*, harbouring 79 species of *Desulfovibrio* spp. and members of the *Desulfobacteraceae* family. A large number of SRP belong to the lineage *Clostridia*, with several species of the *Desulfosporosinus* and *Desulfotomaculum* genera. These bacteria have the ability to form endospores (Stackebrandt *et al.* 1997; Hattori *et al.* 2000; Sass *et al.* 2004).

The remaining bacterial lineages *Nitrospirae*, *Thermodesulfobacteria* and *Thermodesulfobiaceae* only contain thermophilic SRP (Jeanthon *et al.* 2002; Mori *et al.* 2003; Sekiguchi *et al.* 2008). Sulphate reducers belonging to the archaeal lineages were found at submarine hydrothermal systems and in acidic hot springs and could be classified into the phyla *Euryarchaeota* and *Crenarchaeota* (Stetter *et al.* 1987; Burggraf *et al.* 1990; Itoh *et al.* 1999).

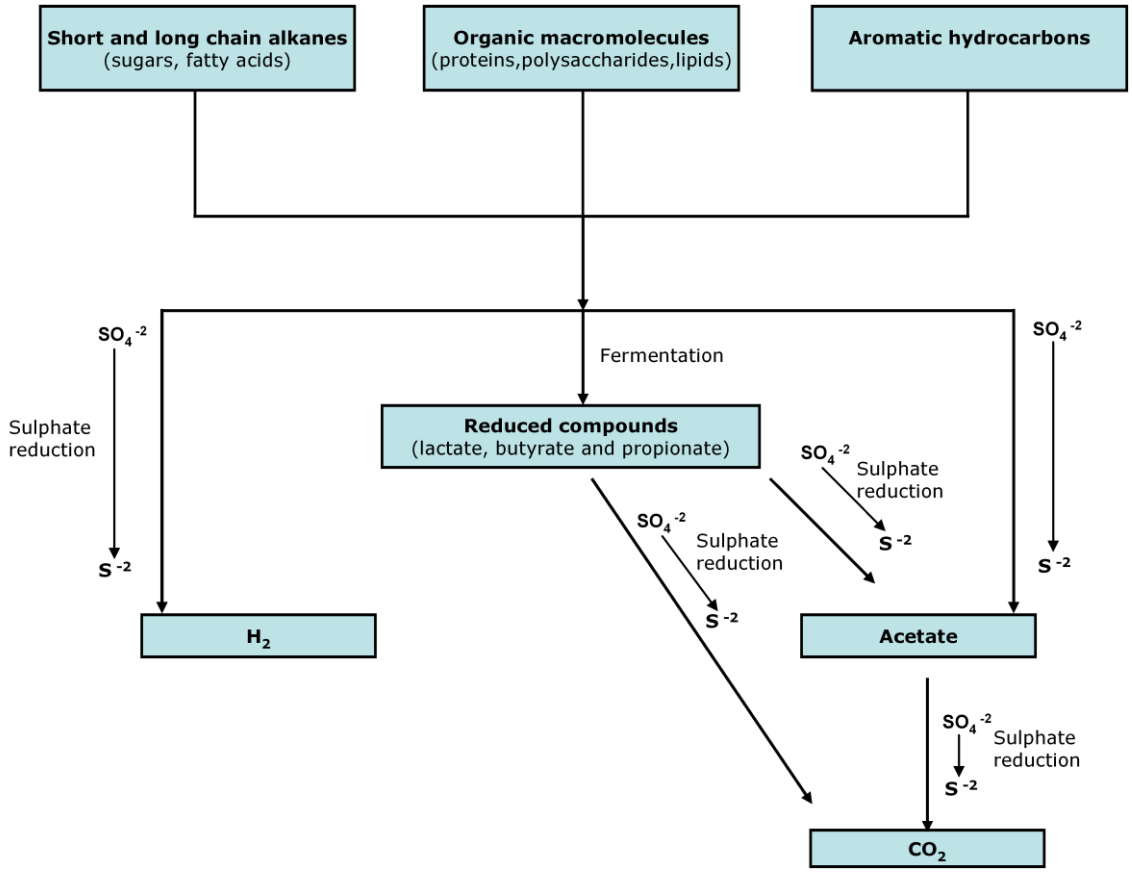
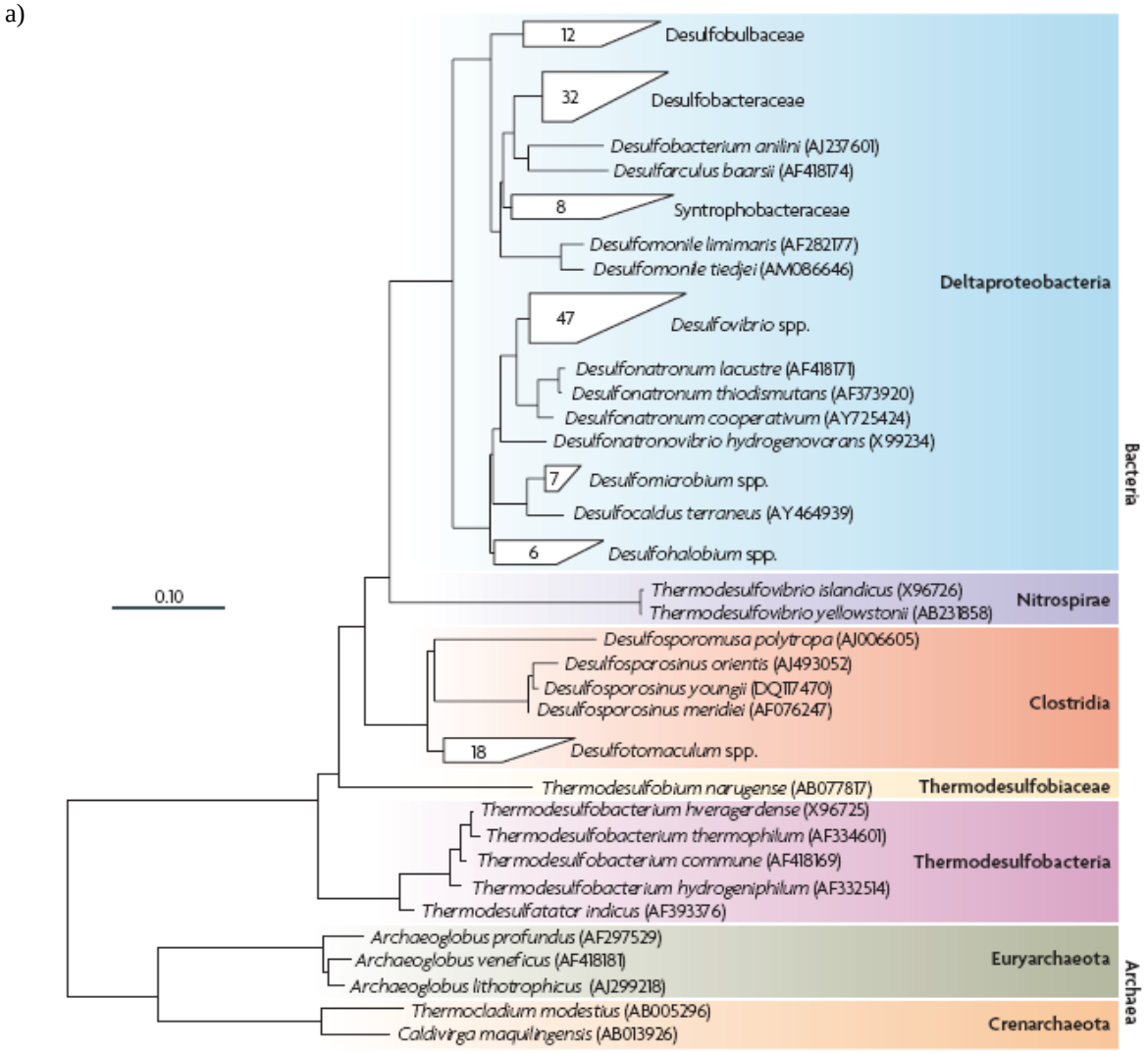



Fig.2 Pattern of microbial degradation of complex organic matter in anoxic environments in the presence of sulphate (modified from Muyzer and Stams 2008).

Apart from 16S rRNA studies, functional gene based phylogenetic studies based on the A and B subunit of dissimilatory (bi-) sulphite reductase (*dsrAB*) have been employed to unravel the diversity and evolution of SRP (Fig.3). It could also be shown that some species e.g. *Desulfotomaculum* acquired *dsrAB* by lateral gene transfer (Klein *et al.* 2001; Zverlov *et al.* 2005).

a)




b)

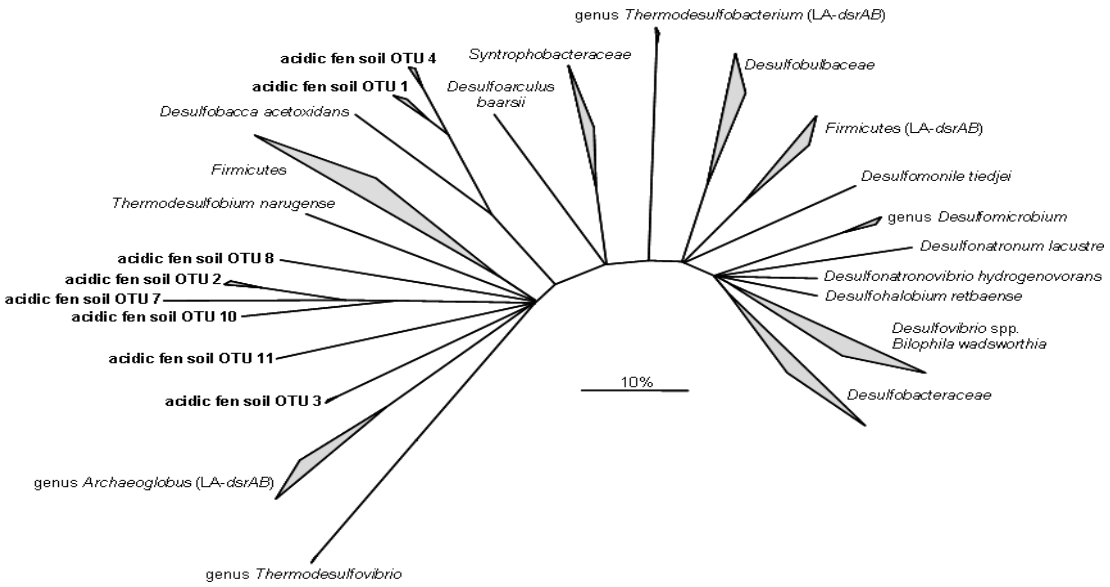



Fig.3 Phylogenetic trees of SRP based on their a) 16S rRNA and b) *DsrAB* sequences. Scale bar shows estimated 10% sequence difference (Muyzer and Stams 2008, Wagner 2005).

## 1.3 Dissimilatory reduction of sulphate

SRP use sulphate as the terminal electron acceptor for growth. The $E^{0'}$ of the redox couple sulphate-sulphite is −516 mV, which is too negative to allow reduction by the electron acceptors ferredoxin or NADH (−420 mV respectively −310 mV) present in SRP (Madigan *et al.* 2003). Therefore, sulphate has to be activated with adenosine-5'-triphosphate (ATP) by ATP sulphurylase. The resulting activated product, adenosine-5'-phosphosulphate (APS) is then converted to (bi-) sulphite, by APS reductase (−60 mV) (Fig.4). In this reaction two electrons are transferred to the sulphur ion and adenosine-5'-monophosphate (AMP) is released. The released AMP is converted by ATP-dependent adenylate kinase into two molecules of ATP. This makes the activation of sulphate an energy-consuming step at the expense of two ATP.

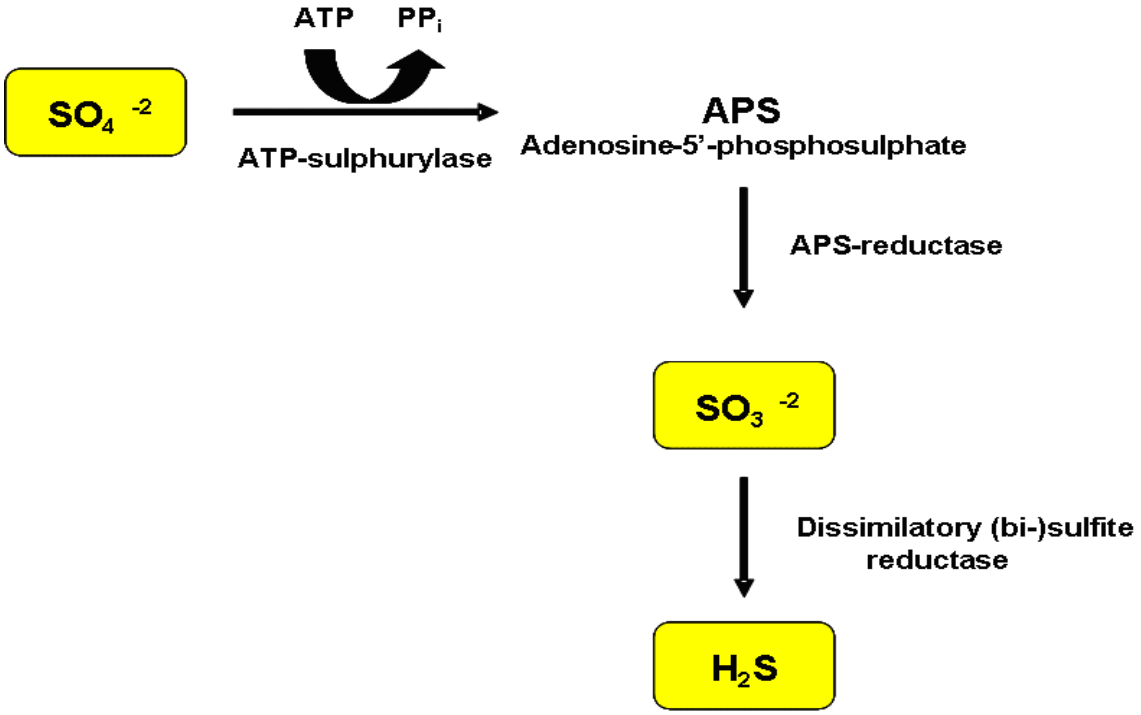



Fig.4 Dissimilatory reduction of sulphate (modified from Madigan 2003).

The resulting sulphite is further reduced to sulphide. The exact mechanism remains unclear, whether it is one six-electron-step or three two-electron-steps through trithionate and thiosulphate (Fitz and Cypionka 1990; Broco *et al.* 2005). In any regard, dissimilatory (bi-) sulphite reductase (*dsrAB*) is the essential catalysing enzyme of this step, expressed by all known sulphate reducers (Rabus *et al.* 2006). To obtain energy out of these redox reactions, electron transfer is coupled to a membrane bound electron transport chain, leading to a proton motive force that is used to form ATP via ATPase (Odom and Peck 1984; Crane and Getzoff 1996). Taking into account the uptake and

activation of sulphate, the net yield of ATP would therefore be one quarter of an ATP molecule per sulphate molecule reduced (Thauer *et al.* 2007). Besides sulphate, many other compounds are used by SRP as electron acceptors. Those range from other sulphur compounds like thiosulphate, sulphite and sulphur to even nitrate and nitrite (Moura *et al.* 1997). Also heavy metals such as iron (Lovley *et al.* 1993a), uranium (Lovley *et al.* 1993b), chromate (Lovley and Phillips 1994) and arsenate (Macy *et al.* 2000) were found to be electron acceptors for certain SRP. However, it remains unclear whether these processes are coupled to growth. Beside their biological role, this ability makes SRP useful candidate organisms for removal of heavy metals from contaminated soil, groundwater or waste water as already applied, e.g. in the mining industry (van Houten *et al.* 2006).

## 1.4 Habitats of SRP

Due to their versatile use of different electron donors and electron acceptors, SRP are ubiquitously distributed in natural and engineered environments. Although sulphate reduction is an anaerobic process, SRP have also been found in oxic-anoxic transition zones as they are capable of dealing with oxygen stress (Cypionka *et al.* 1985; Bade *et al.* 2000). SRP have been detected in environments ranging from -2°C to 100°C as well as under highly acidic (pH 2) (Koschorreck *et al.* 2003) or basic conditions (pH 10) (Foti *et al.* 2007). Although being detected over this broad range of pH, the best conditions for cultivated SRP were at neutral pH and acidic conditions even restricted growth of certain species (Widdel 1988). In marine sediments, SRP play an essential role in the carbon cycle due to the high availability of sulphate (28 mM) and anoxic conditions. It is estimated that SRP account for up to 50% of the carbon mineralization in coastal sediments (Jorgensen 1982). Many studies regarding SRP were dealing with these habitats (Knoblauch *et al.* 1999; Sahm *et al.* 1999; Dhillon *et al.* 2003; Fishbain *et al.* 2003; Purdy *et al.* 2003; Leloup *et al.* 2007). Despite low sulphate concentrations of 10 – 200 µM in freshwater environments (Ingvorsen *et al.* 1981), SRP have been detected in e.g. groundwater, freshwater lake sediments and waste water treatment plants (Sass *et al.* 1998; Lehman *et al.* 2001; Dar *et al.* 2005; Ben-Dov *et al.* 2007). They are also found in terrestrial ecosystems, e.g. in rice field soil (Ouattara *et al.* 1999; Scheid and Stubner 2001; Weber *et al.* 2001) and the plant rhizosphere (Hines *et al.* 1999). Moreover they live in syntrophy with methanotrophic archaea (Boetius *et al.* 2000) and even as symbionts in the marine worm *Olavius argavensis* (Dubilier *et al.* 2001). In this thesis, activity of SRP in peatlands was the focus of investigation. The studied site, the Schlöppnerbrunnen fen in the Lehstenbach catchment (Bavaria, Germany), has already been the focus of several studies (Loy *et al.* 2004; Wentrup 2007; Hamberger *et al.* 2008; Wust *et al.* 2009).

## 1.5 Wetlands, climate and SRP

Natural and agricultural wetlands account for 20 to 40% of the annual emission of the powerful greenhouse gas methane (Fung *et al*. 1991; Hein *et al.* 1997). Regular flooding and low abundance of electron acceptors like e.g. nitrate in these areas is favouring growth of methanogenic species making wetlands their favoured habitat (Horn *et al.* 2003). Interestingly, emission of methane is decreased up to 78% when SRP are actively growing (Lovley and Klug 1983; Gauci *et al.* 2004; Schimel 2004). Both functional groups of mcrobes can compete for the same substrates (i.e. acetate, hydrogen), which they require for growth. Due to a higher energy yield of sulphate reduction, compared to methanogenesis, SRP out compete these microorganisms even at low sulphate concentration of about 100 µM (Lovley and Klug 1983).

Apart from these findings, other developments make sulphate reduction an important research topic regarding climate change. Geo-engineering attempts to dampen the greenhouse effect by $SO_2$ deposition in the stratosphere, would increase sulphur deposition as well as predicted increase in industrial activity in developing countries, especially China (Gauci *et al.* 2004; Rasch *et al*. 2008). In this context, a catchment area of the Fichtelgebirge in the northeast of Bavaria, the Schlöppnerbrunnen site, was found to be a suitable model system (Fig.5).

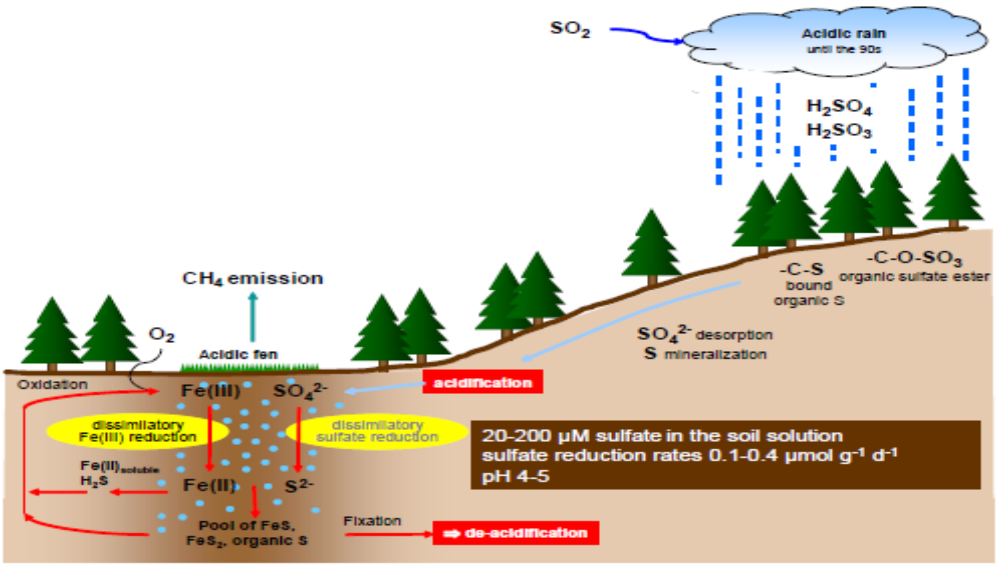



Fig.5 The Schlöppnerbrunnen model system. The figure illustrates the flow of sulphur and ongoing anaerobic processes in this area in the Fichtelgebirge in Bavaria, Germany (Loy 2006).

Many aspects of this ecosystem were already examined, e.g. methanogenesis (Wust *et al.* 2009) anaerobic carbon flow (Hamberger *et al.* 2008) and sulphate reduction (Loy *et al.* 2004; Duller 2005; Wentrup 2007). Most of the sulphur compounds found at the Schlöppnerbrunnen site result from acidic rain being a consequence of anthropogenic

activities in the area of industrial build up in 1950 – 1987 (Novak *et al.* 2007). Nowadays, this accumulated sulphur is gradually released in the catchment after snowmelt or heavy rain falls and collected in fen systems located at lower elevations, like for example Schlöppnerbrunnen I an II. Studies investigating sulphur isotope ratios proved that sulphate reduction is an ongoing process thereby providing an excellent environment for studies about SRP in acidic wetlands (Alewell and Novak 2001).

## 1.6 Aims of this study

Although biogeochemical cycling is of importance in terrestrial ecosystems, the biology of sulphate reduction is not very well understood. Earlier studies at the investigated site discovered a great diversity of potential SRP on the basis of *dsrAB* phylogeny (Fig.6) (Loy *et al.* 2004). Following studies, applying FISH and qPCR (Duller 2005; Wentrup 2007), tried to reveal abundance and identity of these new species. Relatively high amounts of microorganisms harbouring these novel *dsrAB* were detected, but could not be connected to sulphate reduction. In this study, a differential display stable isotope probing (SIP) approach was applied to identify active SRP at the studied site. In the recent past, SIP has been used extensively to connect phylogeny and activity of uncultivated microorganisms *in situ*, giving new insights into the utilisation of various substrates such as phenol (Manefield *et al.* 2007) and various monosaccharides (Hamberger *et al.* 2008). Applying SIP even gave insights in the syntrophic relationship mediating the oxidation of propionate in rice field soil (Lueders *et al.* 2004b).

For this study, soil samples taken from the Schlöppnerbrunnen II site were incubated up to 6 months with $^{13}$C-labelled substrates and with or without sulphate. Soil slurries were sampled at different time points and nucleic acids were extracted. Through isopycnic centrifugation and gradient fractionation, $^{13}$C-labelled nucleic acids of active microorganisms were obtained. Those were identified by terminal restriction fragment length polymorphism (T-RFLP) and clone library analysis of 16S rRNA and *dsrAB* marker genes was applied.

The main objectives of this study were:

To identify the key players of sulphate reduction in the observed area by comparing the isotopically labelled nucleic acids of the microbial community via 16S rRNA screening of sulphate induced samples with non-sulphate induced samples.

Connecting the new discovered species found by *dsrAB* analysis of the Schlöppnerbrunnen (Loy *et al.* 2004) with active sulphate reducers by comparing the two isotopically labelled fractions on the level of the *dsrAB* gene.

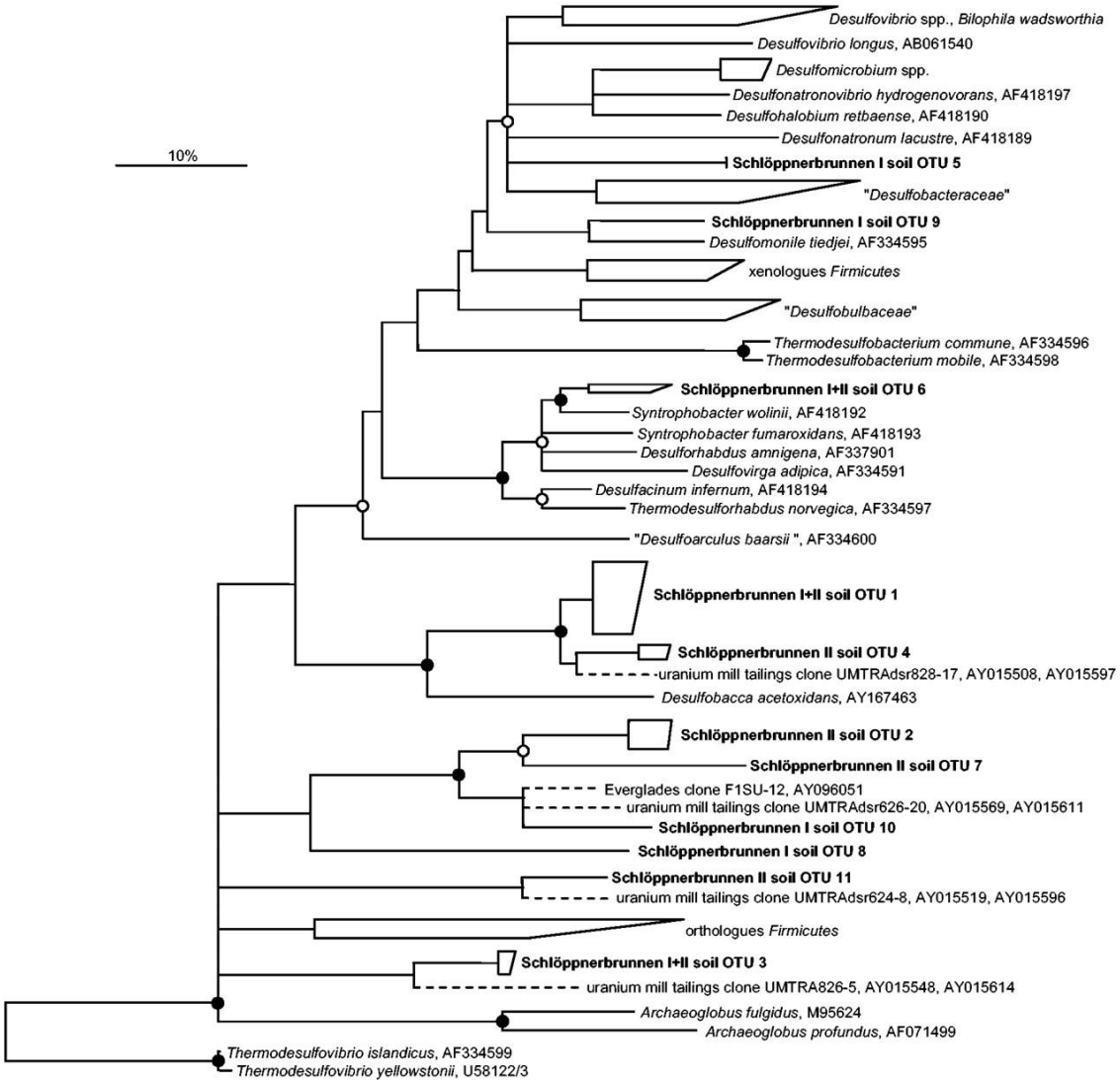



Fig.6 *DsrAB* tree of potential sulphate reducing microorganisms obtained from Schlöppnerbrunnen I and II. Scale bar indicates 10% sequence difference as inferred from distance matrix analysis (Loy *et al.* 2004).

# 2 Material and Methods

All nucleic acid samples, investigated in this study, were prepared by Michael Pester from the Department of Microbial Ecology, University of Vienna as explained in the following section (2.1).

## 2.1 Provided samples

### 2.1.1 Sampling site

Soil samples were taken at the Schlöppnerbrunnen II sampling site (50°08'38''N, 11°51'41''E), located in the Lehstenbach catchment (Bavaria, Germany). The catchment is a 4.2 km² wide forested area, of which 90% is covered with Norway spruce (*Picea abies*). Soils in the catchment have developed from weathered granitic bedrock and are predominantly cambisols and cambic podsols. The temperature level is around 5°C in average, with a mean precipitation of 900 to 1,160 mm per m² and year. The sampling site is one of many fens, distributed throughout the catchment, with a soil pH of approximately 3.9 to 4.2 (Loy *et al.* 2004). Samples were taken from a depth of 10-20 cm in November 2007, sealed and transported to the laboratory in a cooling device. Samples were stored at 4°C until further experimental usage.

### 2.1.2 Sample incubation

Prior to incubation with $^{13}C$ labelled substrates, 30 g of soil from the corresponding samples were put in 125 ml glass bottles and gased with 100% $N_2$, to establish anoxic conditions. Additionally, 60 ml of anoxic fen soil water was added to the sample after filter sterilisation (0.2 µM) and immediately sealed with butyl rubber septa. For depletion of internal electron acceptors and donors, samples were preincubated without any addition of substrate for 28 days at 14°C in the dark. After pre-incubation, a $^{12}C$ substrate mix of lactate, acetate, propionate (100 µM each), and formate (200 µM) was added twice over a period of 2 weeks to determine substrate turnover times. In addition, three soil slurries were supplemented once with 200 µM sulphate. In all incubations, lactate and formate were readily turned over within 2 days, while acetate and propionate needed 4 and 6 days for turnover after the first and second substrate addition, respectively. To isotopically label active prokaryotes, a mixture of fully labelled $^{13}C$-substrates was added weekly. The mixture consisted of lactate, acetate, propionate (100 µM each), and formate (200 µM). In addition, sulphate was added to an end-concentration of 100–200 µM. The concentrations were adjusted to resemble the natural concentrations of the

sampling site, being 20–240 µM for sulphate and 80–190 µM for the carbon species (Loy *et al.* 2004; Schmalenberger *et al.* 2007). Soil slurries without sulphate addition served as controls. Upon substrate addition, soil slurries were shaken to ensure complete mixing. In total, 6 bottles, 3 with sulphate added and 3 without sulphate added were prepared as described.

### 2.1.3 Soil DNA and RNA extraction

Total nucleic acids were extracted from frozen samples (–80°C) by grinding in liquid nitrogen and following the procedure described by Lueders and co-workers (Lueders *et al.* 2004a). Minor modifications were applied, including a humic acid precipitation step with 7.5 M Na-Acetate as described by Bodrossy and co-workers (Bodrossy *et al.* 2006). DNA was separated from RNA using the All Prep DNA/RNA Mini Kit (Qiagen, Hilden, Germany).

## 2.2 General Material and Methods

Except noted otherwise, all liquids and media used in this study were prepared using double distilled, UV-light treated and filtered water of a water purification facility system (Milli-Q®, Millipore, Billerica, MA, USA). In addition, used water was treated with diethylpyrocarbonate (DEPC) to get rid of possible contaminations with RNAses ($H_2O_{DEPC}$). Moreover, all liquids and heat resistant materials were sterilised in a water vapour high-pressure autoclave (H+P, München, Germany) at 121°C and a pressure of $1.013\times10^5$ Pa for 20 min. Heat sensitive liquids and substances were filter sterilised, using 0.2 µm filters (Qualilab®, Merck Labor und Vertrieb GmbH, Bruchsal, Germany) and added to the respective solutions after autoclaving. All of the solutions were kept in bottles, which were baked at 180°C overnight to avoid RNAse contamination.

Table 1: Software

| Software | URL | Reference |
|---|---|---|
| ARB software package | http://www.arb-home.de/ | Ludwig *et al.* 2004 |
| Basic local alignment search tool | http://www.ncbi.nlm.nih.gov/BLAST | Altschul *et al.* 1990 |
| Chromas Pro 1.42 | http://www.technelysium.com.au/ ChromasPro.html | unpublished |
| DOTUR | http://schloss.micro.umass.edu/ software/dotur.html | Schloss *et a.l.* 2005 |
| Finch TV 1.4 | http://www.geospiza.com/Products/ finchtv.shtml | unpublished |
| Peak Scanner 1.0 | http://www.appliedbiosystems.com/ peakscanner | unpublished |
| SINA Webaligner | http://www.arb-silva.de/aligner/ | Pruesse *et al.* 2007 |

| | | |
|---|---|---|
| TRF Cut | http://www.mpi-marburg.mpg.de/downloads/ | Ricke *et al.* 2005 |
| UniFrac | http://bmf.colorado.edu/unifrac/ | Lozupone *et al.* 2006 |

Table 2: Technical equipment

| Equipment | Manufacturer |
|---|---|
| Centrifuges:<br>Mikro 22 R<br>Rotina 35<br>Mikro 120<br>Optima L-XP series | <br>Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany<br><br>Beckman Coulter Inc., Fullerton CA, USA |
| DNA sequencer Applied Biosystems 3130 | Applied Biosystems, Lincoln CA, USA |
| Digital refractometer: DR 301- 95 | A.KRÜSS Optronic GmbH,Hamburg,Germany |
| Gel carriage:<br>Sub-Cell GT UV-Transparent Gel Tray | <br>Bio-Rad Laboratories, Inc., Hercules CA, USA |
| Gel electrophoresis:<br>Sub Cell®-GT | <br>Bio-Rad Laboratories, Inc., Hercules CA, USA |
| Gel Documentation System Media System<br>FlexiLine 4040 | <br>Biostep, Jahnsdorf, Germany |
| Heat Block:<br>VWR Digital heat block | <br>VWR Int. , Vienna, Austria |
| Infinite® 200 microplate reader | Tecan Group Ltd., Männedorf, Switzerland |
| Laminar flow hood Safe 2010 Model 1.2 | Holten, Jouan Nordic, Allerød, Denmark |
| NanoDrop® ND-1000 | NanoDrop Technologies, Wilmington DE, USA |
| Microwave MD6460 | Microstar |
| Magnetic stirrer:<br>RCT basic<br>Variomag® Maxi | <br>IKA® Werke GmbH, Schwabach,Germany<br>Variomag®, Dayton Beach, FL, USA |
| Milli-Q® Ultrapure Water Purification System | Millipore . Billerica MA,USA |
| PCR thermocyclers:<br>iCycler<br>System Mastercycler gradient | <br>Bio-Rad Laboratories, Inc., Hercules CA, USA<br>Eppendorf AG, Hamburg, Germany |
| pH-Meter WTW inoLab Level 1 | Wissenschaftlich-Technische Werkstätten GmbH, Weilheim,Germany |
| Pipettes:<br>Pipetman® P2 – P1000<br>Eppendorf Research® pipettes 1 – 1000 µl | <br>Gilson international, Wien, Austria<br>Eppendorf AG, Hamburg, Germany |
| Platform shaker Innova 2300 | New Brunswick Co., Inc., Madison NJ, USA |
| Power supply for gel electrophoresis:<br>PowerPac Basic | <br>Bio-Rad Laboratories, Inc., Hercules CA, USA |
| Peristaltic pump:<br>WPI AL 2000 Programmable syringe pump | <br>WPI Inc., Sarasota FL, USA |
| Scales:<br>Sartorius BL 6100<br>Ohaus Analytical Plus | <br>Sartorius AG, Goettingen, Germany<br>Ohaus Corporation, Florham Park NJ, USA |
| Transilluminator UST-30M-8E (312 nm) | Biostep GmbH, Jahnsdorf, Germany |

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|---|---|
| Ultracentrifuge Rotor: Vti 90 | Beckman Coulter Inc., Fullerton CA, USA |
| Ultraviolet Sterilizing PCR Workstation | Peqlab Biotechnology GmbH, Germany |
| Vortex Genie 2 | Scientific Industries, New York, USA |
| Waterbath: DC10 Thermo | Haake, Karlsruhe, Germany |
| Water vapour high pressure autoclaves:<br>Varioclav 135S | H+P, München, Germany |

Table 3: Consumables

| Item | Company |
|---|---|
| Centrifuge tubes, various sizes | Greiner Bio-One GmbH, Frickenhausen, Germany |
| DURAN® bottles, various sizes | Schott Glas, Mainz, Germany |
| Eppendorf reaction tubes, various sizes | Eppendorf AG, Hamburg, Germany |
| Microseal "A" film<br>Whatman UNISEAL | MJ Research, Waltham, MA, USA<br>GE Healthcare, Piscataway NJ,USA |
| Microtiter plates:<br>Eppendorf® twin.tec PCR Plates 96<br>Greiner® 96 well plates black flat<br>U96 MicroWell™ Plates, 0.5 ml | Eppendorf AG, Hamburg, Germany<br>Greiner Bio-one GmbH, Frickenhausen, Germany<br>Nunc life science, Roskilde, Denmark |
| Needles:<br>Braun Sterican 23 gauge<br>Braun Sterican 26 gauge | B. Braun Melsungen AG, Melsungen, Germany |
| Optiseal™Ultracentrifude tubes 4.9ml | Beckman Coulter Inc., Fullerton CA, USA |
| PCR tubes, 0.2 ml | Biozym Scientific GmbH, Hess. Oldendorf, Germany |
| Petri dishes 94/16 | Greiner Bio-one GmbH, Frickenhausen, Germany |
| Sterile filter, 0.22 µm pore size | Qualilab®, Merk Labor und Vertrieb GmbH, Bruchsal, Germany |
| Tips, various volumes | Biozym Scientific GmbH, Hess. Oldendorf, Germany |
| Well Caps | Nunc life science, Roskilde, Denmark |

Table 4: Chemicals

| Chemical | Company |
|---|---|
| Agar | Sigma-Aldrich Chemie GmbH, Steinhausen, Deutschland |
| Agarose, electrophoresis grade | Biozym Scientific GmbH, Hess. Oldendorf, Germany |
| Boric acid | Fluka Chemie AG, Buchs, Switzerland |
| Bovine Serume Albumin (BSA) | Fermentas GmbH, St.Leon-Rot, Germany |
| Bromphenolblau | Sigma-Aldrich Chemie GmbH, Steinhausen, Deutschland |

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|---|---|
| Caesium Chloride | Sigma-Aldrich Chemie GmbH, Steinhausen, Deutschland |
| CaesiumTrifluoroacetate (CSTFA) | GE Healthcare, Piscataway NJ,USA |
| Diethylpyrocarbonate (DEPC) | Sigma-Aldrich Chemie GmbH, Steinhausen, Deutschland |
| Deionised formamide | Carl Roth GmbH & Co., Karlsruhe, Germany |
| Ethanol absolute per analysis (p.A.) | Merck KgaA, Darmstadt, Germany |
| Ethidium bromide (EtBr) | Fluka Chemie AG, Buchs, Switzerland |
| Ethylene-di-amine-tetra-acetic acid (EDTA) | Sigma-Aldrich Chemie GmbH, Steinhausen, Germany |
| Ficoll | Sigma-Aldrich Chemie GmbH, Steinhausen, Germany |
| Glycerol | Carl Roth GmbH & Co., Karlsruhe, Germany |
| Glycogen | Fermentas GmbH, St.Leon-Rot, Germany |
| Hydrogen peroxide ($H_2O_2$), 30% | Carl Roth GmbH & Co., Karlsruhe, Germany |
| Hydrochloric acid (HCl) | Carl Roth GmbH & Co., Karlsruhe, Germany |
| Isopropanol (2-propanol) p.A. | Carl Roth GmbH & Co., Karlsruhe, Germany |
| Kanamycin | Sigma-Aldrich Chemie GmbH, Steinhausen, Germany |
| Polyethyleneglycol-6000 (PEG-6000) | Sigma-Aldrich Chemie GmbH, Steinhausen, Deutschland |
| Potassium acetate (KCl) | Merck KgaA, Darmstadt, Germany |
| Sodium chloride (NaCl) | Carl Roth GmbH & Co., Karlsruhe, Germany |
| Sodium hydroxide (NaOH) | Carl Roth GmbH & Co., Karlsruhe, Germany |
| SYBR® Green I | Cambrex Bio Science, Rockland, Inc., Rockland, ME,USA |
| Tris | Carl Roth GmbH & Co., Karlsruhe, Germany |
| Tryptone | Oxoid LTD., Hampshire, England |
| X-Gal (5-brom-4-chlor-3-indolyl-β-D-galactopyranoside | Fermentas GmbH, St.Leon-Rot, Germany |
| Xylencyanol | Sigma-Aldrich Chemie GmbH, Steinhausen, Germany |
| Yeast extract | Oxoid LTD., Hampshire, England |

Table 5: Molecular Biology Kits

| Kit | Company |
|---|---|
| MinElute PCR Purification Kit | QIAgen, Hilden, Germany |
| One Tube Access RT-PCR System Kit | Promega Corporation, Madison WI, USA |
| Quant-iT ™ PicoGreen® dsDNA Assay kit | Invitrogen Corporation, Carlsbad, CA, USA |
| Quant-iT™ RiboGreen® RNA Assay Kit | Invitrogen Corporation, Carlsbad, CA, USA |
| QIAquick PCR purification kit | QIAgen, Hilden, Germany |
| QIAquick® gel extraction kit | QIAgen, Hilden, Germany |
| TOPO TA Cloning® | Invitrogen Corporation, Carlsbad, CA, USA |
| TOPO XL Cloning® | Invitrogen Corporation, Carlsbad, CA, USA |

## 2.3 Buffers, media and solutions

### 2.3.1 General solutions

DEPC treated water

Diethylpyrocarbonate (DEPC) (conc.) 0.1% (v/v)

$H_2O_{bidist}$ ad 1000 ml

stirred overnight (o.n.) under a fume hood followed by autoclaving

### 2.3.2 General solutions for ultracentrifugation

Caesium chloride (CsCl) solution (7.163 M)

| | |
|---|---|
| CsCl | 603.0 g |
| $H_2O_{DEPC}$ | 500 ml |

0.1 M NaOH solution

| | |
|---|---|
| NaOH | 4 g |
| $H_2O_{DEPC}$ | 1000 ml |

PEG – 6000 30% / 1.6 M NaCl solution

| | |
|---|---|
| Polyethylene glycol 6000 | 150 g |
| NaCl | 46.8 g |
| $H_2O_{DEPC}$ | 500 ml |

Gradient buffer

| | |
|---|---|
| Tris | 0.1 M |
| KCl | 0.1 M |
| EDTA | 1 mM |

### 2.3.3 Buffers, solutions and standards for gel electrophoresis

Modified TAE buffer (ready solution by Millipore)

50×TAE, modified

| | |
|---|---|
| Tris | 2 M |
| EDTA | 5 mM |

TBE buffer

10×TBE

| | |
|---|---|
| Tris (890 mM) | 162.0 g $l^{-1}$ |
| Boric acid (890 mM) | 27.5 g $l^{-1}$ |
| EDTA (20 mM) | 9.3 g $l^{-1}$ |

$H_2O_{bidist}$ ad 1000 ml

pH 8.3–8.7

Loading buffer

| | |
|---|---|
| Ficoll | 25% (w/v) |
| Bromphenol blue | 0.5% (w/v) |
| Xylencyanol | 0.5% (w/v) |
| EDTA | 50 mM |

Ethidium bromide stock solution

10 mg $ml^{-1}$ Ethidium bromide (EtBr) in $H_2O_{bidist}$

Ethidium bromide staining solution

EtBr-stock solution 1:10,000 diluted in $H_2O_{bidist}$

SYBR® Green I staining solution

SYBR® Green I stock solution 1:10,000 diluted in $H_2O_{bidist}$

DNA ladder (Kbl)

GeneRulerTM 1 kb (Fermentas, St. Leon-Rot, Germany)

### 2.3.4 Culture media for Escherichia coli (*E. coli*) strains

Luria Bertani medium (LB medium)

| | |
|---|---|
| Tryptone | 10.0 g $l^{-1}$ |
| Yeast extract | 5.0 g $l^{-1}$ |
| NaCl | 5.0 g $l^{-1}$ |

$H_2O_{bidist}$ ad 1000 ml

pH 7.0–7.5

15 g $l^{-1}$ of agar were added for solid media before autoclaving

LB-Kan medium

Kanamycin stock solution (Kan)

Kanamycin 100 mg $ml^{-1}$ dissolved in $H_2O_{bidist}$

Kanamycin stock solution was added to LB media after autoclaving and due to its heat sensitivity, LB media was cooled down to approximately 50°C, prior to adding of Kan to a final concentration of 140 µg $ml^{-1}$.

LB + 7% Glycerol freezing solution

LB media

Glycerol 7% (v/v)

Before freezing *E. coli* strains at – 80°C for conservation, they were grown o.n. in LB + 7% Glycerol solution at 37°C in 96 well plates and sealed with corresponding well caps (Nunc life science, Roskilde, Denmark).

Blue/white screening medium for TOPO TA cloning®

For blue / white screening of positively transformed cells, 40 µl of X-Gal solution (40 mg $ml^{-1}$ Fermentas GmbH, St.Leon-Rot, Germany), was spread on LB-Kan selective media plates before plating the transformed cells.

### 2.3.5 Culture media for *Halobacterium salinarum*

| | |
|---|---|
| Casamino acids | 7.50 g |
| Yeast extract | 10.0 g |
| $Na_3$-Citrate | 3.0 g |
| KCl | 2.0 g |
| $MgSO_4$ | 20.0 g |
| $FeSO_4$ | 0.05 g |
| $MnSO_4$ | 0.20 mg |
| NaCl | 250.00 g |

Fill up to 1000 ml adjust to pH 7.4, then autoclave

## 2.4 Methods

### 2.4.1 Quantitative analysis of nucleic acids

#### 2.4.1.1 NanoDrop® analysis

Quantitative analysis using the NanoDrop® ND-1000 device, was performed according to the manufacturers manual, by pipetting 1.0–1.5 µl of the nucleic acid solution to the measurement pedestal. Concentrations were measured with UV-Vis at λ = 260 nm. Additionally, ratios of 260/230 nm and 280/260 nm were evaluated, to assess the purity of the sample.

#### 2.4.1.2 Picogreen® analysis

Quantitative measurements of dsDNA concentrations made with the Quant-iT ™ PicoGreen® dsDNA Assay kit were done according to the manufacturer's protocol and measured with the Infinite® 200 microplate reader. PicoGreen® is a fluorescent dye intercalating into dsDNA, which is excited at λ = 480 nm when bound and an emission maximum at λ = 520 nm. A provided dsDNA standard was diluted in different quantities (ng $\mu l^{-1}$) of the desired range for preparing a standard curve, which was measured in duplicates, and plotted against its fluorescence intensities. Fluorescence values of DNA samples were detected simultaneously, and concentrations determined with the regressions curve of the evaluated standard.

### 2.4.2 Qualitative nucleic acid analysis by agarose gel electrophoresis

#### 2.4.2.1 Qualitative nucleic acid fragment analysis

1% (w/v) agarose gels were made by mixing agarose (1 g × 100 ml $^{-1}$) with 1×TBE buffer and melting the solution in a microwave oven. The solution was poured into a gel tray with combs and after polymerisation, the tray was transferred into an electrophoresis unit filled with 1×TBE buffer. The nucleic acid solutions (3–50 µl) were mixed in 1:2-5 ratios with loading buffer and pipetted into the wells, after removing the combs, as well as the nucleic acid size standard. For separation of nucleic acids, a voltage of 100–130 V was applied for 30–90 min, depending on the desired resolution of fragments. For detection of the fragments, the gel was put into EtBr staining solution for 30 – 60 min and visualised by putting the gel onto a UV light emitting (λ = 312 nm) transilluminator. Resulting patterns were recorded and digitalised using a gel documentation system.

#### 2.4.2.2 Qualitative nucleic acid fragment analysis followed by gel purification

1% (w/v) agarose gels were made as described above (2.4.2.1.), with the exception of using 1×modified TAE buffer for the gel as well as for filling the gel electrophoresis unit. Voltage used was the same as above with a running time of 90 min for adequate fragment separation. To detect the fragments the gel was put in SYBR® Green I staining solution for 45 min and visualised with a transilluminator. The desired fragments were cut out by a scalpel and nucleic acids were extracted using the QIAquick® gel extraction kit according to the manufacturers protocol, except that the final elution step was done in 30 µl $H_2O_{DEPC}$.

### 2.4.3 Amplification of specific nucleic acid fragments by PCR

For further analysis, specific DNA regions were amplified via polymerase chain reaction (PCR). This three-step reaction was carried out using Taq polymerase. This enzyme also attaches 3'–dATP overhangs to amplified fragments, which facilitate further cloning using the TOPO TA cloning® kit. All used primers in this study were obtained from Thermo Fisher Scientific Inc., Waltham MA, USA

#### 2.4.3.1 Amplification of 16S rRNA

Reaction mix (25 µl) for one PCR

| | |
|---|---|
| dNTP - Mix (2 mM/dNTP) | 2.5 µl |
| Ex-Taq Polymerase Buffer (10 x) | 2.5 µl |
| $MgCl_2$ (25 mM) | 2.0 µl |
| Forward primers (50 pmol $\mu l^{-1}$) | 0.5 µl |
| Reverse primers (50 pmol $\mu l^{-1}$) | 0.5 µl |
| Taq Polymerase (5 units $\mu l^{-1}$) | 0.1 µl |
| Template | 1.0-2.0 µl |
| $H_2O_{bidist}$ | ad 25 µl |

All reagents except $H_2O_{bidist}$ and primers were obtained from Fermentas GmbH, St.Leon-Rot, Germany. When performing multiple reactions, all reagents except templates were mixed together, followed by pipetting 24 µl of the mixture in each 0.2 ml PCR tubes. Negative control PCR were run with $H_2O_{bidist}$ as template. 16S rRNA genes of bacteria and archaea were amplified with primers shown in Tab. 6. Conditions for the

PCR reaction are shown in Tab.7 and Tab.8. Analysis of amplicons was performed by agarose gel electrophoresis as described in 2.4.2.

Table 6: Primers used for 16S rRNA gene fragment amplification.

| Primer | Sequence (5' - 3') | T (°C) | Reference |
|---|---|---|---|
| 616V[a] | AGA GTT TGA TYM TGG CTC | 54 | (Edwards *et al.* 1989) |
| 907R[b] | CCG TCA ATT CMT TTG AGT TT | 54 | (Muyzer *et al.* 1995) |
| Ar109f[a] | ACK GCT CAG TAA CAC GT | 55 | (Grosskopf *et al.* 1998) |
| Ar912rt[b] | GTG CTC CCC CGC CAA TTC CTT TA | 55 | (Lueders and Friedrich 2002) |

[a] forward primers (616V: universal bacterial primer; Ar109f: universal archaeal primer)

[b] reverse primers (907R: universal bacterial primer; Ar912rt: universal archaeal primer)

Table 7: Conditions for bacterial 16S rRNA gene fragment amplification

| PCR step | T (°C) | Time | Number of cycles |
|---|---|---|---|
| Initial denaturation | 94 | 2 min. | 1 |
| Denaturation | 94 | 30 sec | |
| Annealing | 54 | 30 sec | 23/30 |
| Elongation | 72 | 1 min | |
| Final elongation | 72 | 10 min | 1 |

Table 8: Conditions for archaeal 16S rRNA gene fragment amplification

| PCR step | T (°C) | Time | Number of cycles |
|---|---|---|---|
| Initial denaturation | 94 | 2 min. | 1 |
| Denaturation | 94 | 30 sec | |
| Annealing | 54 | 30 sec | 23 |
| Elongation | 72 | 1 min 30 sec | |
| Final elongation | 72 | 10 min | 1 |

### 2.4.3.2 M13 screening PCR

After cloning (section 2.4.7) of sequence fragments (16S rRNA, *dsrAB*), *E. coli* colonies, potentially harbouring plasmids with the desired sequences, were screened via M13 PCR, following the TOPO TA(XL) Cloning® kits (Invitrogen Corporation, Carlsbad, CA, USA) protocol. Reactions were prepared as described in 2.4.3.1, with the modification of using the double volume of each component of the reaction mix (50 µl). The plasmid templates needed for M13 screening were obtained using two different protocols.

**Direct colony PCR (DCPCR)** (Zon *et al.* 1989)

After overnight incubation at 37°C of transformed *E.coli* cultures, single colonies were picked using sterile toothpicks and directly resuspended in 49 µl PCR reaction mix, followed by PCR using an extended first denaturation step of 5 min., to promote lysis of *E. coli* cells.

**Fast plasmid preparation by boiling**

*E. coli* colonies, obtained as described above, were re-suspended in 50 µl of $H_2O_{bidist}$ and then boiled at 100° C for 10 min. After keeping the reaction on ice for 30 sec, it was centrifuged for 1 min at 4°C at maximum speed, to separate soluble DNA from cell debris. 1 µl of supernatant was used for M13 PCR under described conditions.

Table 9: Primers used for M13 screening PCR

| Primer | Sequence (5' - 3') | T (°C) | Reference |
|---|---|---|---|
| M13 F[a] | GTA AAA CGA CGG CCA G | 60 | TOPO cloning kit (Invitrogen Corporation, Carlsbad, CA, USA) |
| M13 R[b] | CAG GAA ACA GCT ATG AC | 60 | TOPO cloning kit (Invitrogen Corporation, Carlsbad, CA, USA) |

Table 10: Conditions for M13 screening PCR

| PCR step | T (°C) | Time | Number of cycles |
|---|---|---|---|
| Initial denaturation | 95 | 4-10 min | 1 |
| Denaturation | 95 | 30 sec | 30 |
| Annealing | 60 | 30 sec | |
| Elongation | 72 | 1min 15sec (16S rRNA)<br>1min 45sec (*dsrAB*) | |
| Final elongation | 72 | 10 min | 1 |

### 2.4.3.3 Touchdown hot-start PCR amplification of *dsrAB*

Two genes coding for subunit A and B of dissimilatory (bi-)sulphite reductase (*dsrAB*) were amplified by touchdown hot-start PCR (Don *et al.* 1991). Touchdown hot-start PCR decreases amplification of unspecific sequences by using higher annealing temperatures in the first cycles of PCR followed by a decrease of temperature in the following cycles. A higher temperature at the beginning focuses binding of primers to perfectly matching target sequences. In subsequent rounds of amplification, the desired amplicon outnumbers unspecific products due to the exponential nature of PCR amplification. To further, increase the specificity the enzyme was added after heating up the prepared reaction mixtures to 95°C for the first denaturation step. Reaction conditions for touchdown Hot Start PCR are shown in Table 13.

A mixture of several forward and reverse primers was used to cover most known *dsrAB* sequences (Tab. 11, 12). Reaction mixtures were prepared the same as described in 2.4.3.1 with the modification of using 50 µl of the reaction mix and 0.3 µl of Taq Polymerase. Quality analysis was done as described in 2.4.2.2 by agarose gel electrophoresis followed by gel extraction of desired fragments via gel extraction.

Table 11: Dsr1F forward primer mixture solution for use in *dsrAB* PCR amplification

| Primer | conc. (pmol/µl) | Sequence (5' - 3') | Reference |
|---|---|---|---|
| DSR1F | 10 | ACS CAC TGG AAG CAC G | (Wagner *et al.* 1998) |
| DSR1Fa | 10 | ACC CAY TGG AAA CAC G | (Loy *et al.* 2004) |
| DSR1Fb | 10 | GGC CAC TGG AAG CAC G | (Loy *et al.* 2004) |
| DSR1Fc | 10 | ACC CAT TGG AAA CAT G | (Zverlov *et al.* 2005) |
| DSR1Fd | 10 | ACT CAC TGG AAG CAC G | (Zverlov *et al.* 2005) |
| DSR1Fe | 10 | GTT CAC TGG AAA CAC G | (Loy, unpublished) |
| DSR1Ff | 10 | AGC CAC TGG AAA CAC G | (Loy, unpublished) |
| DSR1Fg | 10 | GGC CAC TGG AAA CAT G | (Loy, unpublished) |
| DSR1Fh | 10 | GGC TAT TGG AAG CAC G | (Loy, unpublished) |

Table 12: Dsr4R reverse primer mixture solution for use in *dsrAB* PCR amplification

| Primer | conc. (pmol/µl) | Sequence (5' - 3') | Reference |
|---|---|---|---|
| DSR4R | 10 | GTG TAG CAG TTA CCG CA | (Wagner *et al.* 1998) |
| DSR4Ra | 10 | GTG TAA CAG TTT CCA CA | (Loy *et al.* 2004) |
| DSR4Rb | 10 | GTG TAA CAG TTA CCG CA | (Loy *et al.* 2004) |
| DSR4Rc | 10 | GTG TAG CAG TTK CCG CA | (Loy *et al.* 2004) |
| DSR4Rd | 10 | GTG TAG CAG TTA CCA CA | (Zverlov *et al.* 2005) |
| DSR4Re | 10 | GTG TAA CAG TTA CCA CA | (Zverlov *et al.* 2005) |
| DSR4Rf | 10 | GTA TAG CAR TTG CCG CA | (Loy, unpublished) |
| DSR4Rg | 10 | GTG AAG CAG TTG CCG CA | (Loy, unpublished) |

Table 13: Conditions for *dsrAB* PCR amplification

| PCR step | T (°C) | Time | Number of cycles |
|---|---|---|---|
| Initial denaturation | 95 | 3 min | 1 |
| Denaturation | 95 | 30 sec | |
| Annealing | 58−48 | 30 sec | 1 per °C |
| Elongation | 72 | 1 min 30 sec | |
| Denaturation | 95 | 30 sec | |
| Annealing | 48 | 30 sec | 25 |
| Elongation | 72 | 1 min 30 sec | |
| Final elongation | 72 | 10 min | 1 |

#### 2.4.3.4 Amplification of 16S rRNA fragments by RT-PCR

The One Tube Access RT-PCR System Kit (Promega Corporation, Madison WI, USA) was used for reverse transcription (RT) of 16s rRNA. RT is done by a RNA dependent DNA-polymerase, which is synthesising single stranded (ss) DNA out of ssRNA molecules. The resulting complementary DNA (cDNA) then serves as a template for further amplification using standard PCR in the same reaction tube.

**Reaction mix (25 µl) for one RT-PCR**

| | |
|---|---|
| dNTP - Mix (10 mM/dNTP) | 0.5 µl |
| AMV/*Tfl* 5X Reaction Buffer | 5.0 µl |
| $MgCl_2$ (25 mM) | 1.0 µl |
| Forward primers (50 pmol $\mu l^{-1}$) | 0.5 µl |
| Reverse primers (50 pmol $\mu l^{-1}$) | 0.5 µl |
| AMV Reverse Transcriptase (5u $\mu l^{-1}$) | 0.5 µl |
| *Tfl* DNA Polymerase (5u $\mu l^{-1}$) | 0.5 µl |
| Template | 2.0 µl |
| Nuclease free water | ad 25 µl |

All solutions except templates and primers were provided with the manufacturer's kit. Multiple reactions were prepared as described in section 2.4.3.1 and the primers de-

scribed in Table 6 were used. The reaction conditions are shown in Table 7 and 8 with an extra reverse transcription step prior to PCR of 45 minutes at 45°C. Following qualitative analysis was done by agarose gel electrophoresis. To check for possible DNA contaminations of templates and reagents, two negative controls were included each time, one without template and one without AMV Reverse transcriptase.

### 2.4.4 Isopycnic centrifugation of nucleic acids

#### 2.4.4.1 Refractrometric density measurements

For density measurements of corresponding CsCl/CsTFA solutions, refractive indices (nD) of 50 µl aliquots were measured using a digital refractometer (A.KRÜSS Optronic GmbH, Hamburg, Germany). To determine densities out of nD values a calibration curve was set up by weighing 100 µl aliquots of fractions and measuring corresponding nD values. Results are shown in the appendix section. All refractometric measurements were performed at 23°C, weighing at 27°C.

#### 2.4.4.2 Isopycnic centrifugation of DNA (Neufeld *et al.* 2007b)

To screen differentially isotopically labelled DNA from the conducted SIP experiments, CsCl based isopycnic centrifugation was performed. CsCl solutions needed for adequate separation of DNA and stock solutions were prepared as described in section 2.3.2 resulting in a final density of ~ 1.890 g $ml^{-1}$. For setting up the gradient, the samples were mixed together with gradient buffer (GB) and CsCl solution according to the following formula:

$$\text{GB/DNA volume (ml)} = \{\text{CsCl stock density (g ml}^{-1}\text{)} - \text{desired final density (g ml}^{-1}\text{)}\} \\ *\text{volume of CsCl stock added (ml)}* 1.52$$

In this experiment, 4.9 ml centrifuge tubes were used and for each tube a gradient medium with a final density of 1.725 g $ml^{-1}$ was prepared. As suggested by Neufeld *et.al* (2007), gradient medium was prepared in excess (6.0 ml) and the desired DNA/GB volume was 1.2 ml. The amount of DNA added to GB varied due to a maximum of 5 µg DNA that can be loaded onto each gradient at a time and different DNA concentrations of examined SIP DNA samples. After mixing the components in a 15 ml tube, the calculated final density of the medium was confirmed empirically with refractometric measurement. Centrifuge tubes were filled completely with the mixture, and corresponding pairs were balanced to ± 1 mg prior of placing them in the Vti 90 rotor. Centrifuging was performed with 177,000 × *g* for about 70 h. For gradient fractionation, the tube was carefully pierced at the bottom and at the top with a sterile needle. For gradient fractionation, $H_2O_{DEPC}$ was pumped in with a flow rate of 750 µl $min^{-1}$ and drops at the bottom were collected for 20 sec each fraction. Prior to gradient fractionation the pump-

ing tube was cleaned once with 0.1 M NaOH and twice with 70 % EtOH and subsequently flushed with $H_2O_{DEPC}$ to clean residual EtOH. For setting up a density gradient curve, 50 µl of each fraction were measured by refractometry as explained in section 2.4.4.1.

#### 2.4.4.3 Isopycnic centrifugation of RNA (Whiteley *et al.* 2007)

For centrifugation of SIP-RNA samples, the following CsTFA gradient medium with a final density of 1.8 g $ml^{-1}$ was prepared:

| | |
|---|---|
| CsTFA stock solution (2 g $ml^{-1}$) | 4.8025 ml |
| $H_2O_{DEPC}$ | 954.3 µl |
| Di Formamide | 204.6 µl |
| RNA (max 500 ng) | ad 6 ml |

The following procedures for centrifuging and fractionation were the same as described in section 2.4.4.2 except that centrifugation was performed at 128,000 × *g*.

### 2.4.5 Nucleic acid precipitation

After centrifugation and fractionation of isopycnic gradients, nucleic acids were re-extracted for further analysis, using the following protocols.

#### 2.4.5.1 DNA precipitation out of CsCl gradient medium after Neufeld (2007)

Each DNA fraction was amended with two volumes of PEG 6000 solution and 1 µl of glycogen (20 µg/µl) and incubated at 4°C for 2 h–10 h. After precipitation, DNA was pelleted by centrifugation (4°C at 13000 × *g* for 30 min) and supernatant was discarded. The DNA was subsequently washed with 500 µl ice cold 70% ethanol and centrifuged (4°C at 13000 × *g* for 10 min). After removal of ethanol the DNA pellet was air dried for 15 min and resuspended in 20 µl $H_2O_{DEPC}$.

#### 2.4.5.2 RNA precipitation out of CsTFA gradient medium after Whiteley (2007)

Each RNA fraction was amended with two volumes of ice cold isopropanol (2-Propanol) and 1 µl of glycogen (20 µg/µl) followed by centrifugation (4°C at 13000 × *g* for 30 min). The supernatant was discarded and the resulting RNA pellet was washed with 500 µl ice cold 70% ethanol and centrifuged again (4°C at 13000 × *g* for 10 min). Ethanol was removed, the resulting RNA pellet was air dried for 15 min and resuspended in 20 µl $H_2O_{DEPC.}$

### 2.4.6 Terminal restriction fragment length polymorphism (T-RFLP) analysis

For analysis of the microbial communities in the SIP incubations, T-RFLP analysis was performed (Liu *et al.* 1997). T-RFLP is a rapid analysis method for assessing compositions of microbial communities and is based on length differences in terminal 16S rRNA restriction fragments. Nucleic acid samples were PCR amplified using fluorescently labelled primers, followed by endonuclease digestion.

Prior to experimental T-RFLP analysis of 16S rRNA amplicons, *in silico* analysis using TRF-CUT (Ricke *et al.* 2005), a tool implemented in the ARB software package (Ludwig *et al.* 2004), was performed to identify the optimal restriction enzymes. PCR amplification was performed as described in section 2.4.3.1. Used primers and conditions were the same, except that the 616V and Ar912rt primers were labelled with 6-FAM, a fluorescent dye. Prior to restriction digestion, PCR products were purified using the QIAquick PCR purification kit (QIAgen, Hilden, Germany). Qualitative and quantitative analysis of purified PCR products was performed with agarose gel electrophoresis and NanoDrop photometry respectively. For enzyme restriction, 40 ng (single sequences) or 100 ng (SIP fractions) were digested for 3 h with the appropriate restriction enzymes (Tab. 14) according to the manufacturer's instructions.

Table 14: Used restriction enzymes for this study

| Enzyme | Manufacturer | Target group |
|---|---|---|
| Msp I | Fermentas GmbH, St.Leon-Rot, Germany | *Bacteria* |
| Rsa I | Fermentas GmbH, St.Leon-Rot, Germany | *Bacteria* |
| Taq I | Fermentas GmbH, St.Leon-Rot, Germany | *Archaea* |

Fragment were analysed with an automated DNA sequencer (Applied Biosystems 3130). Prior to loading on the sequencer, the reaction mixture was purified by using a Sephadex SF-50 column (Sigma-Aldrich, St.Louis, MO, USA). Then 5 µl of the resulting liquid were mixed with 10 µl High Dye formamide (Applied Biosystems Inc, CA, USA) and 0.25 µl of Rox marker 1000 (Applied Biosystems Inc.). Afterwards the mixture was heated to 95°C for 3 min followed by immediate cooling on ice for 3 min. Resulting T-RFLP patterns were evaluated using Peak Scanner 1.0 software (Applied Biosystems Inc). X and y-axes were standardised to accurately compare the different pat-

terns of the fractions. The x-axis showing base pairs, was scaled ranging from right after the primer peak, (20 bp for 616V, 23 bp for Ar912rt) to 1000 bp and the y axis was scaled to the top according to the highest peak.

### 2.4.7 Cloning of PCR products

#### 2.4.7.1 Cloning of 16S rRNA PCR products

For cloning of amplified 16S rRNA gene sequences, the TOPO TA Cloning® kit (Invitrogen Corporation, Carlsbad, CA, USA) was used. Ligation of fragments is catalysed by a topoisomerase, which connects the 16S rRNA fragments with the linearised vector. The *kan*$^r$ and *lacZα* harbouring vector plasmid was then transformed into *E. coli* TOP 10 cells.

Fresh PCR products (2.4.6) were prepared with the following reagents according to the following scheme:

| | |
|---|---|
| PCR-product | 2.0-4.0 µl |
| Salt solution | 1.0 µl |
| Vector (pCR®II) | 1.0 µl |
| $H_2O_{bidist}$ | ad 6 µl |

The reaction was incubated at room temperature (RT) for 20 min before transformation. For transformation, chemically competent *E.coli* TOP 10 cells were thawed on ice and 2 µl of the ligation reaction mixture were pipetted into the reaction tube containing the cells. After additional 30 min on ice, the *E. coli* cells were immediately transferred into a 42°C hot water bath and incubated for 30 sec. Following heat shock the cells were amended with 250 µl SOC medium incubated at 37°C and shaken horizontally at 200 rpm.

Meanwhile kanamycin containing LB-agar plates were pre-heated in the 37°C room and 40 µl of X-Gal solution (40 mg ml$^{-1}$) were distributed on the plate prior of spreading transformed cells. From each transformation reaction 25, 50, 75 and 100 µl were plated out and incubated at 37°C o.n. Vector insert positive colonies were identified via M13 PCR (2.4.3.2).

#### 2.4.7.2 Cloning of *dsrAB* amplicons

The TOPO XL cloning® kit (Invitrogen Corporation, Carlsbad, CA, USA) was used for cloning of 1.9 kbp *dsrAB* PCR fragments. Ligation procedure was the same as decribed in section 2.4.7.1, but different proportions of ligation reagents were used:

| | |
|---|---|
| PCR-product | 8 µl |
| Vector (pCR® XL-TOPO®) | 1.5 µl |

After 5 min of the ligation reaction, 1.5 µl of TOPO cloning® stop solution (6×) provided by the manufacturer was added. Transformation was performed as described in section 2.4.7.1 using 2 µl of the ligation reaction mixture.

Transformed cells were plated out on kanamycin containing plates to select for positive plasmid inserts (TOPO XL harbours *kan*$^r$). Individual colonies were then analysed for correct fragments via M13 PCR (2.4.3.2.).

### 2.4.8 DNA sequencing

Sequencing was done using the automated sequencer DNA Sequencer Applied Biosystems 3130 following the manufacturer's instructions. For 16S rRNA sequencing either primer TopoSeq-F or TopoSeq-R (Tab.15) having their priming site on the TOPO-TA cloning vector was used. *DsrAB* was sequenced with the primer mix DSR1F or DSR4R (Tab.11+12).

Table 15: Primers used for sequencing of 16S rRNA fragments

| Primer | Sequence (5' - 3') | T (°C) |
|---|---|---|
| TOPOSeq-F | AGC TTG GTA CCG AGC T | 60 |
| TOPOSeq-R | GTA AAA CGA CGG CCA GT | 60 |

Resulting partial forward and reverse sequences were proofread using FinchTV 1.4 (Geospiza, Inc) and merged with Chromas Pro 1.42 (Technelysium Pty Ltd). The output files were exported in the FASTA format.

### 2.4.9 Comparative sequence analysis

For sequence analysis, the ARB software package (Ludwig *et al.* 2004), and its plug-ins were used. The software package includes tools for phylogenetic calculations, sequence alignment, probe and primer design, T-RFLP *in silico* analysis as well as import and export of sequence data.

#### 2.4.9.1 Sequence alignment

16S rRNA alignment

The sequenced and proofread 16S rRNA sequences (2.4.8) were merged into a multi-FASTA file and were then imported into the SINA Web aligner (http://www.arb-silva.de/aligner/) for automated alignment of sequences according to the latest SILVA database release (Pruesse *et al.* 2007). In this procedure sequences are arranged in a way that homologous regions are aligned for further comparative analysis, e.g. phylogenetic analysis. The resulting ARB compatible output file was imported, the alignment proof-read compared to the latest available database and if needed corrected manually.

*DsrAB* alignment

The retrieved *dsrAB* sequences were imported into ARB and aligned using the integrated Fast Aligner. Alignment was done according to the latest *dsrAB* database (Dsr_AB_dome_Cecillias_Endversion) provided by Doris Steger (Department of Microbial Ecology, University of Vienna) containing 3264 publicly available *dsrAB* sequences. The first alignment was based on the nucleic acid sequence, which was then translated into an amino acid sequence for correct protein alignment. Automated alignments were inspected visually and adjusted manually.

#### 2.4.9.2 Phylogeny

Phylogenetic trees of *dsrAB* were calculated using different software packages implemented in ARB. Calculations were performed using several different treeing methods in combination with different, integrated conservation filters to exclude highly variable regions in the examined sequence alignments. In order to determine the root of the tree, outgroup sequences were included in the tree calculations (Fig. 24).

For 16S rRNA phylogeny all found sequences were used for calculating trees with the Neighbour-Joining (Saitou and Nei 1987) algorithm to get a quick overview of the phylogeny based on the 96_SILVA dataset (www.arb-silva.de). Following that, operational taxonomic units (OTUs) were calculated using DOTUR (Schloss and Handelsman 2005). To reduce computing time, only one sequence of each OTU was used for later calculations, as well as their closest relative according to BLAST (Altschul *et al.* 1990) and close relatives out of the first neighbour joining tree. Different trees using neighbour-joining (Saitou and Nei 1987), maximum parsimony (Felsenstein 1995) and maximum likelihood (Stamatakis *et al.* 2002) algorithms were calculated and a consensus tree was constructed manually out of these data.

The phylogeny of *dsrAB* clone sequences was calculated based on previously released sequences from the studies of Loy (Loy *et al.* 2004) and Zverlov (Zverlov *et al.* 2005)

as references. Phylogenetic trees based on deduced amino acid sequences were computed using the FITCH algorithm included in the ARB implemented PHYLIP (Felsenstein 1995) software. Additionally the indel filter and KIMURA (Kimura 1980) correction were applied.

#### 2.4.9.3 Test for chimeric sequences

Due to the possibility of regarding chimeric sequences as new species when calculating phylogenetic trees, all sequences where checked in ARB. Therefore, NJ trees out of the obtained forward and reverse sequences were calculated independently. If the forward and reverse sequences of the same clone were placed to different taxons, they were regarded as of chimeric origin and not used for further phylogenetic analysis.

#### 2.4.9.4 Rarefaction

Despite calculating different OTUs, rarefaction curves of 16S rRNA and *dsrAB* libraries, were calculated as well using DOTUR (Schloss and Handelsman 2005).

#### 2.4.9.5 Statistical comparison of different habitats

UNIFRAC

To reliably compare community composition of the investigated environments, UNIFRAC a web-based online tool, was used. This tool employs several multivariate statistic tools, using sequence data, to determine the significance of difference between multiple environments. Additionally the lineages contributing to this difference can be identified (Lozupone *et al.* 2006).

COVERAGE

Coverage calculations estimate to what extent the hypothetical genpool of a sample is covered by the sequences obtained. Following formula is used (Good 1953):

$$C_{coverage} = [1 - (n1/N)] * 100\%$$

n1 = number of OTUs represented by a single sequence

N = number of all sequences in the gene library

# 3 Results

## 3.1 Gradient centrifugation of nucleic acids

Soil DNA and RNA extractions, from each time point (2 weeks, 2 months, 6 months) of the SIP incubations (± sulphate), were centrifuged and re-extracted according to the protocols described in section 2.4.4 and 2.4.5. To determine separation efficiency based on GC content and $^{13}$C labelling, different mixtures of $^{12}$C and fully labelled $^{13}$C-nucleic acids of *E. coli* and $^{12}$C-nucleic acids of *Halobacterium salinarum* were centrifuged (Fig.7+8). DNA and RNA concentrations were measured by Picogreen® and Ribogreen® assays respectively, and densities were calculated out of calibration curves that were established prior to the experiment (Appendix 5.1).

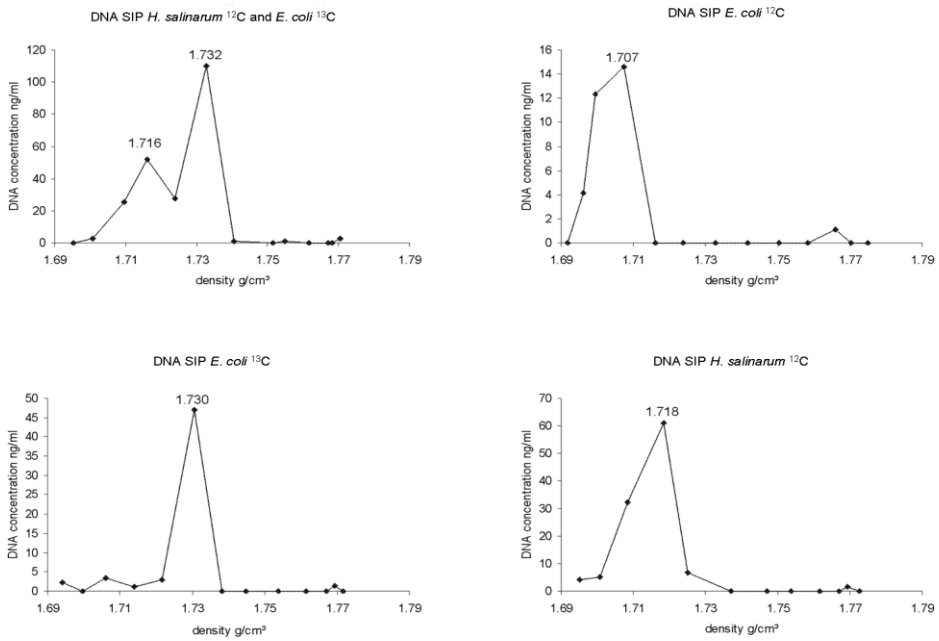


Fig.7 CsCl density gradients of different mixtures of *E. coli* $^{12}$C- and $^{13}$C- and *H. salinarum* $^{12}$C-DNA. Respective densities of peak maxima are indicated in g cm$^{-3}$.

$^{12}$C labelled DNA from *E. coli* with a GC content of 51 mol% (Blattner *et al.* 1997) peaked at 1.707 g cm$^{-3}$. $^{12}$C *H. salinarum* DNA with a GC content of 68 mol% (Ng *et al.* 2000) peaked at 1.716 in the mixture with *E. coli* DNA and at 1.718 g cm$^{-3}$ when centrifuged alone. These values matched recently determined density values of $^{12}$C

*E. coli* (1.710 g cm$^{-3}$) and $^{12}$C *M. extorquens* (GC% 66; 1.719 g cm$^{-3}$) DNA (Lueders et al. 2004a). In contrast, density values of $^{13}$C *E. coli* DNA peaked at 1.730 and 1.732 g cm$^{-3}$ showing great difference to a calculated value of 1.750 g cm$^{-3}$ for 100% $^{13}$C-labelled *E. coli* DNA (Buckley *et al.* 2007). This lower density indicates that the used $^{13}$C labelled *E. coli* DNA was not fully labelled. Nevertheless, separation of $^{12}$C-labelled *E. coli* DNA, $^{12}$C-labelled *H. salinarum* DNA and partially labelled *E. coli* $^{13}$C-DNA was successful.

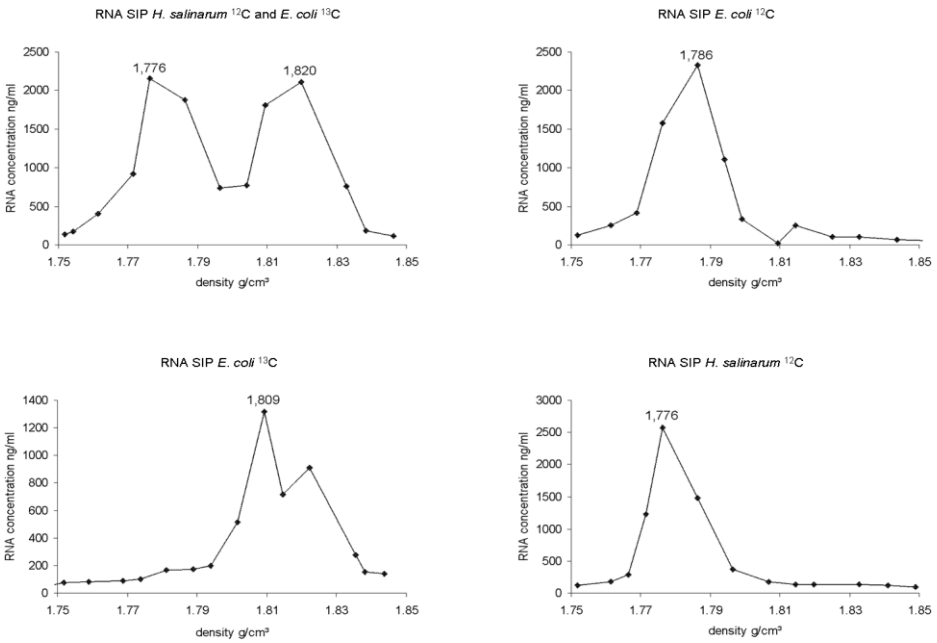


Fig.8 CsTFA density gradients of different mixtures of *E. coli* $^{12}$C and $^{13}$C and *H. salinarum* $^{12}$C RNA. Respective densities of peak maxima are indicated in g cm$^{-3}$

For RNA, GC content shows no effect regarding buoyant density, making $^{13}$C content the decisive component for separation (Lueders *et al.* 2004a). $^{12}$C labelled *E. coli* RNA peaks at 1.786 g cm$^{-3}$ resembling the published value (1.785 g cm$^{-3}$) (Lueders *et al.* 2004a). *H. salinarum peaks* at 1.776 g cm$^{-3}$ and fully $^{13}$C labelled *E. coli* RNA peaks at 1.809 and 1.820 g cm$^{-3}$. The lower buoyant density for the centrifugation with $^{13}$C *E.coli* RNA only is presumably due to a loss of RNA while reextracting it from the CsTFA fractions. This is indicated by an up and down of measured RNA concentrations of the gradient after the peak maxima (Fig.8). After extrapolation of the curve, the estimated peak maxima would be at 1.814 g cm$^{-3}$. This resembles the value for fully $^{13}$C labelled RNA of *M. extorquens* at 1.815 g cm$^{-3}$ (Lueders et al. 2004a). As for DNA, separation worked with the setup used, but unlike for DNA no great differences to literature values of buoyant density of fully $^{13}$C labelled *E.coli* RNA was observed.

## 3.2 T-RFLP analysis of density resolved nucleic acids

T-RFLP analysis of the incubations was performed to examine differences in the microbial community composition due to treatment with and without sulphate. In addition to changes over time, differences between the active ($^{13}$C-labelled) and dormant ($^{12}$C-labelled) microbial population were monitored. Prior to T-RFLP analysis of the incubated samples, the reliability of the method was assessed. Clones p7k23f, p4k1f and p2k9f harbouring 16S rRNA sequences from earlier studies of the Schlöppnerbrunnen fen (Wentrup 2007) were amplified using fluorescently labelled bacterial primers (2.4.3.1). Forty nanogram of PCR product was digested with endonuclease MspI and T-RFLP profiles were analysed. No undigested fragments or fragments of the wrong size could be detected in any of the profiles making the method applicable for analysis of the incubated samples (Fig.27- appendix section).

### 3.2.1 Bacterial T-RFLP analysis of DNA-SIP incubations

DNA templates of all fractions were amplified using the fluorescently labelled bacterial primer pair, resulting in a fragment of approximately 880 bp length. Starting with the 2 weeks incubation, differences in yield of PCR reactions were evaluated performing amplifications with 23 and 30 cycles (Fig. 9). When performing 30 cycles of PCR, products can be visualised even at fraction densities of 1.746 g cm$^{-3}$. Although fully $^{13}$C-labelled DNA of *M. extorquens* (GC% 66) has a buoyant density of 1.757 g cm$^{-3}$, not captured by our gradient, it is unlikely that after two weeks of incubation any of the species found in the fen sample are $^{13}$C-labelled largely due to the relatively low amount of substrates applied. This leads to the assumption that PCR products found at 1.746 g cm$^{-3}$ are caused by applying too many PCR cycles. Choosing too many cycles could possibly lead to an over amplification of certain 16S rRNA fragments due to PCR saturation effects, resulting in a misinterpretation of the bacterial community composition (Sipos *et al.* 2007). Therefore, 23 cycles instead of 30 PCR cycles were used for the following gradients.

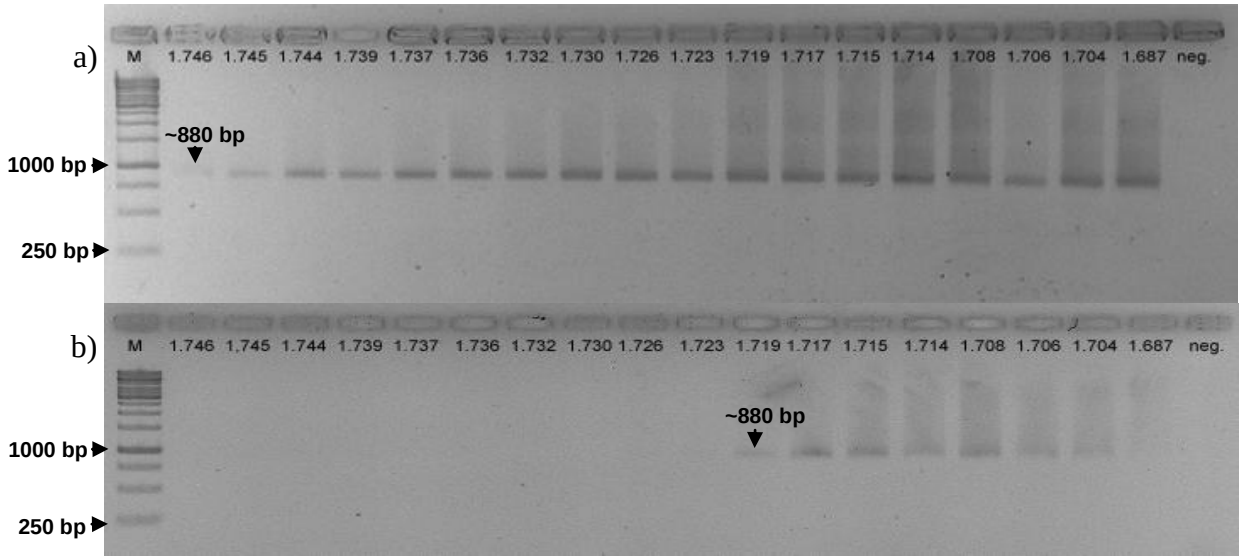


Fig.9 Bacterial 16S rRNA gene PCR products from 2 week incubations amplified with a) 30 cycles b) 23 cycles. Neg. indicates negative control and numbers given are densities of corresponding fractions in g $cm^{-3}$. Arrows indicate corresponding fragment and DNA ladder lengths.

100 ng of each PCR product was used to perform restriction enzyme digestion using *MspI*. After 2 weeks no clear differences in peak distribution in both gradients as well as throughout the gradients themselves could be observed. The major T-RFs of the heaviest fractions at 1.723 g $cm^{-3}$ of the sulphate-incubated gradient were at 90 and 147 bp. In addition minor peaks at 136, 285, 438, and 489 bp appear. Peaks in the heavy control fraction were the same except a small peak appearing at 429 bp not visible in the sulphate incubated heavy fraction. The light fractions (1.713 g $cm^{-3}$ and 1.714 g $cm^{-3}$) of both gradients harboured T-RFs at 877, 896, and 912 bp. Additionally, a peak at 262 bp appears to be more prominent in the 'lighter' fractions from both gradients ($\leq$ 1.714 g $cm^{-3}$). Other major and minor peaks were the same as in the heavy fractions (Fig.10.)

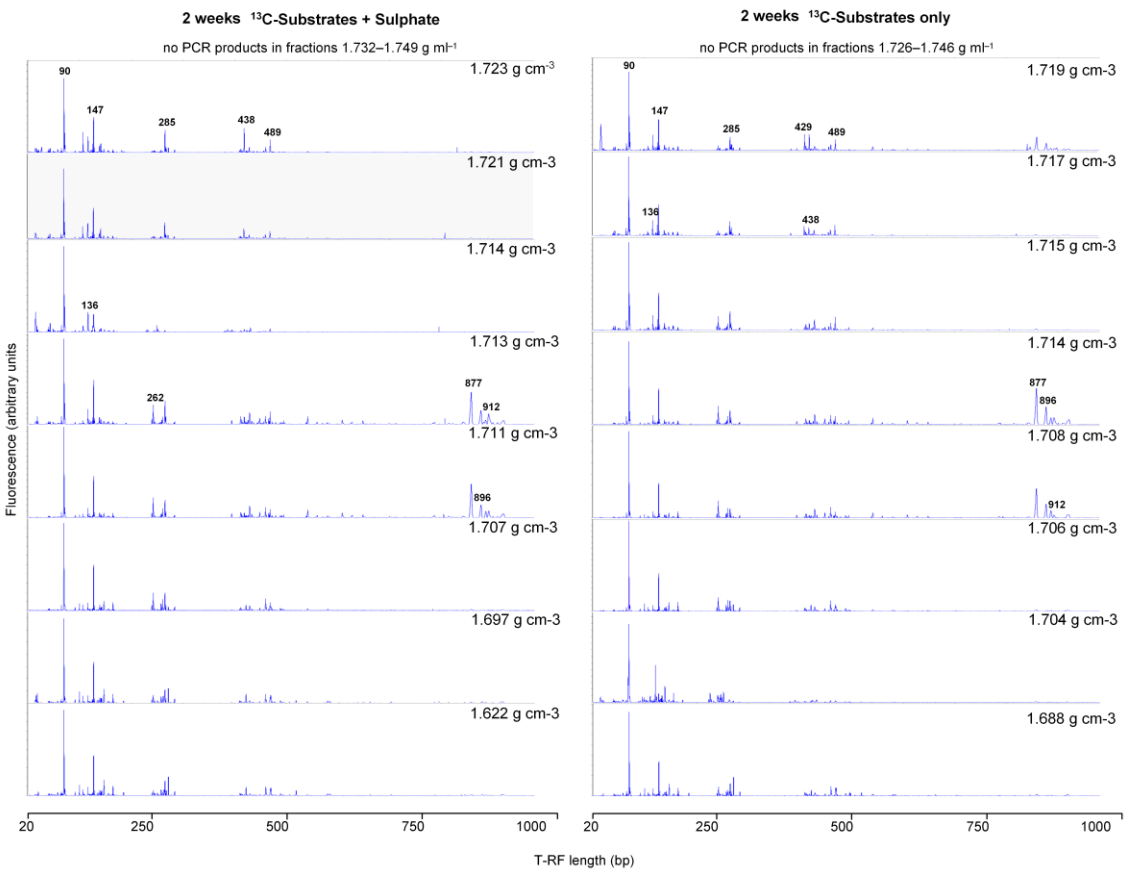


Fig.10 T-RFLP profiles of density resolved DNA from 2 week incubations with and without sulphate. CsCl densities and bp lengths of important T-RFs (as mentioned in the text) are given.

For the 2 month incubations, the T-RFLP profiles obtained show a different picture then the 2 weeks incubation. T-RFLP profiles were obtained at densities of 1.727 g $cm^{-3}$ in the sulphate-amended incubations, indicating a higher content of incorporated $^{13}C$. Peak distribution between the control and the sulphate incubation as well as between heavy and light fractions differ to the 2 weeks incubations. In the heavy, sulphate induced fraction at 1.727 g $cm^{-3}$, major peaks are visible at 105 and 136 bp, which are not detected, or of low abundance in the control. Contrary, the peaks at 126, 292 and 438 bp were more prominent in the heavy control fraction (1.719 g $cm^{-3}$), although they were also present in the sulphate induced fraction. The peak at 89 and 90 bp was high in both gradients. Except the 90bp T-RF, all others were hardly detectable in the light fractions. A peak at 147 bp, which was also detected after 2 weeks, was distributed through all density fractions, appearing smaller in the sulphate-induced incubations. T-RFs between 877 and 912 bp detected in the lighter fractions also appear in the heavy control fraction at 1.719 g $cm^{-3}$ (Fig.11).

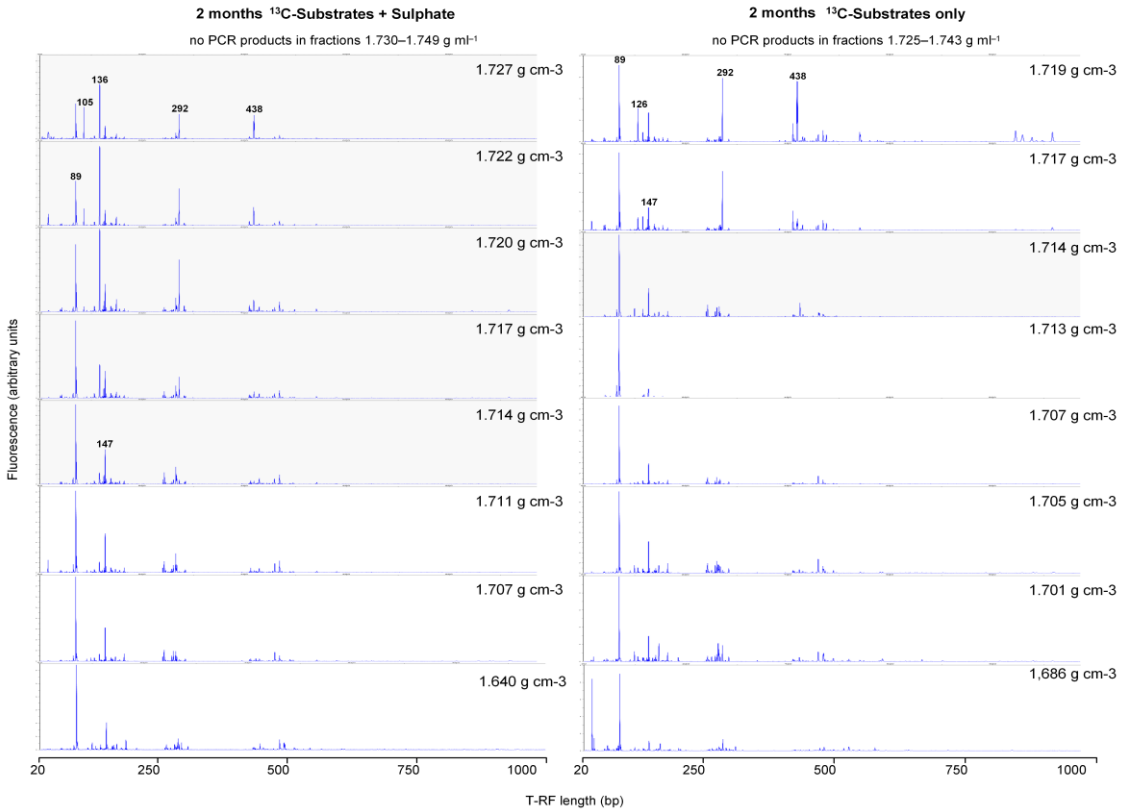


Fig.11 T-RFLP profiles of density resolved DNA from 2 months incubations with and without sulphate. CsCl and bp lengths of important T-RFs (as mentioned in the text) are given.

In the 6 months incubations, the obtained T-RF pattern changes again. T-RFLP profiles were obtained in both gradients starting at a density of 1.728 g $cm^{-3}$. The largest peak in the heavy, sulphate added incubation was still at 136 bp and peaks at 90, 105, 438, and 517 bp are visible, but to a relatively smaller extent. Here, T-RFs ranging from 865-945 bp, as detected in the two weeks incubations, also appeared. In the light fractions (1.719 g $cm^{-3}$ and less) the 136 bp peak was smaller while peaks at 90 and 147 bp were relatively high. The sulphate amended incubation fraction at 1.718 g $cm^{-3}$ showed a prominent peak at 489 bp, which could not be seen in this extent in other fractions of this incubation. At this density, T-RFs between 878 and 945 bp were found that were detected again at densities of 1.708 and 1.707 g $cm^{-3}$. In the heavy control incubation (1.725 g $cm^{-3}$), major peaks were at 430 and 438 bp as well as smaller peaks at 105 bp. T-RF fragments ranging from 865 to 917 bp could seen in all density fractions. At lower densities, an additional peak at 90 bp appeared compared to T-RFs found at 1.725 g $cm^{-3}$. At 1.721 g $cm^{-3}$ the peaks at 105 and 438 bp decreased but a T-RF at 289 bp could be seen. In the next less denser fractions, peaks at 147, 262 and 489 bp got more prominent, as well as the peak at 90 bp which was relatively larger. In the following lighter fractions, this peak was by far the largest one. Only the peaks at 147, 262 and 289 bp remained relatively prominent throughout the rest of the light density gradients (Fig.12).

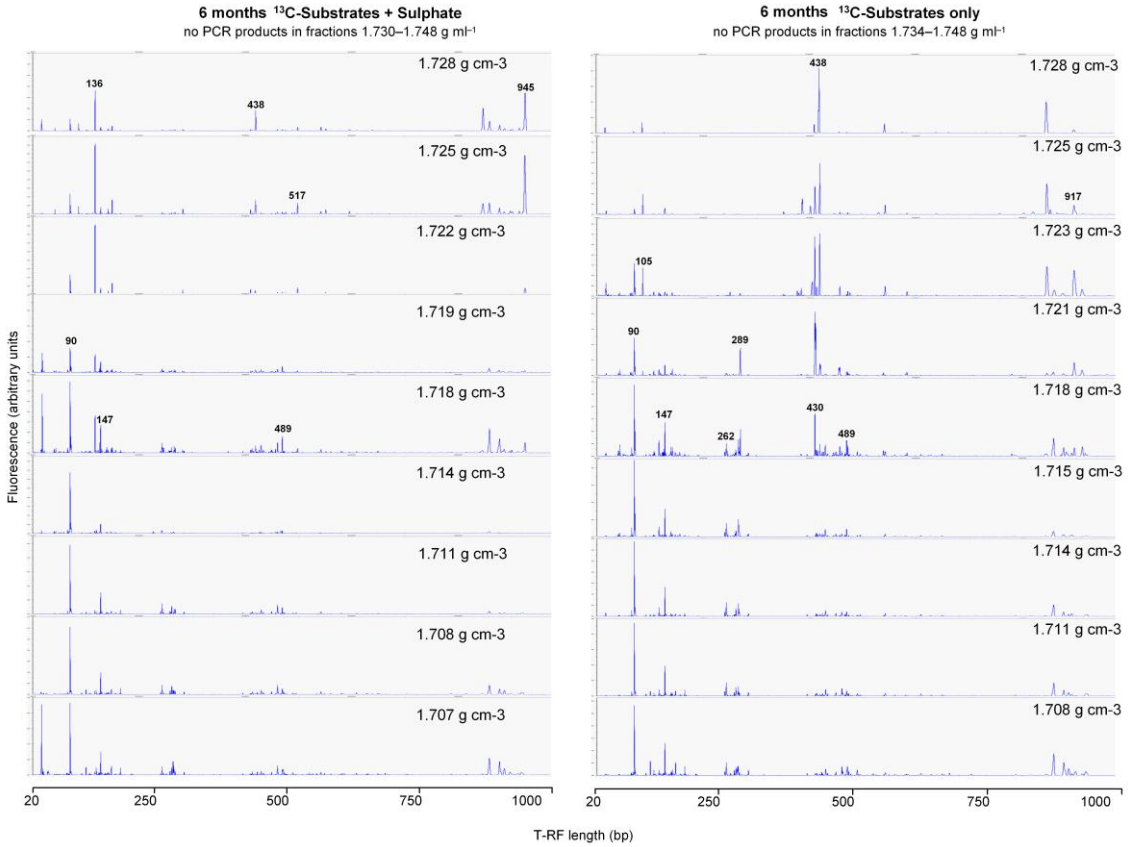


Fig.12 T-RFLP profiles of density resolved DNA from 6 months incubations with and without sulphate. CsCl densities and bp lengths of important T-RFs (as mentioned in the text) are given.

Before constructing a gene library, T-RFLP profiles of the sulphate induced and control incubation were compared to T-RFLP profiles from unincubated fen soil from a depth of 10-20 cm (Fig.13). The major peak at 90 bp could be found in the zero control up to densities of 1.722 g $cm^{-3}$, which might be caused by species with different GC content represented by this T-RF. For peaks at 126, 292, and 438 bp in the $^{13}C$ only control as well as for the peaks at 105 and 136 bp in the sulphate amended incubation $^{13}C$ labelling clearly occurred as indicated by absence or appearance only at lighter densities in the untreated zero control.

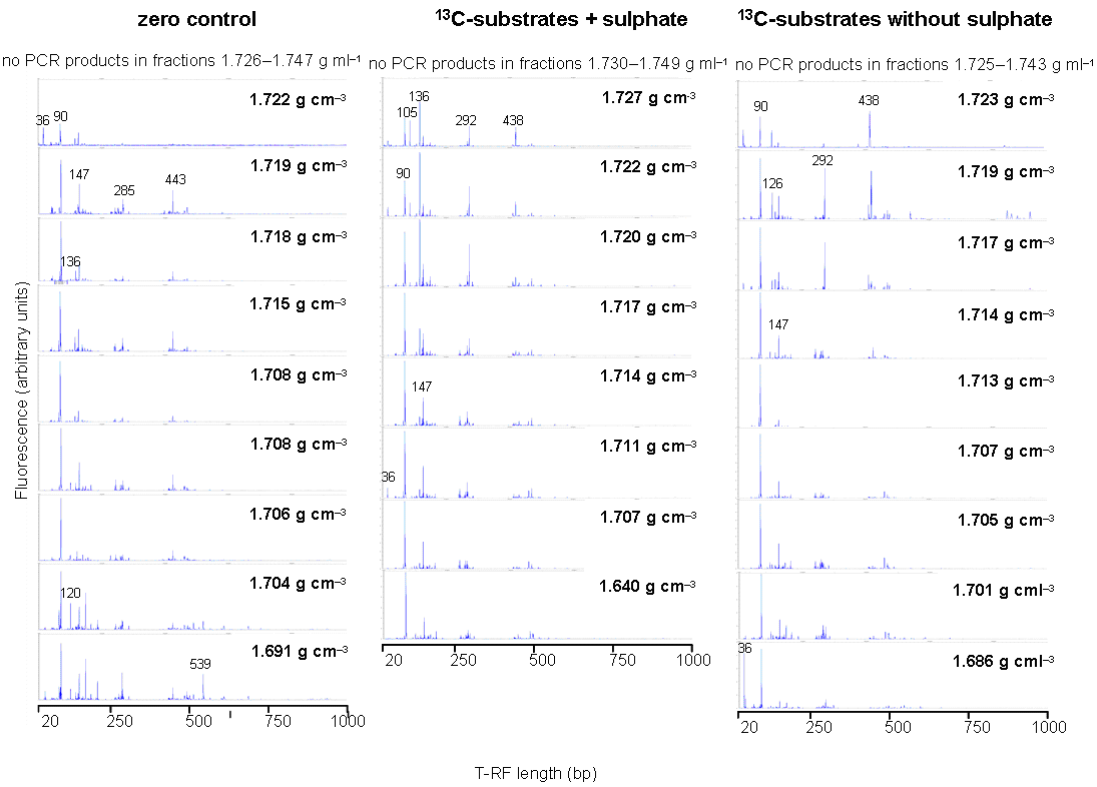


Fig.13 T-RFLP profiles of 2 months incubations compared to untreated zero control. CsCl densities and bp lengths of important T-RFs (as mentioned in the text) are given.

Furthermore, T-RFLP analysis was also performed with all DNA extracts prior to centrifugation to control for consistency of dominant peaks compared to the centrifugations (Fig.14). All dominant peaks could be found in the whole extracts as well. Peaks at 120, 147 and 443 bp, which could be found in relatively high extent in the zero control are relatively lower or can not be detected anymore, indicating that the respective microorganisms do not utilize the offered substrates under the given conditions. Interestingly, the peak at 136 bp, which was very prominent at heavier densities in the sulphate induced 2 months incubation, could also be found at low intensity in the zero control, the 2 months and the 6 months $C^{13}$ incubations without sulphate. In the zero control the 136 bp peak could only be found until densities of 1.718 g $cm^{-3}$ and in low abundance opposed to its dominant appearance at heavier densities in the 2 months sulphate induced incubations. This indicates an important role in sulphate reduction for these species.

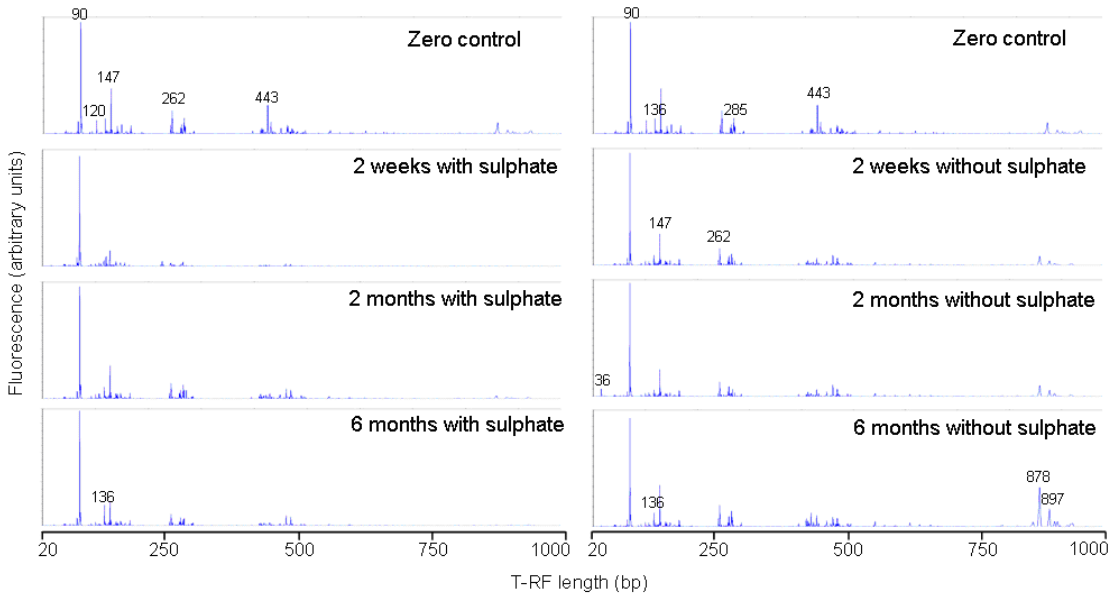


Fig.14 T-RFLP profiles of DNA whole extracts of all time points. The bp lengths of important T-RFs (as mentioned in the text) are given.

### 3.2.2 Archaeal T-RFLP analysis of DNA SIP incubations

2 months incubations were also analysed for archaeal 16S rRNA sequences, using the primers and conditions described in section 2.4.3.1, resulting in approximately 800 bp fragments. According to the results of the previous amplification of bacterial 16S rRNA, 23 PCR cycles were used. The highest densities where PCR products could be obtained were at 1.721 g $cm^{-3}$ and 1.719 g $cm^{-3}$ (Fig.15). 100 ng of PCR products were digested with the restriction enzyme *TaqI* and resulting fragments were identified by capillary gel electrophoresis. Coinciding with weak bands visible on the agarose gel, there was no sufficient fluorescence intensity above densities of 1.717 g $cm^{-3}$. This indicates less $^{13}C$ labelling of the archaeal community compared to the bacterial community after 2 months. The resulting T-RFLP profiles of all densities showed only few peaks with no differences between the sulphate induced and control-incubation, as well as throughout the gradients. A major peak at 184 bp and a smaller peak at 216 bp were present in every fraction. In both of the 'light' fractions at 1.707 g $cm^{-3}$ and below, additional peaks at 390, 740 bp, 852bp (+sulphate) and 861bp (-sulphate) appeared. Only in the heaviest fractions of the control incubation, two additional T-RFs at 28 and 40 bp are visible (Fig.16).

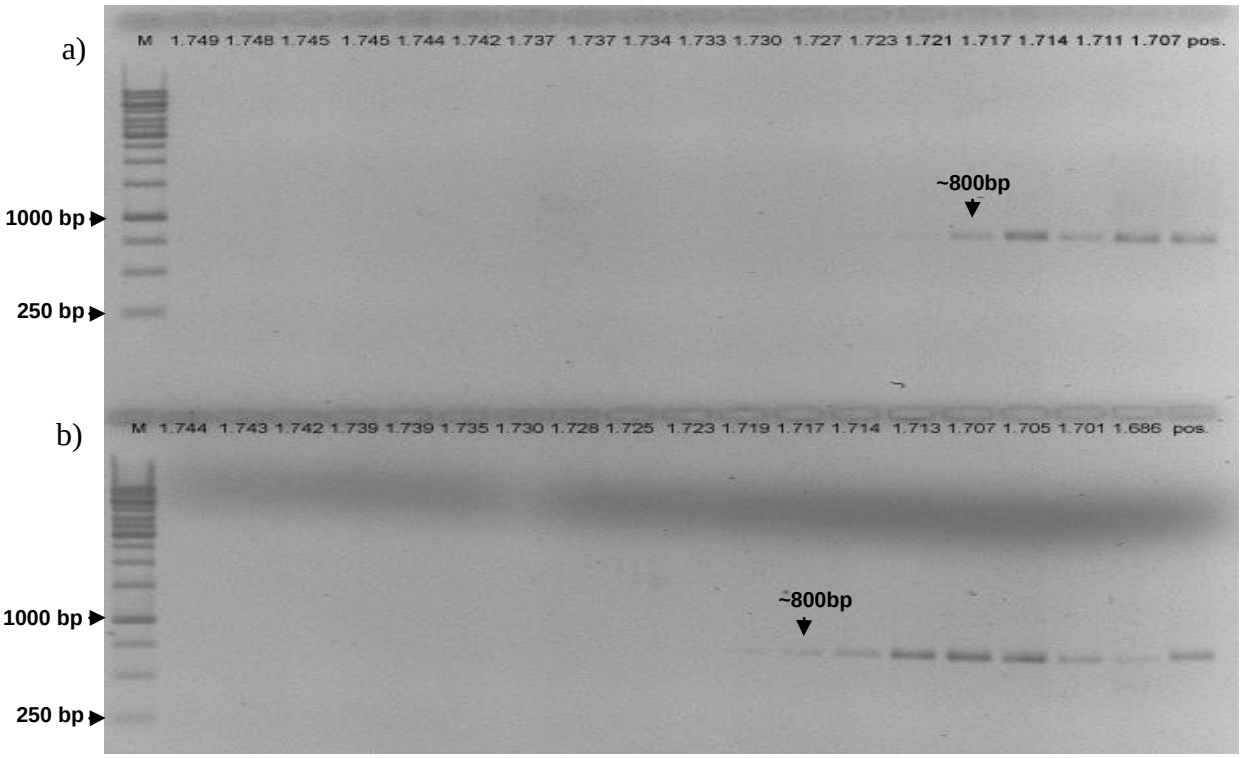


Fig.15 Archaeal 16S rRNA PCR products from 2 months incubations a) with b) and without sulphate added. Pos. indicates positive controls and numbers given are densities in g $cm^{-3}$. Arrows indicate corresponding fragment and DNA ladder lengths. Negative control is not shown.

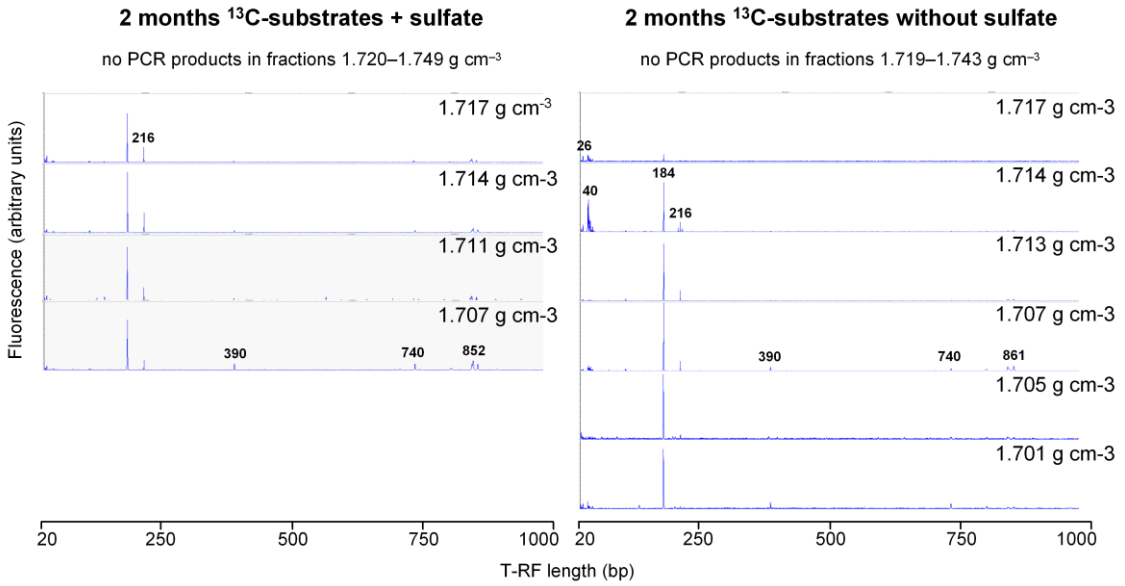


Fig.16 Archaeal T-RFLP profiles of density resolved DNA from 2 months incubations with and without sulphate. CsCl densities in g $cm^{-3}$ are numbers in brackets. The bp lengths of important T-RFs (as mentioned in the text) are given.

### 3.2.3 Bacterial T-RFLP analysis of RNA-SIP incubations

Samples incubated for two weeks were additionally subjected to RNA-SIP analysis. RT-PCR was performed using the same bacterial primers as for DNA-SIP (2.4.3.4), resulting in approximately 880 bp fragments (Fig.17). Due to problems with the temperature correction of the refractometer, no valid density values could be obtained here.

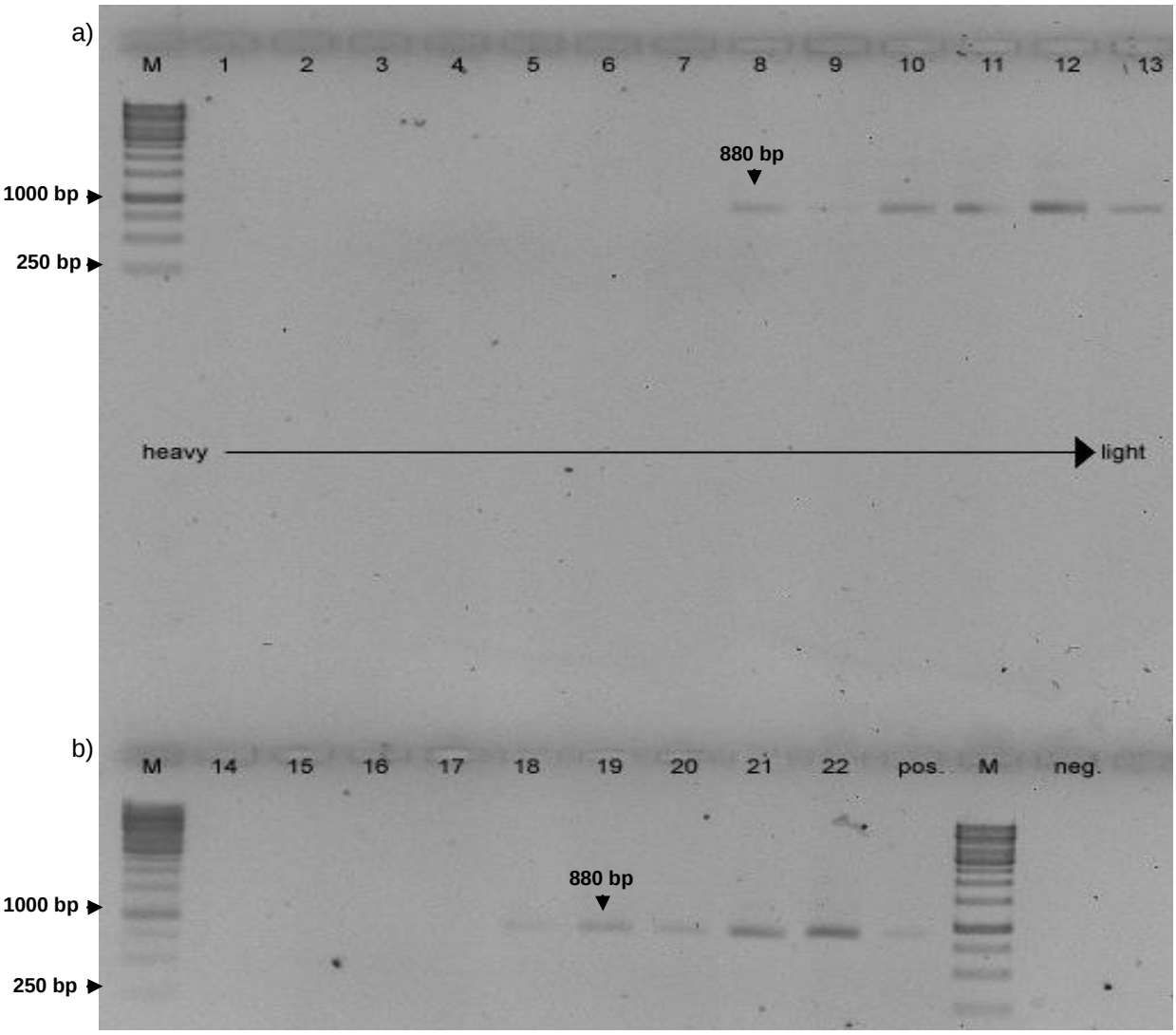


Fig.17 RT-PCR products from 2 week incubations a) with b) and without sulphate added. Pos. indicates positive control, Neg. negative control. Arrows indicate corresponding fragment and DNA ladder lengths.

Digestion for T-RFLP was performed as done for DNA-SIP with restriction enzyme *MspI*. T-RFLP profiles could even be obtained from fraction 17 without any visible PCR product on the agarose gel. Peak distribution and variety showed great differences to the corresponding 2 week DNA samples analysed. Starting with the heaviest fraction of the sulphate amended incubation (8), major T-RFs are at 97 and 124 bp. In the lighter fractions the most prominent peaks are at 130, 198, and 236 bp. In the lightest fractions,

additional peaks at 90 and 147 bp appeared and the T-RFs mentioned above get relatively smaller or disappear. In the control incubation, the heaviest fraction (17) only showed major peaks at 97 and 130 bp. At the following fraction peaks at 90, 145, and 198 bp appeared, while others get smaller. At lighter densities starting with fraction 19, the peaks stayed the same, except at 198 bp and 236 bp. They were hardly detectable in fractions 19 and 20 and showed up again at fraction 21. Another peak at 323 bp could be found in fractions 19 to 22. Additionally, all fractions harbour T-RFs, ranging from 864 to 936 bp at different extents (Fig.18).

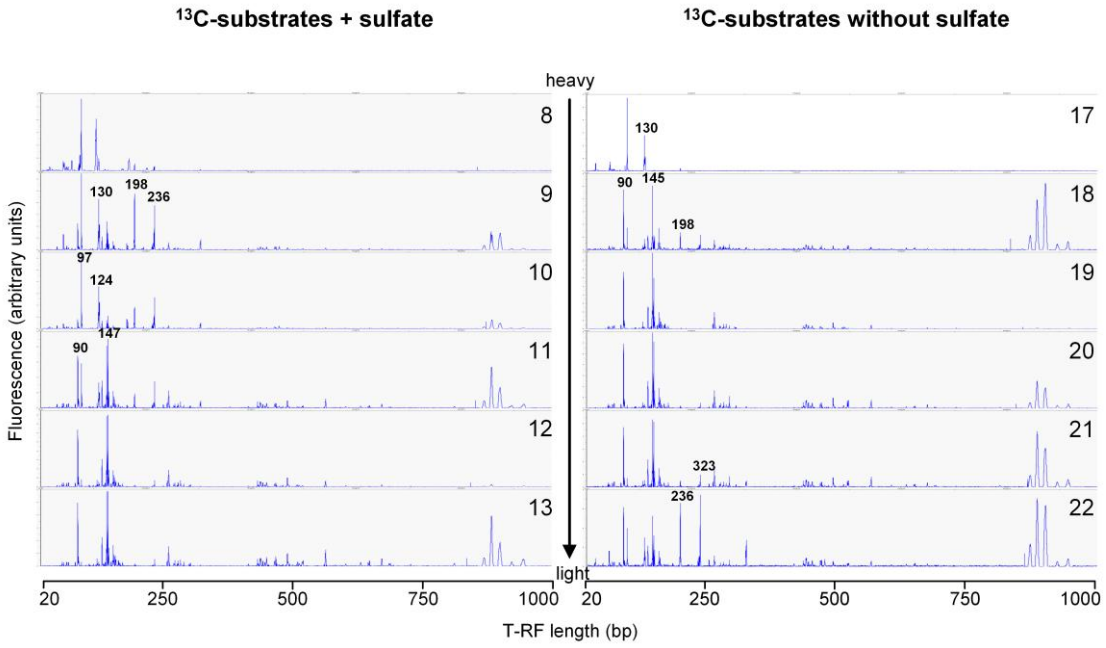


Fig.18 Bacterial T-RFLP profiles of density resolved RNA from 2 weeks incubations with and without sulphate. The bp lengths of important T-RFs (as mentioned in the text) are given.

## 3.3 Amplification, cloning and sequencing of 16S rRNA genes and *dsrAB*

Having shown with T-RFLP-fingerprinting that after two months of incubation communities of active bacteria in the two setups differ from each other, the corresponding heavier fractions at 1.720 g $cm^{-3}$ (+sulphate) and 1.719 g $cm^{-3}$ (-sulphate) were used for further cloning. 16S rRNA genes and *dsrAB* were PCR amplified using unlabelled primers as described in section 2.4.3.1 and 2.4.3.3 and subsequently cloned. Prior to cloning of *dsrAB* amplicons, fragments of the appropriate size (~1.9 kbp) were separated from unspecific PCR products via agarose gel electrophoresis, cut out and extracted (Fig.19). Topo XL® clones with a positive insert were grown on selective media

and were used for further analysis via M13 screening (2.4.3.2). Clones harbouring the right insert were sequenced.

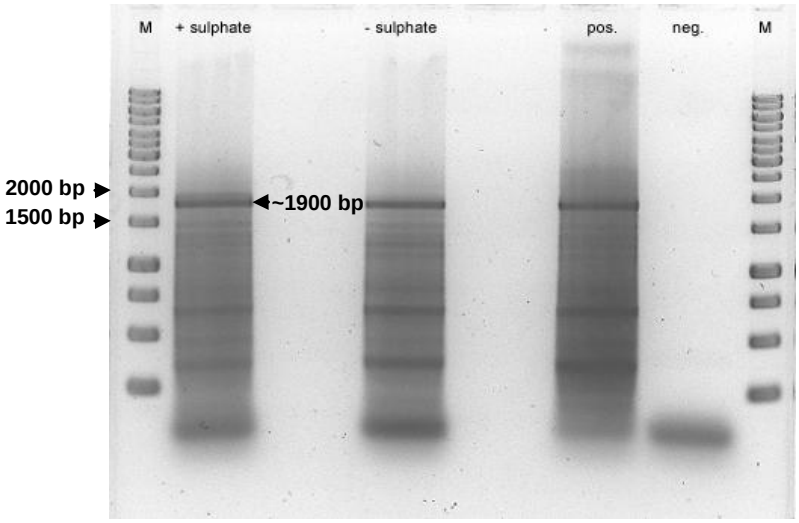


Fig.19 *DsrAB* PCR products from 2 months heavy fractions. Arrows indicate corresponding fragment length of marker and cloned fragment. Pos. indicates positive control, Neg. negative control

## 3.4 Sequence analysis

### 3.4.1 Bacterial 16S rRNA analysis

Phylogenetic analysis of proofread sequences confirmed the observed differences between the two examined fractions. Most of the clones of the sulphate amended incubation were affiliated with the genus *Desulfosporosinus* (20%), known gram-positive sulphate reducers (Stackebrandt *et al.* 1997). Numerous clones of *Acidobacteria* subgroup 1 (16%); subgroup 2 (7%); subgroup 3 (7%) and one clone of subgroup 8 were detected as well. Members of the families *Rhodospirillaceae* (8%), *Acidaminococcaceae* (6%), *Acidimicrobiaceae* (5%) and *Acetobacteraceae* (4%) were also among the prominent members in the heavy DNA fraction from the sulphate-amended incubation (Fig. 20). Furthermore, sequences of several other phyla were detected like members of the *Proteobacteria*, *Actinobacteria*, *Nitrospira*, *Spirochaetes*, *Planctomycetes* and OP10.

The control incubation showed a different composition of 16S rRNA genes. No *Desulfosporosinus* and *Acetobacteraceae* related clones were detected. Members of the *Acidobacteria* subgroup 1 (21%) represented the most abundant ones in the control and members of the subgroups 2 (3%), subgroup 3 (6%), subgroup 6 (1%) and subgroup 13 (1%) could be identified as well. Additionally, sequences affiliated to *Acidaminococca-*

*ceae* (13%), *Rhodospirillaceae* (14%), *Azospirillum* (10%), *Acidimicrobiaceae* (6%) and *Geobacter* (5%) were found to a higher extent in the $^{13}C$ control without sulphate (Fig. 20). Other clones with sequences belonging to the phyla *Proteobacteria*, *Bacteroidetes*, *Spirochaeta*, *Planctomycetes*, *Cyanobacteria*, OP10, OD1 and TG1, could be identified as well.

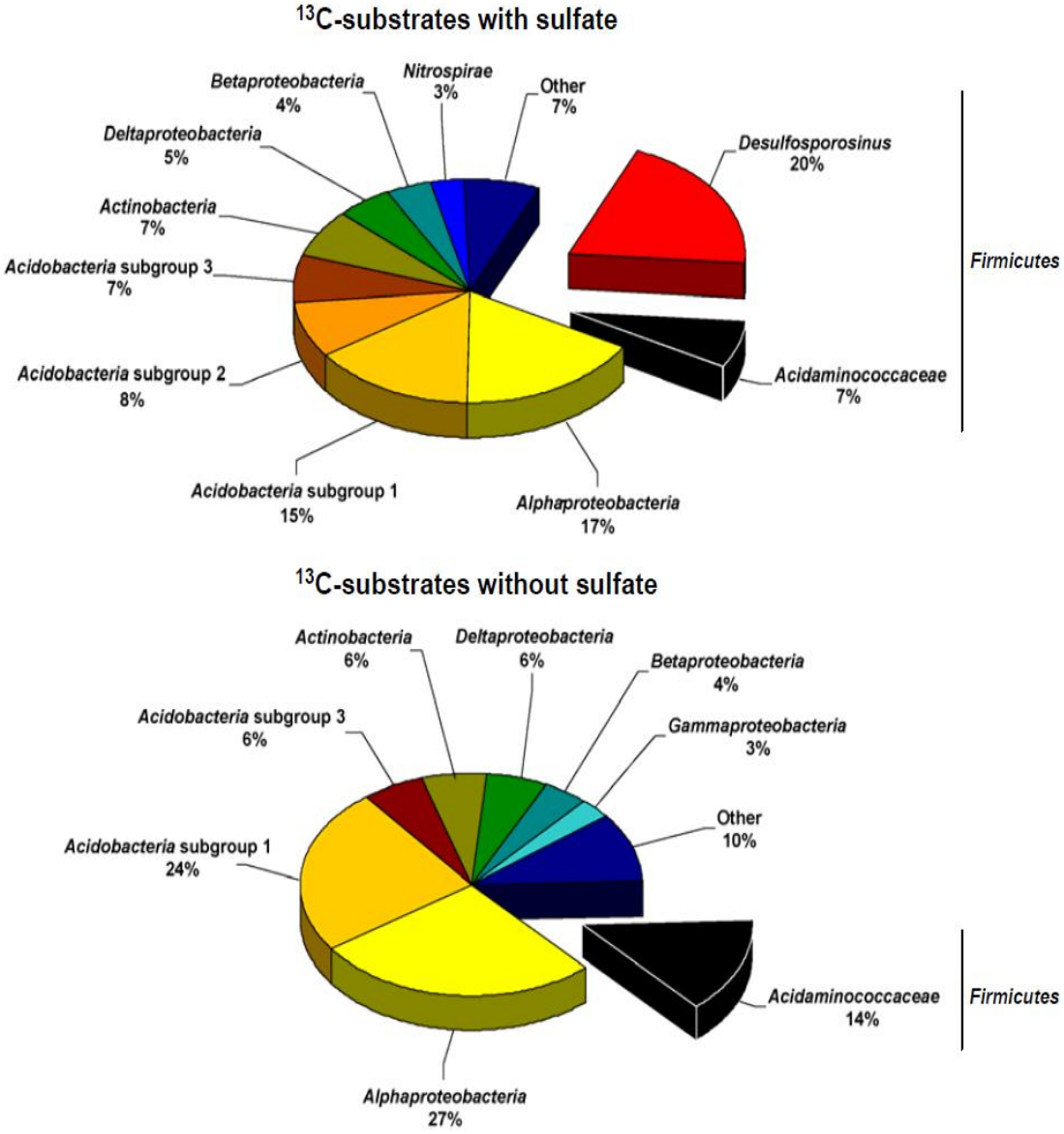


Fig.20 Relative abundance of major bacterial lineages in the 16S rRNA gene clone libraries obtained from the 2 months incubations in the presence and absence of sulphate. For details and representing T-RFs see table 17 in the appendix section.

In addition to T-RF fragment length assignment of analysed sequences *in silico*, T-RF lengths of corresponding single clones were empirically determined (Fig.28 - appendix section). They were PCR amplified and 40 ng of product was digested with *MspI*. Differences of empirically determined sizes to *in silico* values result from the size determi-

nation algorithms used in automated sequencing devices. These algorithms assume that the migration time of fragments increases linearly with size, which is not true, making size calling of fragments erroneous (Shyu *et al.* unpublished). In addition, the fluorophores used for T-RFLP influence migrational behaviour of fragments (Tu *et al.* 1998). The most abundant peaks corresponded with the number of clones of equivalent size (Tab.17-appendix section). Peaks consisting of T-RFs of different microorganisms at 136 bp (*Desulfosporosinus* and *Acidobacteria subgroup* 3) and 437 bp (*Rhodospirillaceae* and *Rhizobiales*) of the sulphate amended heavy fraction were further examined by T-RFLP analysis with the enzyme *RsaI*. Peaks could be resolved and data shows that the dominant members of the resolved peaks are of the genus *Desulfosporosinus* (136 bp) and the family of *Rhodospirillaceae* (437 bp). These findings are in accordance with the frequency of clones found in the clone library (Fig.21, Table 17 appendix section).

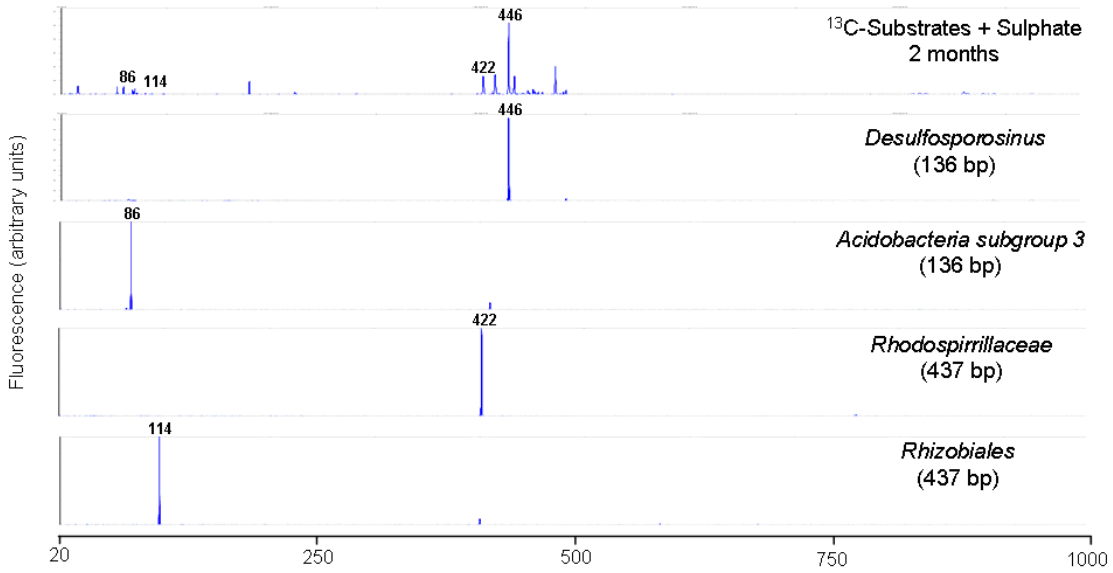


Fig.21 *RsaI* resolved T-RFLP profile of the heavy sulphate induced fraction (1.720 g $cm^{-3}$) and corresponding single clones. Fragment lengths of resolved peaks are given in bp. Fragment lengths of *MspI* digests of single clones are given in brackets.

To determine the number of OTUs from each of the fractions, bacterial 16S rRNA sequences with a similarity of ≥ 99%, approximately defining species level phylotypes (Stackebrandt 2006), were clustered together using the software DOTUR (Schloss and Handelsman 2005). The heavy fraction from the sulphate-induced incubation consisted of 54 OTUs compared to 47 for the control fraction. In addition, both 16S rRNA gene clone libraries were subjected to rarefaction and coverage calculations using different sequence similarity thresholds for OTU determination (Fig. 22 and Tab.16).

Table 16: Goods coverage results for 16S rRNA gene sequences of the 2 months incubations with different similarity thresholds.

| Sequence similarity threshold | Coverage (+ sulphate) | Coverage (-sulphate) |
|---|---|---|
| 90% | 60.71% | 40.74% |
| 95% | 32.55% | 31.57% |
| 97% | 31.91% | 30.23% |
| 99% | 27.78% | 21.27% |

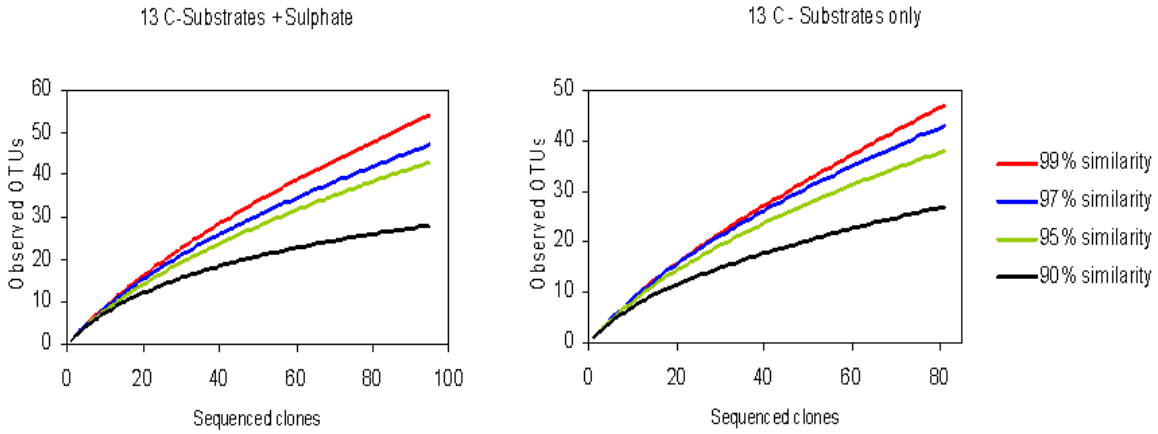


Fig.22 Rarefaction curves of the analysed heavy fractions of the 2 months incubations

Except for 90% of sequence similarity for OTU calculation, the resulting curves did not reach the plateau phase. In accordance with low coverage values this indicates a high diversity of microorganisms in the incubations not sufficiently sampled yet.

UNIFRAC divergence analysis of the heavy fractions resulted in a p-value of 0.05 indicating minor divergence. Additionally, lineage specific divergence analysis (G-test) confirmed the *Desulfosporosinus* genus being the genus responsible for this minor divergence (branch length threshold = 0.117791) (Lozupone *et al.* 2006).

### 3.4.2 *DsrAB* analysis

In addition, phylogenetic analysis of *dsrAB* was performed examining the heavy fractions already used for construction of the 16S rRNA gene libraries. OTU assignment with DOTUR revealed 13 distinct OTUs for the sulphate-amended fraction, compared to 9 for the incubation without added sulphate. OTUs were defined with a sequence similarity threshold of ≥ 90% based on earlier studies (Loy *et al.* 2004). Additionally, rarefaction and coverage analysis was performed. A curve not reaching the plateau and small coverage values were gained, but only 26 clones of the sulphate induced fraction and 20 of the control fraction were sequenced (Fig.23).

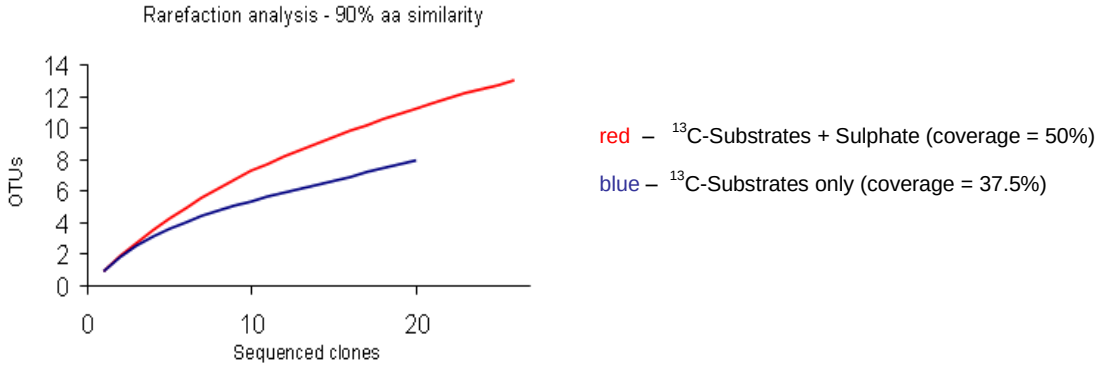


Fig.23. Number of *dsrAB* OTUs observed calculated with the rarefaction method and coverage values for analysed gene libraries of heavy fractions of 2 months incubations.

Phylogenetic analysis revealed that most of the new sequences cluster closest to the novel deep branching *dsrAB* lineages, namely OTU 2, 7 and 10 found by Loy and co – workers (Fig.24) (Loy *et al.* 2004). Contrastly to 16S rRNA gene libraries no significant differences between the sulphate amended incubations and the control incubations could be observed since each observed OTU was present in both fractions. Furthermore, no *dsrAB* sequence was clustering close to *Desulfosporosinus orientis* or relatives, compared to the findings of the 16S rRNA data. Sequences found in both fractions, cluster close to *Thermosinus carboxydivorans* a bacteria known for the utilisation of thiosulphate (Sokolova *et al.* 2004). Sequences from each OTU were used for construction of phylogenetic trees (Fig.24).

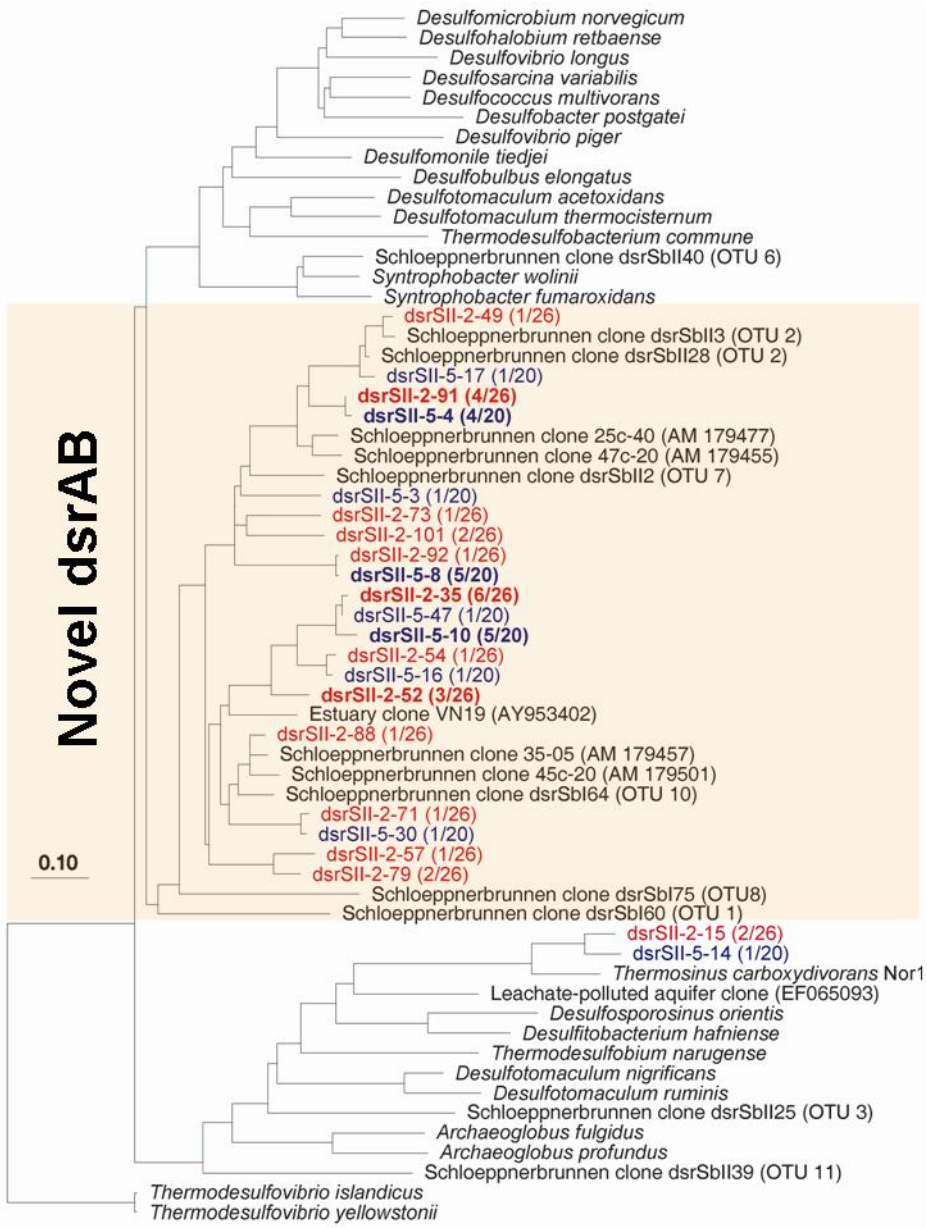


Fig. 24 *DsrAB* tree based on 318 deduced amino acids showing the affiliation of the OTUs detected within this study from the sulphate-induced incubation (red) and control incubation (blue). Clone numbers are given in parentheses. Coloured section shows *dsrAB* OTUs specific for fen soils found in this and a previous study (Loy *et.al.* 2004).

# 4 Discussion

Examining the microorganisms that are actively involved in sulphate reduction in the investigated acidic model fen, the Schlöppnerbrunnen peatland was the main aim of this study. Several recent studies, based on SIP e.g. (Hamberger *et al.* 2008), 16S rRNA (Kraigher *et al.* 2006) as well as *dsrAB* phylogenetic analysis (Loy *et al*. 2004; Wentrup 2007) have already allowed insights into the diversity and metabolic capability of the microorganisms inhabiting this area. However, until now no studies have been conducted, employing differential display SIP focussing on SRP. Based on previous studies the applied concentrations of sulphate and carbon were relatively low, resembling the conditions at the investigated area (Hamberger *et al.* 2008). This helps reducing communtiy shift biases in SIP introduced by applying too high concentrations of substrates (Neufeld et al. 2007a).

To calibrate the centrifugation system prior to the actual fen soil experiments, 100% $^{13}C$ labelled *E. coli* DNA was used. I never observed a greater difference than 0.02 g $cm^{-3}$ to unlabelled *E. coli* DNA, which is only half the value of fully $^{13}C$ labelled DNA as published by Lueders in 2004. One reason may be that when growing the bacteria, part of the initially used $^{12}C$ -LB-medium was transferred and contaminated the $^{13}C$ media. Additionally, $^{13}C$ growing media was not kept airtight, therefore natural $^{12}CO_2$ was incorporated during *E.coli* nucleotide synthesis, lowering $^{13}C$ content of *E.coli* DNA to a small extent. Nonetheless, separation of gradients worked and we proceeded with the centrifugation of the soil incubation experiments.

Although the system was calibrated, I could not establish a gradient with densities higher than 1.749 g $cm^{-3}$ as published in earlier studies (Lueders et al. 2004a). This may have several reasons as e.g. the use of a different rotor than in the study by Lueders, therefore having different *g* forces influencing the gradients shape (Neufeld *et al.* 2007). Nevertheless, we covered most of the density range allowing us to gain insights into SRP activity in the investigated area.

For bacteria, T-RFLP profiles of all time points were screened and clear differences between the sulphate amended- and control incubations could be observed over time. After two weeks of substrate incubation, only minor changes were detected between the $^{13}C$-labelled and unlabelled fractions of both incubations. This was not surprising, considering that relatively low concentrations of labelled substrates were added weekly and that SRP are generally slow growing bacteria, due to the lower energy yield of dissimilatory sulphate reduction (Thauer *et al.* 1977). However, certain parts of the bacterial

community incorporated labelled carbon after this short period, indicated by the appearance of certain peaks in the T-RFLP profiles at heavier densities e.g. 285bp at 1.723 g $cm^{-3}$ compared to 1.720 g $cm^{-3}$ for the zero control.

T-RFLP profiles from the RNA-SIP of the 2 week incubation showed great variety to the corresponding DNA profiles as well as between the control and the sulphate-induced incubation. On the RNA level, the effects of isotope labelling can be observed earlier than on the DNA level due to fast and direct incorporation of labelled carbon, not dependent on cell division. This has already been demonstrated in several studies, e.g. with experiments on phenol degrading microorganisms (Manefield *et al.* 2002). In general, when examining a complex microbial community with SIP after a short incubation time, results have to be interpreted with care due to gene doses effects of differences in *rrna* gene copy number and transcription regulation among different bacteria (von Wintzingerode *et al.* 1997). In addition, the buoyant densities of the RNA fractions in our study could not be determined due to temperature correction problems while measuring refractive indices. Therefore, a closer look at the later time points of the DNA-SIP experiments was taken.

At the later time points, clear differences in community composition were observed. A peak at 136 bp, dominating in the two months as well as in the 6 months heavier fractions of the sulphate induced incubations, could be identified to consist almost exclusively of 16S rRNA gene sequences affiliated with the genus *Desulfosporosinus*. Activity was clearly demonstrated by comparing density values with the untreated zero control where this peak could be observed to a relatively small extent at 1.722 g $cm^{-3}$. In the examined sulphate amended fraction this peak was dominating. In addition, the density of the fraction observed was at 1.727 g $cm^{-3}$ demonstrating labelling with heavy $^{13}C$ isotopes (Fig.13). Interestingly, a peak at 105 bp, which could only be found in the sulphate added heavy fractions after two months, appeared in the control incubation without sulphate after six months as well. Possibly, it was also present in minor quantities in the control incubation after two months, but results of the 16S rRNA clone library, constructed with the two heavy fractions at 1.719 g $cm^{-3}$ or 1.720 g $cm^{-3,}$ could not prove that point. Other observed major peaks were present in both fractions to a different extent.

Despite observing more significant differences after 6 months, the shorter incubation time was chosen for sequence analysis to reduce bias affiliated with possible cross feeding effects, which have to be considered when interpreting results of later time points (Dumont and Murrell 2005; Okabe *et al.* 2005). In addition, to the construction of the 16S rRNA gene library of the bacterial consortia, T-RFLP profiles of the archaeal community after 2 months were analysed as well. Archaeal sulphate reducers of the

genera *Euryarcheota* and *Crenarcheota* (Achenbach-Richter *et al.* 1987; Itoh *et al.* 1998; Itoh *et al.* 1999) are known, but analysis showed no differences between the two incubations. This leads to the conclusion that if any sulphate reducing archaea are found in this habitat, their activity is low compared to the bacterial community (Fig.10, 13).This does not seem to be surprising as all known sulphate reducing archaea were found at hot environments e.g. hot springs and hydrothermal vents (Stetter *et al.* 1987; Burggraf *et al.* 1990; Itoh *et al.* 1999). Therefore, no deeper analysis of the archaeal community was performed.

Some peaks of the observed fractions, RNA as well as DNA, showed different sized T-RFs, with lengths of ≥860 bp. It is suggested that these originate from uncut PCR products of unknown sequences having no restriction site for the enzymes used. Although performing T-RFLP analysis of known 16S rRNA sequences *in silico* prior to the *in vitro* experiments, choice of the appropriate restriction enzyme remains critical when applying T-RFLP (Engebretson and Moyer 2003; Schutte *et al.* 2008). Surprisingly, none of these longer fragments could be found in any of the two gene libraries constructed, but the fractions cloned did not show high occurrence of these peaks. This coincides with the notion that clone libraries preferentially cover high abundant species (Ward *et al.* 1992). For further sequence analysis, a gene library of fractions showing higher occurrence of these species, could be constructed and analysed.

Analysis of the gene libraries of the heaviest fractions from incubations with or without sulphate, confirmed the results gained with T-RFLP. The proportion of genera in the clone library, correlated with the abundance of the observed major peaks (Fig.10, Tab.17). Coinciding with sulphate addition, the most abundant genus in the corresponding heavy fraction was identified to be *Desulfosporosinus*. *Desulfosporosinus* species are known Gram-positive members of the *Firmicutes* phylum, capable of sulphate reduction. Although being classified as Gram-positive, these spore forming bacteria, stain Gram-negative due to their unusually thin peptidoglycan layer (Spring and Rosenzweig 2006). Several species within the genera have been described to be active in acidic soils (Church et al. 2007; Kupka et al. 2007), capable of fermenting several organic substrates including lactate, which was used in this study. All of the used carbon species are incompletely oxidized to acetate by *Desulfosporosinus* (Campbell and Postgate 1965; Stackebrandt *et al.* 1997; Robertson *et al.* 2001).

Furthermore, the second lineage only appearing in the sulphate-induced incubation is the *Acetobacteraceae*. This lineage of the class of the A*lphaproteobacteria* mainly consists of species of the genus *Acidocella*. Surprisingly, *Acidocella* spp. are reported to grow only in pure culture in aerobic media (Hallberg and Johnson 2001). Nonetheless, recent experiments with a mixture of two pure cultures in an anoxic fermenter under

acidic conditions (pH 3.8 – 4.2), resembling the low pH conditions of the investigated fen, showed a possible syntrophic relationship between *Desulfosporosinus orientis* and *Acidocella aromatica* (Kimura *et al.* 2006). Syntrophic relationships are common with anaerobic microorganisms, but little is known about syntrophic living aerobic microorganisms (McInerney *et al.* 2009). The authors of the fermenter study assume that acetate produced by *Desulfosporosinus orientis* is metabolized to molecular hydrogen and carbon dioxide by *Acidocella aromatica*. Results of the Kimura study strongly support that this novel type of syntrophy is existing in natural environments, but further experiments confirming these findings are necessary, e.g. CARD and RAMAN FISH as well as q-PCR to specifically prove activity and occurrence of the species involved. It is interesting, that after 6 months the 105 bp peak, identified to be *Acetobacteraceae*, is also appearing in the heavy fraction of the control incubation. It is suggested that the acetate applied and acetate produced by other microorganisms than *Desulfosporosinus*, is utilised by these bacteria. Possibly other types of syntrophic relationships, e.g. with methanogenic species have developed which allow anaerobic growth of *Acidocella* (McInerney *et al.* 2008).

Interestingly, no other known sulphate-reducing bacteria except single clones that affiliated with *Desulfobulbacaeae* and *Desulfovibrio* were found in the gene library. Although *dsrAB* and 16S rRNA gene microarray data of previous other studies indicate several *dsrAB* harbouring microorganisms in the examined area e.g. *Desulfobacca acetoxidans* and *Syntrophobacter wolinii* related species, the 16S rRNA data of this study show no important role of these microorganisms under the experimental conditions (Loy *et al.* 2004; Wentrup 2007). Possibly other sulphate reducing microorganisms are outgrown by *Desulfosporosinus* due to a higher affinity to the substrates used here or several species possessing *dsrAB* are yet not identified as SRP. Surprisingly, *dsrAB* analysis, which will be discussed in detail later, gave results different to the 16S rRNA data. Under these conditions *Desulfosporosinus* is the main sulphate reducer, but SIP experiments with other substrates than used here, could give additional insights into microbial activity of SRP.

Although the main focus of this study was to find the main sulphate reducing microorganisms in the Schlöppnerbrunnen fen via comparative SIP analysis, several other bacterial groups could be identified to actively assimilate the $^{13}$C-labelled substrates added under these conditions. In both of the fractions the dominant phylum were the *Acidobacteria* correlating with results of recent studies revealing the high abundance of this phylum in the Schlöppnerbrunnen habitat (Wentrup 2007). Little is known about these bacteria and only few cultivated and described species are known, although it is suggested that this phylum harbours a high diversity of bacterial species (Barns *et al.*

2007). As in other studies about *Acidobacteria* most of the members found here can be assigned to the subgroups 1, 2 and 3, known to favour acidic soil pH (Sait *et al.* 2002; Mannisto and Haggblom 2006; Sait *et al.* 2006). Two representatives of subgroup 1, *Acidobacterium capsulatum* and *Terriglobus roseus*, have been cultivated and both have been identified to be acidophilic (Kishimoto and Tano 1987; Kishimoto *et al.* 1991; Hiraishi *et al.* 1995; Eichorst *et al.* 2007; Meisinger *et al.* 2007). In this context, it is interesting that although being described as members of soil microbial populations, so far no activity under anoxic conditions has been reported for members of subgroup 3 as indicated by the T-RFLP data from this study. Additionally, single sequences of the subgroups 6, 8 and 13 have been found, but little is known about them (Barns *et al.* 2007).

Following, the other major lineages found in this study are described briefly.

Another lineage of the *Firmicutes* phylum, prominent in both incubations (6% in the sulphate-induced incubation and 13% in the control) is the *Acidaminococcaceae*. They are known as anaerobic members of the order *Clostridiales*, which have been found in the investigated area (Wentrup 2007; Wust *et al.* 2009), as well as in other moderately acidic soil habitats (Kuhner *et al.* 2000; Matthies *et al.* 2001; Lee *et al.* 2007). They are known fermenters of several organic compounds including lactate, utilised in this study, which can be degraded to acetate by e.g. *Psychrosinus fermentans* and *Pelosinus fermentans* (Shelobolina *et al.* 2007; Sattley *et al.* 2008).

Despite the already described *Acetobacteraceae*, the metabolic diverse phylum of the *Proteobacteria* is also represented by numerous genera in this study. Clearly dominant, especially in the control incubation, are members of the *Rhodospirillaceae* family and several *Azospirillum* species. Compared to only 1% of the clone library sequences in the sulphate-induced incubation, 10% of all retrieved sequences in the control clustered close to the *Azospirillum* genus. They are known nitrogen-fixing bacteria, associated with plants and were already isolated from various soils (Paredes-Cardona *et al.* 1988; Tapia-Hernandez *et al.* 1990; Doroshenko *et al.* 2007; Young *et al.* 2008; Zhou *et al.* 2009). The observed decreased abundance of *Azospirillum* species in the sulphate amended incubation may be due to the toxic effect of sulphate on the nitrogenase activity of these bacteria (Rao and Venkateswarlu 1985). Other members of the *Rhodospirillaceae* were also found to be active in acidic soils, fermenting several organic compounds, for example the aerotolerant bacteria *Telmatospirillum siberiense* (Sizova *et al.* 2007).

*Betaproteobacteria* found in equal amounts (3%) in both incubations are of the *Chromobacterium* genus. *Chromobacterium* species were already isolated from forest soil and showed growth on various organic compounds including many sugars like lactose.

They are aero-tolerant, but are producing a violet dye under oxic conditions, presumably to diminish effects of oxygen radicals (Martin *et al.* 2007). Prominent in both incubations too, are species of *Geobacter,* known anaerobic *Deltaproteobacteria* capable of oxidizing acetate, formate and hydrogen with humic substances serving as electron acceptors. These compounds can be found in the investigated area (Coates *et al.* 2002; Cervantes *et al.* 2003; Shrestha *et al.* 2009). *Gammaproteobacteria* of the genus *Hydrogenophaga* were only identified in the control incubation. Members of this genus are chemo-organotrophic or chemolithoautotrophic, gaining their energy via the oxidation of hydrogen and $CO_2$ as a carbon source (Wen *et al.* 1999; Spring *et al.* 2004). There are no reports about sulphate inhibition of these bacteria, but presumably sulphate favours growth of certain species that outcompete *Hydrogenophaga*.

Few clones (5-6%) found in this habitat under both conditions belong to the family *Acidimicrobiaceae*. Members are widespread in acidic soil environments but many of them are reported to be sulphide oxidizers (Okibe and Johnson 2004; Cleaver *et al.* 2007; Jenkins *et al.* 2009). It remains unclear whether the identified members are active in sulphate reduction, due to their equivalent occurrence in both experimental setups.

For a more detailed view into the phylogeny of active sulphate reducers, *dsrAB* libraries of both fractions, already used for 16S rRNA analysis, were constructed with surprising results. Not a single sequence was found which clustered close to already known *Desulfosporosinus dsrAB* gene sequences. Furthermore, all sequences found clustered next to the novel *dsrAB* sequences found previously by Loy and co-workers (Loy *et al.* 2004), except one sequence having high similarity to the *Thermosinus carboxydivorans dsrAB* sequence. This is rather unusual, because studies on this bacterium revealed that it could not grow on sulphate, neither on one of the carbon substrates used. It grows on thiosulphate, ferric iron and several sugars and is capable of utilising carbon monoxide, producing hydrogen, carbon dioxide and acetate (Sokolova *et al.* 2004). Nonetheless, the majority of sequences clustered next to the novel *dsrAB* OTUs 2, 7 and 10 (71.64 – 96.67 % sequence similarity) and no significant differences between fractions with or without sulphate added could be determined. The OTUs 2, 7 and 10 are all from the same lineages described by Loy and colleagues (Loy *et al.* 2004). Except OTU 10 all these sequences were found in the same sampling site as for this study, although soil samples from a bigger depth were analysed (22.5 – 30 cm). Clones related to these sequences were already identified in uranium mill tailing groundwater and Everglades soil (Chang *et al.* 2001; Castro *et al.* 2002). It is suggested, that these novel *dsrAB* OTUs derive from either novel bacterial or archaeal phyla or organisms that are yet not known to be capable of sulphate reduction. This has been confirmed by analysis of their gene sequence, finding conserved catalytic sequence motifs indicating that they are no

pseudo-genes (Loy *et al.* 2004). Still it remains unclear why all lineages identified were also found under non sulphate conditions. This may be a result of sequencing only 51 clones altogether, and rarefaction and coverage calculations showed that further sequencing is necessary for better coverage. Another possibility maybe, that although the bacteria are harbouring the gene for dissimilatory sulphate reduction, they are not actively using the gene as reported for *Pelotomaculum* strain MGP. It is suggested that this strain recently adopted to a syntrophic lifestyle with a hydrogenotrophic methanogen and therefore lost its ability of sulphate reduction (Imachi *et al.* 2006).

The non-emergence of sequences affiliated with *Desulfosporosinus* could have several reasons. First of all, the database for calculating phylogenetic trees only comprised a single sequence from *Desulfosporosinus orientis* that is covered by the primers used. It is very unlikely, but possibly *dsrAB* sequences of the other *Desulfosporosinus* species known, do not cluster close to the variant in the database due to lateral gene transfer, described for SRP (Zverlov *et al.* 2005). Secondly, the *dsrAB* sequence of other *Desulfosporosinus* species are possibly not covered by the primer mix used and these are possibly the dominant species identified with 16S rRNA data. Sequencing and further analysis of the *dsrAB* genes of pure cultures of known *Desulfosporosinus* species could give clearing insights. (Note added: new primers specific for the *Desulfosporosinus/Desulfitobacterium* cluster, developed after this thesis could obtain sequences clustering close to *Desulfosporosinus* out of the examined soil samples.)

# 5 Summary

Despite their importance for carbon mineralization in wetlands, little is known about sulphate-reducing prokaryotes (SRP) in these habitats. In addition, by out-competing methanogenic microorganisms, which are highly active in these environments, they can significantly reduce emission of the greenhouse gas methane.

For this study the Schlöppnerbrunnen, a fen located in the Fichtelgebirge (Germany) at the German-Czech border was chosen. This area has been exposed to acidic rain and sulphur deposition due to intensive soft coal burning in the time of the former socialist regimes. Earlier 16S rRNA gene and dsrAB diversity studies at this site indicated the presence of a community that consists of known SRP species and microorganisms with novel, deep-branching *dsrAB* SRP. In the present study, a differential display DNA stable isotope probing approach was employed to identify the active SRP in the fen. Soil samples from the fen were incubated with $^{13}C$ substrates for up to 6 months with and without sulphate. Subsequently, nucleic acid samples from different time points were separated based on their $^{13}C$ content and these "heavy" nucleic acids were subjected to further downstream analysis by T- RFLP and clone library analysis of 16S rRNA genes and *dsrAB.*

Data obtained from the 2 months samples revealed that members of the genus Desulfosporosinus are the main sulphate reducers in this wetland under the conditions employed. These findings could only be observed based on the data obtained through 16S rRNA clone library analysis. In addition, clone sequences that could be affiliated to the genus Acidocella were also enriched in 13C in the sulphate amended incubation. Although Acidocella species only grow aerobically in pure culture, an anaerobic, sulphate reduction-based syntrophy was recently postulated between a Acidocella strain and a Desulfosporosinus strain in co-culture. A similar lifestyle might be the basis for co-occurrence of the Desulfosporosinus and Acidocella species in the Schlöppnerbrunnen fen. Contrary to 16S rRNA data no dsrAB that was affiliated to Desulfosporosinus was found, but this was discovered to be due to biased PCR primers. Furthermore, dsrAB clone library analysis indicated no distinct activity of the "novel" dsrAB harbouring microorganisms and thus their physiology and identity remains unknown, making further research necessary.

# Zusammenfassung

Neben den vielfältigen ökologischen Funktionen von Feuchtgebieten als Wasserspeicher und Lebensraum einer hochspezialisierten Flora und Fauna gehören sie auch zu den wichtigsten $CO_2$ Speichern der Erde. Sulfatreduzierende Mikroorganismen haben einen großen Anteil an der Metabolisierung von Kohlenstoff und Schwefel in diesen Habitaten, jedoch ist sehr wenig über ihre Physiologie und Aktivität bekannt. Außer ihrer wichtigen Rolle im Schwefel- und Kohlenstoffzyklus, wurde auch beobachtet dass durch ihre Aktivität methanogene Mikroorganismen in ihrem Metabolismus gehemmt werden und dadurch der Ausstoß von Methan, einem klimaschädlichen Treibhausgas aus Moorgebieten stark reduziert wird. Für diese Studie wurde das Schlöppnerbrunnenmoor an der deutsch-tschechischen Grenze gewählt, da in dieses Gebiet bis in die 80er Jahre ein hoher Sulfateintrag durch sauren Regen als Folge von industrieller Braunkohleverbrennung stattgefunden hat. Frühere Studien lassen auf eine diverse Gemeinschaft an sulfatreduzierenden Mikroorganismen in dem untersuchten Moor schließen. Zusätzlich zu bereits bekannten Spezies wurden mithilfe genetischer Analysen der *dsrAB* Markergene, welche für die dissimilatorische (Bi)Sulfitreduktase kodieren, mögliche neue sulfatreduzierende Mikroorganismen entdeckt. Die Identifizierung der aktiven Sulfatreduzierer erfolgte in der vorliegenden Studie mittels ‚DNA-stable isotope probing'. Hierbei wurden zur Markierung der Nukleinsäuren aktiver Mikroorganismen Moorbodenproben bis zu 6 Monate lang mit $^{13}C$ markierten Substraten und Sulfat versetzt. Zu verschiedenen Zeitpunkten wurden Proben der Inkubationen entnommen und durch Ultrazentrifugation markierte von unmarkierten Nukleinsäuren getrennt. Danach wurden die markierten Nukleinsäuren mit Hilfe der Analyse von terminalen Restriktionsfragmenten und Klonbibliotheken der 16S rRNS und der *dsrAB* Gene untersucht. Es konnte nachgewiesen werden dass unter diesen Bedingungen der Großteil des Sulfats von Spezies der Gattung *Desulfosporosinus* reduziert wurde. Zusätzlich wurden Sequenzen der Gattung *Acidocella* entdeckt, einem üblicherweise aerob lebenden Bakterium. Frühere Studien lassen jedoch auf eine anaerobe, syntrophe Lebensweise mit *Desulfosporosinus* schließen. Eine Symbiose, die eventuell ebenfalls die Grundlage des gemeinsamen Auftretens dieser beiden Gattungen im untersuchten Moor darstellt. Auf der Ebene von *dsrAB* konnte überraschenderweise keine *Desulfosporosinus* Sequenz entdeckt werden, was sich jedoch als Artefakt der eingesetzten PCR Primer herausstellte. Sulfatreduzierende Aktivität konnte für die neuen *dsrAB*-tragenden Mikroorgansimen nicht nachgewiesen werden. Somit bleibt die Identität und Physiologie dieser neuen Mikroorganismen weiter unbekannt.

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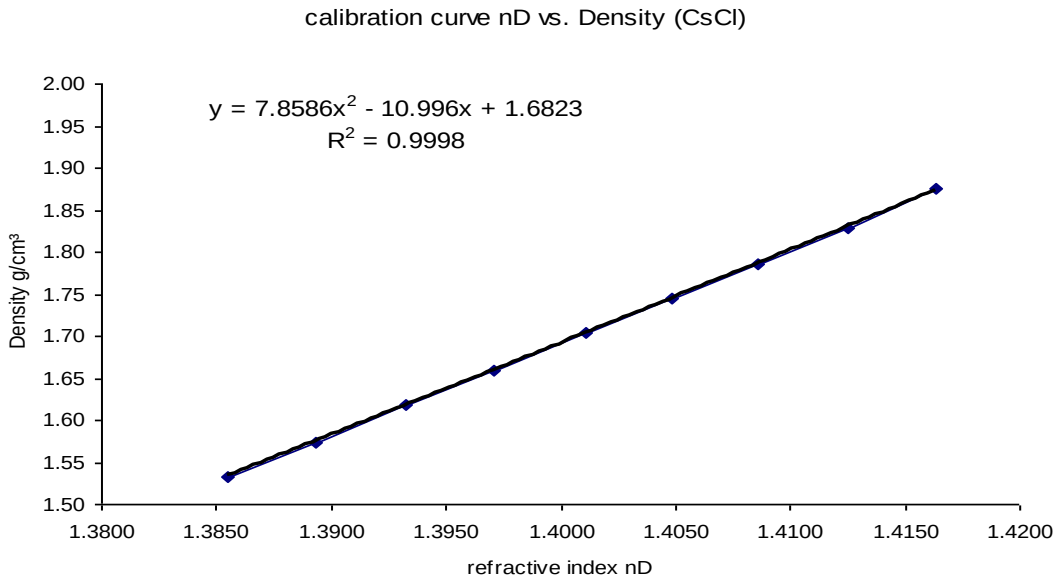
# 7 List of abbreviations

| | |
|---|---|
| % | percent |
| °C | degrees celsius |
| µ | micro |
| 16S rRNA | small subunit of rRNA |
| ARB | software package for phylogenetic analyses |
| ATP | adenosine-5'-triphosphate |
| BLAST | basic local alignment search tool |
| bp | base pair |
| C | carbon |
| cm | centimetre |
| $cm^3$ | cubic centimetre |
| $CO_2$ | carbon dioxide |
| conc. | concentration |
| CsCl | caesium chloride |
| CSTFA | caesium Trifluoroacetate |
| DEPC | diethylpyrocarbonate |
| DNA | desoxyribonuleic acid |
| dNTP | desoxy-nucleotide-tri-phosphate |
| DSR | dissimilatory (bi-)sulphite-reductase |
| *dsrAB* | gene encoding the α- und β- subunit of DSR |
| *E.coli* | *Escherichia coli* |
| $E^0$ | reduction potential |
| EDTA | ethylenediaminetetraacetic acid |
| EtBr | ethidium bromide |
| FAM | fluorescein |
| Fig. | figure |
| FISH | fluorescence *in situ* hybridization |
| g | gram |
| GC | mol% guanine and cytosine |
| h | hours |
| $H_2O$ | water |
| $H_2S$ | hydrogen sulphide |
| kbp | kilo base pairs |
| LB | Luria Bertani |
| M | molar |
| m | milli |

| | |
|---|---|
| $m^2$ | square metre |
| $MgCl_2$ | magnesium chloride |
| min. | minutes |
| mL | millilitre |
| mm | millimetre |
| mV | millivolts |
| N | nitrogen |
| Na | sodium |
| NaCl | sodium chloride |
| NaOH | sodium hydroxide |
| nD | refractive index |
| neg. | negative |
| ng | nanogram |
| nm | nanometre |
| o.n. | over night |
| OTU | operational taxonomic unit |
| p.A. | per analysis |
| PCR | polymerase chain reaction |
| PEG | polyethyleneglycol |
| pH | cologarithm of dissolved hydrogen ions |
| pos. | positive |
| qPCR | quantitative PCR |
| RNA | ribonucleic acid |
| rpm | rounds per minute |
| RT-PCR | reverse transcription PCR |
| S | sulphur |
| sec. | seconds |
| SIP | stable isotope probing |
| $SO_4^{-2}$ | sulphate ion |
| SRP | sulphate reducing prokaryotes |
| Tab. | table |
| TAE | Tris-acetate-EDTA |
| Taq | DNA-polymerase from Thermus aquaticus |
| TBE | Tris-boric acid-EDTA |
| T-RF | terminal restriction fragment |
| T-RFLP | terminal restriction fragment polymorphism |
| Tris | trishydroxymethylaminomethane |
| UV | ultra violet |
| v/v | volume per volume |
| w/v | weight per volume |
| x g | gravitational force |

# 8 Appendix



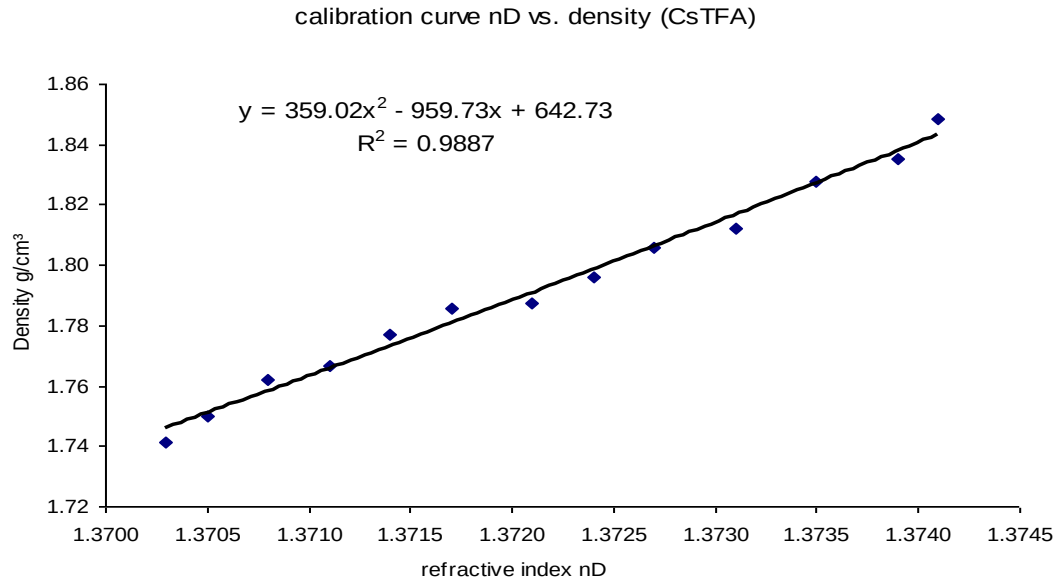
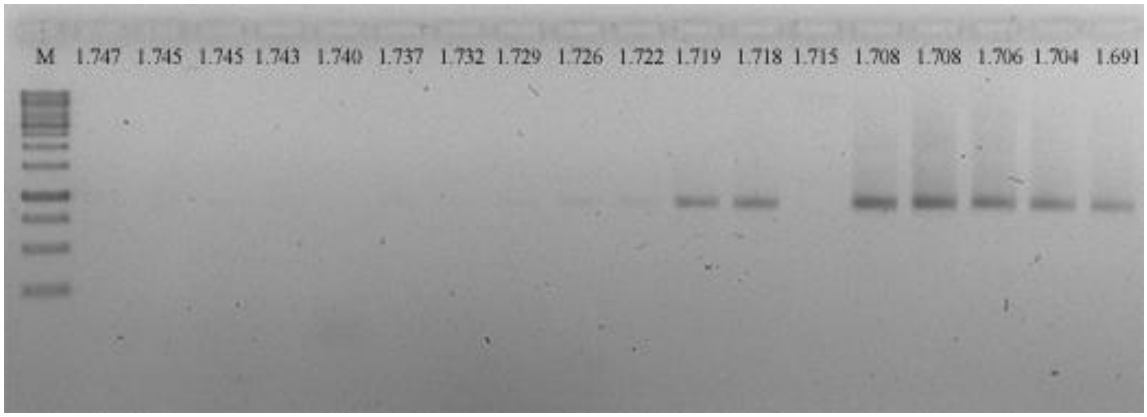
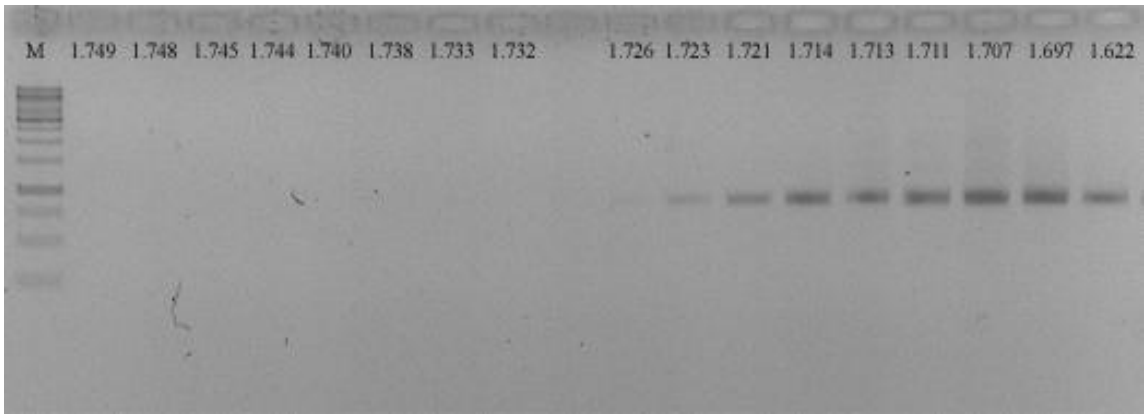




Fig. 25 Calibration curves for calculating densities out of refractive index nD with refractometry from CsCl and CsTFA gradients

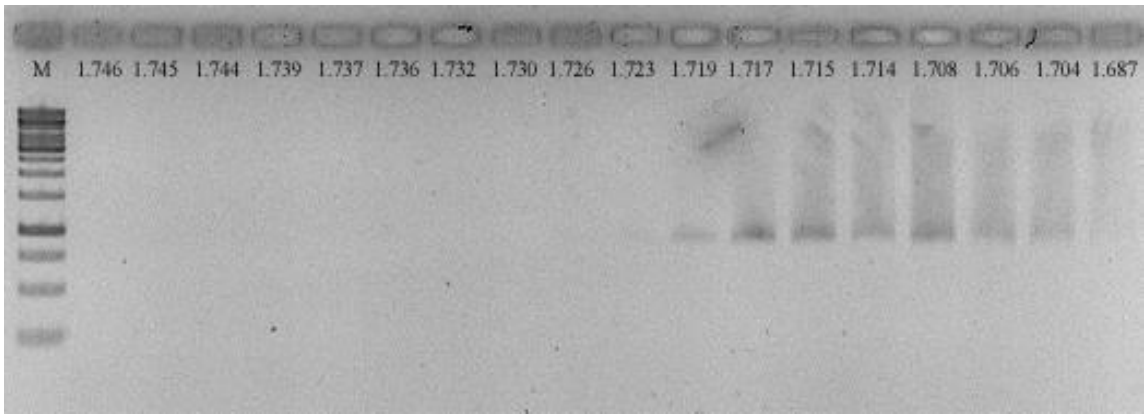
a)


M 1.747 1.745 1.745 1.743 1.740 1.737 1.732 1.729 1.726 1.722 1.719 1.718 1.715 1.708 1.708 1.706 1.704 1.691

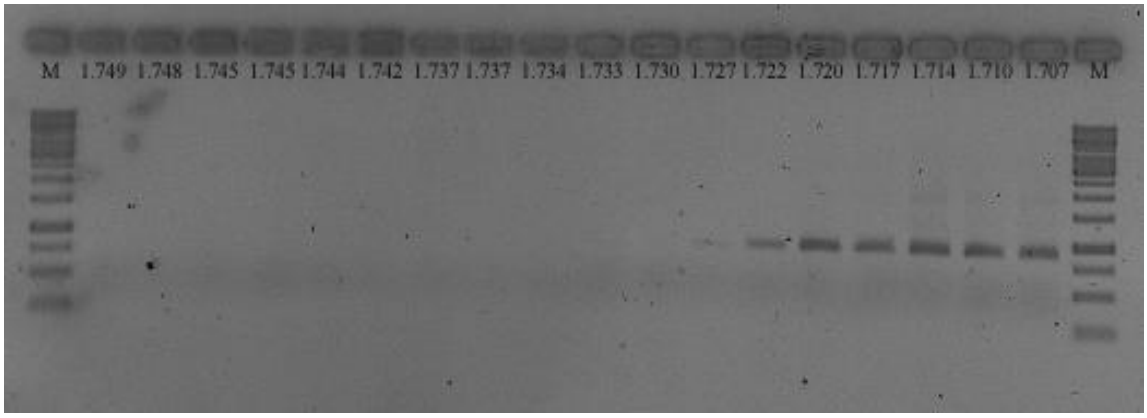
b)


M 1.749 1.748 1.745 1.744 1.740 1.738 1.733 1.732 1.726 1.723 1.721 1.714 1.713 1.711 1.707 1.697 1.622

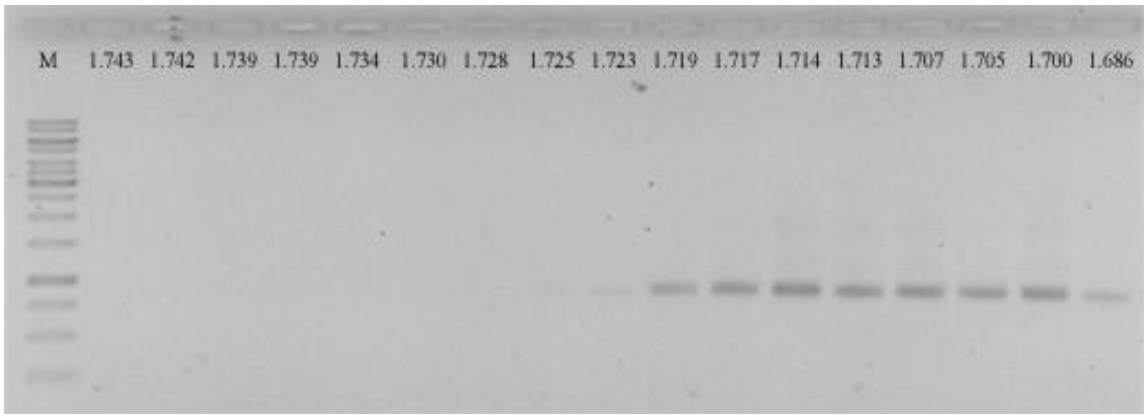
c)


M 1.746 1.745 1.744 1.739 1.737 1.736 1.732 1.730 1.726 1.723 1.719 1.717 1.715 1.714 1.708 1.706 1.704 1.687

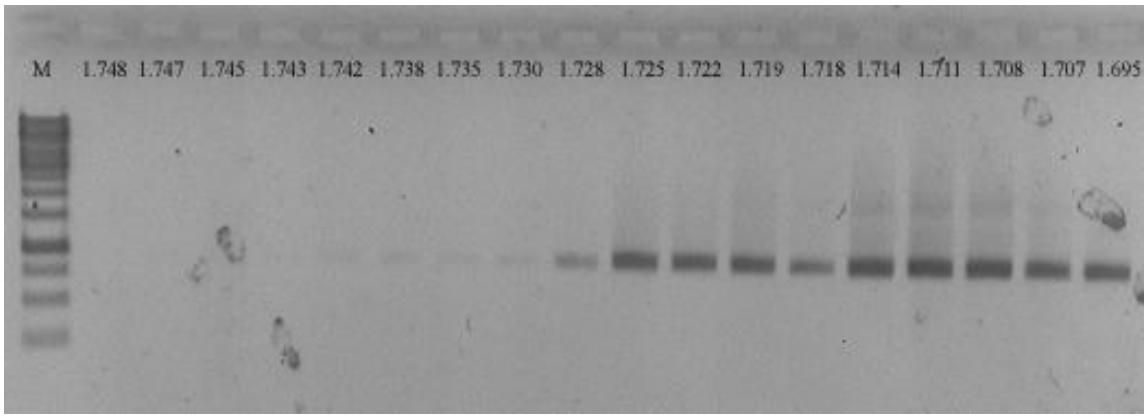
d)


M 1.749 1.748 1.745 1.745 1.744 1.742 1.737 1.737 1.734 1.733 1.730 1.727 1.722 1.720 1.717 1.714 1.710 1.707 M

e)


M 1.743 1.742 1.739 1.739 1.734 1.730 1.728 1.725 1.723 1.719 1.717 1.714 1.713 1.707 1.705 1.700 1.686

f)


M 1.748 1.747 1.745 1.743 1.742 1.738 1.735 1.730 1.728 1.725 1.722 1.719 1.718 1.714 1.711 1.708 1.707 1.695

g)

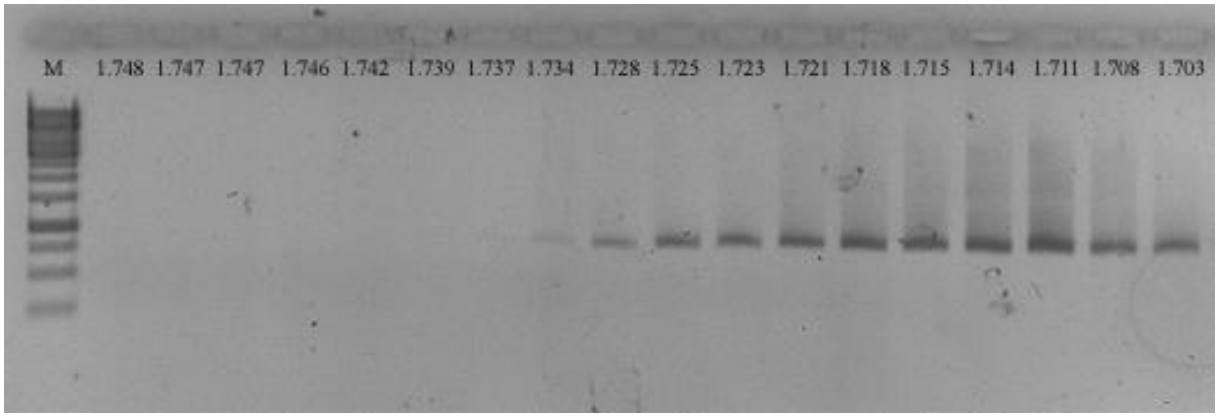



Fig.26 Agarose gel pictures from the amplification of the bacterial 16S rRNA gene out of isopycnic gradient centrifugations from the a) zero control, b) 2 weeks with and c) without sulphate d) 2 months with and e) without sulphate and f) 6 months with and g) without sulphate incubations. Numbers given are densities in g $cm^{-3}$.

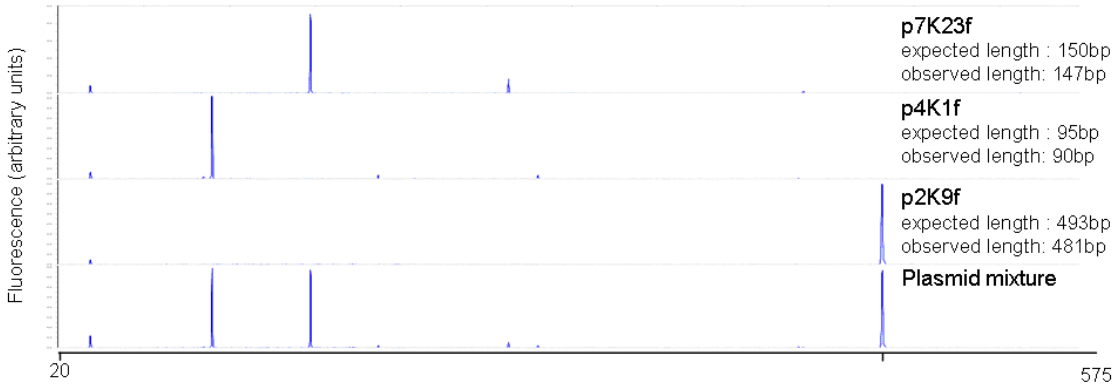



Fig.27 T-RFLP profiles of test plasmids harbouring 16S rRNA sequences digested with restriction enzyme *MspI*

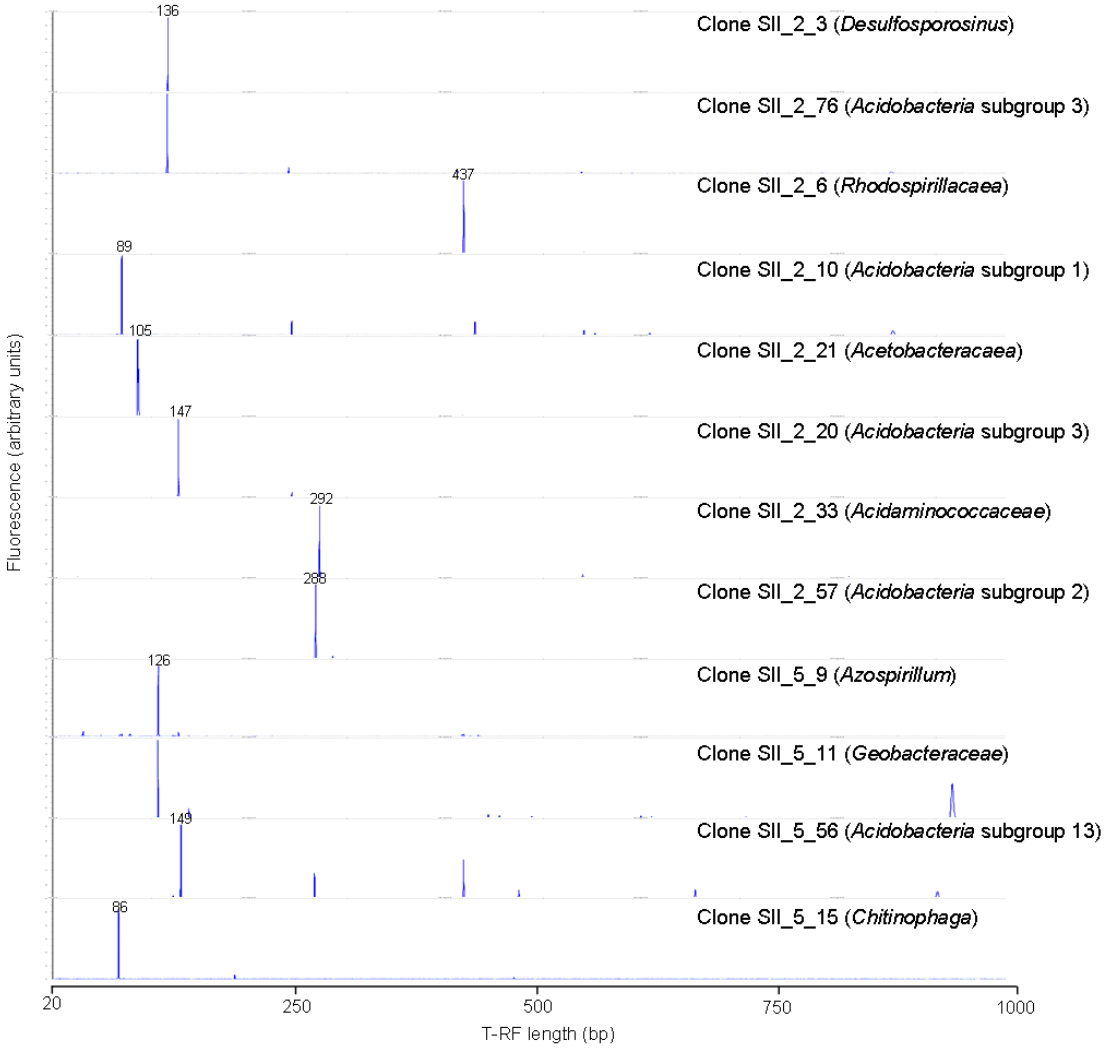



Fig.28 T-RFLP profiles of single clones derived from the 16S rRNA clone library of the 2 months fraction. S_II_2 refers to the sulphate induced incubation, S_II_5 refers to the the control incubation. Numbers given are bp.

Table 17: Phylogenetic affiliations and numbers of bacterial 16S rRNA clones retrieved from 16S rRNA gene-libraries of examined 2 months incubations. T-RF lengths given originate from *in silico* analysis. Corresponding empirically determined T-RF lengths of single clones are shown in italic. If determined, sizes given in the text refer to empiric values.

| | $^{13}$C-substrates + sulfate [a] | | $^{13}$C-substrates only [b] | |
|---|---|---|---|---|
| **Phylogenetic lineage** | **Clones (%)** | **T-RF (bp)** | **Clones (%)** | **T-RF (bp)** |
| *Firmicutes* | | | | |
| *Desulfosporosinus* spp. | 20 | 140 (*136*), 171 | — | — |
| *Acidaminococcaceae* | 6 | 292 (*292*) | 13 | 290, 292 (*292*) |
| *Acidobacteria* | | | | |
| Subgroup 1 | 16 | 94 (*89*), 95, 267 | 21 | 95 (*89*), 97, 265 |
| Subgroup 2 | 7 | 83, 289 (*288*), 499 | 3 | 289 (*288*) |
| Subgroup 3 | 7 | 68,140 (*136)*, 146, | 6 | 140(*136*), 150(*147*), 152, 263 |
| Subgroup 6 | — | 149, 150 | 1 | 292 |
| Subgroup 8 | 1 | 278 | — | — |
| Subgroup 13 | — | — | 1 | 153 |
| *Proteobacteria* | | | | |
| *Alpha-Proteobacteria* | | | | |
| *Acetobacteraceae* | 4 | 110 (*105*) | — | — |
| *Rhodospirillaceae* | 8 | 439 (*437)* | 14 | 439 (*437)* |
| *Azospirillum* spp. | 1 | 445 | 10 | 66, 118, 129(*126*), 130**,** 437 |
| *Rhizobiales* | 2 | 445, 152 | 3 | 118, 160 |
| unclassified | 1 | 67 | 1 | 67 |
| *Beta-Proteobacteria* | | | | |
| *Chromobacterium* | 3 | 81, 430, 494 | 3 | 430, 494, 495 |
| *Gamma-Proteobacteria* | | | | |
| *Nevskia/Hydrogenophaga* | — | — | 3 | 490, 492 |
| *Delta-Proteobacteria* | | | | |
| *Geobacter* spp. | 3 | 130 (*126*), 163 | 5 | 130 (*126*), 161 |
| *Desulfovibrio* spp. | 1 | 505 | — | — |
| *Desulfobulbacaea* | 1 | 164 | — | — |
| uncultured | — | — | 1 | 78 |
| *Actinobacteria* | | | | |
| *Acidimicrobiaceae* | 5 | 70, 82, 139, 170 | 6 | 70, 139, 150 |
| *Acidothermaceae* | 1 | 146 | — | — |
| *Rubrobacteriaceae* | 1 | 142 | — | — |

[a] in total 95 clones; additional clones present in *Nitrospirae*, *Spirochaeta*, *Planctomycetes*, OP10, unclassified
[b] in total 80 clones, additional clones present in *Bacteroidetes*, *Spirochaeta*, *Planctomycetes*, OP10, OD1, *Elusimicrobia*, *Cyanobacteria*

Table 18: All bacterial 16S rRNA gene sequences obtained out of the 2 months incubation clustered into OTUs of 95% sequence similarity. Clones used for calculations of phylogenetic affiliations are in bold print. Affiliations are based on neighbour joining trees calculated in ARB. S_II_2 refers to the sulphate amended incubation, S_II_5 refers to the control incubation.

| OTU name | # of clones | Clones | Phylum | Next Blastn Hit (ACC) | next cultivated representative | Similarity(%) with next cultivated representative |
|---|---|---|---|---|---|---|
| 16S-SIP-5.18 | 6 | SII_5_75,SII_5_17,SII_5_101,SII_5_3, SII_5_36,SII_5_92 | *Acidobacteria* | FJ625318 | *Acidobacteria* bacterium Ellin7137 | 94 |
| 16S-SIP-2.2 | 5 | SII_2_10,SII_2_63,SII_2_105,SII_2_89 SII_2_107 | *Acidobacteria* | FJ625318 | *Acidobacteria* bacterium Ellin7137 | 94 |
| 16S-SIP-2.20 | 5 | SII_2_32,SII_2_87,SII_2_73,SII_2_75, SII_2_40 | *Acidobacteria* | FJ625365 | Candidatus *Koribacter versatilis* | 92 |
| 16S-SIP-5.5 | 4 | SII_5_3,SII_5_55,SII_5_22,SII_5_71 | *Acidobacteria* | FJ625318 | *Acidobacteria* bacterium Ellin7137 | 94 |
| 16S-SIP-2.11 | 3 | SII_2_17,SII_2_56,SII_2_49 | *Acidobacteria* | AB262726 | *Acidobacteria* bacterium Ellin7148 | 88 |
| 16S-SIP-2.14 | 2 | SII_2_20,SII_2_76 | *Acidobacteria* | EF019891 | *Acidobacteria* bacterium KBS 96 | 93 |
| 16S-SIP-2.19 | 2 | SII_2_29,SII_2_82 | *Acidobacteria* | FJ466259 | *Acidobacteria* bacterium Ellin7148 | 94 |
| 16S-SIP-2.23 | 2 | SII_2_37,SII_2_55 | *Acidobacteria* | FJ625310 | Bacterium K-5b2 | 96 |
| 16S-SIP-2.32 | 2 | SII_2_90,SII_2_104 | *Acidobacteria* | FJ466226 | Bacterium Ellin5237 | 96 |
| 16S-SIP-5.7 | 2 | SII_5_6,SII_5_74 | *Acidobacteria* | AM773947 | *Acidobacteria* bacterium Ellin7137 | 92 |
| 16S-SIP-5.17 | 2 | SII_5_31,SII_5_72 | *Acidobacteria* | EU445207 | *Solibacter usitatus* Ellin6076 | 95 |
| 16S-SIP-5.19 | 2 | SII_5_40,SII_5_80 | *Acidobacteria* | FJ625310 | Bacterium K-5b2 | 96 |
| 16S-SIP-5.20 | 2 | SII_5_29,SII_5_83 | *Acidobacteria* | EU360064 | Bacterium K-5b2 | 93 |
| 16S-SIP-2.3 | 1 | SII_2_11 | *Acidobacteria* | AM162430 | *H.foetida* strain TMBS4-T | 91 |
| 16S-SIP-2.22 | 1 | SII_2_34 | *Acidobacteria* | EF492962 | *Solibacter usitatus* Ellin6076 | 94 |
| 16S-SIP-2.25 | 1 | SII_2_43 | *Acidobacteria* | AJ292582 | *Solibacter usitatus* Ellin6076 | 92 |
| 16S-SIP-2.31 | 1 | SII_2_103 | *Acidobacteria* | AM162428 | *Acidobacteria* bacterium Ellin7184 | 94 |
| 16S-SIP-2.33 | 1 | SII_2_106 | *Acidobacteria* | FJ625320 | *Acidobacteria* bacterium KBS 96 | 94 |
| 16S-SIP-2.38 | 1 | SII_2_38 | *Acidobacteria* | EF019053 | *Solibacter usitatus* Ellin6076 | 93 |
| 16S-SIP-2.39 | 1 | SII_2_57 | *Acidobacteria* | FJ624912 | *Acidobacteria* bacterium Ellin7184 | 90 |
| 16S-SIP-2.42 | 1 | SII_2_47 | *Acidobacteria* | EU399672 | *Acidobacteria* bacterium KBS 96 | 92 |
| 16S-SIP-2.37 | 1 | SII_2_91 | *Acidobacteria* | AM773947 | *Acidobacteria* bacterium Ellin7137 | 92 |

| OTU name | # of clones | Clones | Phylum | Next Blastn Hit (ACC) | next cultivated representative | Similarity(%) with next cultivated representative |
|---|---|---|---|---|---|---|
| 16S-SIP-5.1 | 1 | SII_5_1 | *Acidobacteria* | EU335384 | *H.foetida* strain TMBS4-T | 82 |
| 16S-SIP-5.6 | 1 | SII_5_5 | *Acidobacteria* | EF019342 | *Solibacter usitatus* Ellin6076 | 94 |
| 16S-SIP-5.12 | 1 | SII_5_56 | *Acidobacteria* | AB262727 | Bacterium Ellin5227 | 85 |
| 16S-SIP-5.16 | 1 | SII_5_70 | *Acidobacteria* | EF073672 | *Acidobacteria* bacterium KBS 83 | 94 |
| 16S-SIP-5.23 | 1 | SII_5_87 | *Acidobacteria* | EU680438 | *Acidobacteria* bacterium Ellin7184 | 90 |
| 16S-SIP-5.25 | 1 | SII_5_34 | *Acidobacteria* | FJ624909 | *Acidobacteria* bacterium Ellin7184 | 88 |
| 16S-SIP-5.27 | 1 | SII_5_105 | *Acidobacteria* | AM162423 | *Solibacter usitatus* Ellin6076 | 92 |
| 16S-SIP-5.28 | 1 | SII_5_107 | *Acidobacteria* | DQ453805 | *Acidobacteria* bacterium KBS 96 | 92 |
| 16S-SIP-5.33 | 1 | SII_5_17 | *Acidobacteria* | FJ625318 | *Acidobacteria* bacterium Ellin7137 | 94 |
| 16S-SIP-5.36 | 1 | SII_5_33 | *Acidobacteria* | EU150216 | *Acidobacteriaceae* bacterium Gsoil 969 | 95 |
| 16S-SIP-5.11 | 3 | SII_5_57,SII_5_72,SII_5_108 | *Actinobacteria* | EU360030 | *Actinobacterium* BGR 43 | 92 |
| 16S-SIP-2.12 | 2 | SII_2_18,SII_2_80 | *Actinobacteria* | AM162469 | *Actinobacterium* BGR 43 | 95 |
| 16S-SIP-2.10 | 1 | SII_2_7 | *Actinobacteria* | EU881209 | *Acidothermus cellulolyticus* | 88 |
| 16S-SIP-2.16 | 1 | SII_2_24 | *Actinobacteria* | FJ475379 | *Solirubrobacter* sp. BXN5-15 | 93 |
| 16S-SIP-2.26 | 1 | SII_2_69 | *Actinobacteria* | EU360020 | *Actinobacterium* BGR 43 | 92 |
| 16S-SIP-2.35 | 1 | SII_2_85 | *Actinobacteria* | EU360030 | *Actinobacterium* BGR 43 | 92 |
| 16S-SIP-2.41 | 1 | SII_2_101 | *Actinobacteria* | EF446258 | *Actinobacterium* BGR 43 | 92 |
| 16S-SIP-5.26 | 1 | SII_5_102 | *Actinobacteria* | EU044043 | *Actinobacterium* YJF2-33 | 92 |
| 16S-SIP-5.37 | 1 | SII_5_53 | *Actinobacteria* | DQ906070 | *Actinobacterium* BGR 43 | 94 |
| 16S-SIP-5.2 | 11 | SII_5_10,SII_5_77,SII_5_14,SII_5_91, SII_5_20,SII_5_78,SII_5_8,SII_5_27, SII_5_59,SII_5_89,SII_5_21 | *Alphaproteobacteria* | AF524861 | Bacterium K-5b5 | 100 |
| 16S-SIP-2.8 | 8 | SII_2_6,SII_2_65,SII_2_54,SII_2_8, SII_2_51,SII_2_36,SII_2_42 | *Alphaproteobacteria* | DQ094180 | *Telmatospirillum siberiense* | 100 |
| 16S-SIP-5.8 | 6 | SII_5_7,SII_5_79,SII_5_106,SII_5_9, SII_5_47,SII_5_26 | *Alphaproteobacteria* | DQ660857 | *Azospirillum* sp. S07 | 95 |
| 16S-SIP-2.6 | 4 | SII_2_16,SII_2_93,SII_2_96,SII_2_21 | *Alphaproteobacteria* | DQ906080 | *Acidocella aluminiidurans* | 97 |
| 16S-SIP-2.1 | 1 | SII_2_1 | *Alphaproteobacteria* | FM252034 | *Beijerinckiaceae* bacterium BW863 | 100 |

| OTU name | # of clones | Clones | Phylum | Next Blastn Hit (ACC) | next cultivated representative | Similarity(%) with next cultivated representative |
|---|---|---|---|---|---|---|
| 16S-SIP-2.15 | 1 | SII_2_23 | *Alphaproteobacteria* | EF196942 | *alphaproteobacterium* endosymbiont 1b of *Inanidrilus makropetalos* | 91 |
| 16S-SIP-2.28 | 1 | SII_2_77 | *Alphaproteobacteria* | EU266918 | *Pedomicrobium manganicum* | 96 |
| 16S-SIP-2.40 | 1 | SII_2_68 | *Alphaproteobacteria* | EU491294 | *alphaproteobacterium* endosymbiont 1b of *Inanidrilus makropetalos* | 91 |
| 16S-SIP-5.4 | 1 | SII_5_2 | *Alphaproteobacteria* | EF494345 | Bacterium Ellin6089 | 95 |
| 16S-SIP-5.14 | 1 | SII_5_61 | *Alphaproteobacteria* | AM773974 | Bacterium Ellin5299 | 92 |
| 16S-SIP-5.29 | 1 | SII_5_111 | *Alphaproteobacteria* | EU491294 | *alphaproteobacterium* endosymbiont 1b of *Inanidrilus makropetalos* | 91 |
| 16S-SIP-5.31 | 1 | SII_5_96 | *Alphaproteobacteria* | EU881204 | *Phyllobacteriaceae* bacterium AMV1 | 91 |
| 16S-SIP-5.38 | 1 | SII_5_38 | *Alphaproteobacteria* | AM162440 | *Methylocystis* sp. LW5 | 94 |
| 16S-SIP-2.13 | 3 | SII_2_19,SII_2_25,SII_2_74 | *Betaproteobacteria* | AM396358 | *Paludibacterium yongneupense* | 100 |
| 16S-SIP-5.21 | 3 | SII_5_110,SII_5_19,SII_5_85 | *Betaproteobacteria* | AM396358 | *Paludibacterium yongneupense* | 100 |
| 16S-SIP-5.3 | 4 | SII_5_11,SII_5_37,SII_5_67,SII_5_49 | *Deltaproteobacteria* | FJ479238 | *Geobacter bemidjiensis* | 97 |
| 16S-SIP-2.7 | 1 | SII_2_2 | *Deltaproteobacteria* | AY921969 | *Geobacter* sp. Ply1 | 97 |
| 16S-SIP-2.17 | 1 | SII_2_27 | *Deltaproteobacteria* | AY607219 | *Geobacter bemidjiensis* | 97 |
| 16S-SIP-2.27 | 1 | SII_2_70 | *Deltaproteobacteria* | DQ205193 | *Desulfovibrio putealis* strain B7-43 | 97 |
| 16S-SIP-2.30 | 1 | SII_2_67 | *Deltaproteobacteria* | EU542434 | Sulfate-reducing bacterium STP23 | 97 |
| 16S-SIP-2.43 | 1 | SII_2_71 | *Deltaproteobacteria* | AY922036 | *Pelobacter propionicus* | 97 |
| 16S-SIP-5.22 | 1 | SII_5_86 | *Deltaproteobacteria* | EU335338 | *Geobacter* sp. FRC-32 | 84 |
| 16S-SIP-2.4 | 19 | SII_2_12,SII_2_48,SII_2_52,SII_2_35, SII_2_41,SII_2_30,SII_2_94,SII_2_92, SII_2_46,SII_2_4,SII_2_45,SII_2_95 SII_2_3,SII_2_88,SII_2_5,SII_2_84, SII_2_13,SII_2_15,SII_2_22,SII_2_28 SII_5_43,SII_5_68,SII_5_84,SII_5_28, | *Firmicutes* | EU981221 | *Desulfosporosinus* sp. 44a-T3a | 98 |
| 16S-SIP-5.10 | 9 | SII_5_12,SII_5_44,SII_5_76,SII_5_81, SII_5_54 | *Firmicutes* | AB486409 | *Psychrosinus fermentans* strain FCF9 | 96 |
| 16S-SIP-2.21 | 6 | SII_2_33,SII_2_44,SII_2_26,SII_2_50, SII_2_64 | *Firmicutes* | AM159306 | *Pelosinus* sp. UFO1 | 97 |
| 16S-SIP-5.34 | 1 | SII_5_18 | *Firmicutes* | AB486393 | Low G+C Gram-positive bacterium TR1 | 99 |
| 16S-SIP-5.9 | 2 | SII_5_39,SII_5_64 | *Gammaproteobacteria* | FJ625378 | *Gammaproteobacterium* CH43 | 94 |
| 16S-SIP-2.5 | 1 | SII_2_14 | *Nitrospirae* | EF492940 | *Stigmatella aurantiaca* | 95 |

| OTU name | # of clones | Clones | Phylum | Next Blastn Hit (ACC) | next cultivated representative | Similarity(%) with next cultivated representative |
|---|---|---|---|---|---|---|
| 16S-SIP-2.9 | 1 | SII_2_9 | *Nitrospirae* | EF464628 | *Thermodesulfovibrio yellowstonii* | 88 |
| 16S-SIP-2.18 | 1 | SII_2_28 | *Nitrospirae* | FM956249 | *Magnetobacterium bavaricum* | 87 |
| 16S-SIP-5.15 | 1 | SII_5_63 | OD1 | EF516850 | *Oscillatoriales cyanobacterium* UVFP2 | 75 |
| 16S-SIP-2.29 | 2 | SII_2_61,SII_2_62 | OP10 | EU266864 | *Thermincola carboxydiphila* strain 2204 | 82 |
| 16S-SIP-5.24 | 1 | SII_5_32 | OP10 | EF075557 | Candidate division OP10 bacterium Gsoil | 91 |
| 16S-SIP-5.32 | 1 | SII_5_13 | *Planctomyces* | AY963300 | *Planctomycetales* bacterium Ellin7244 | 92 |
| 16S-SIP-2.34 | 1 | SII_2_83 | *Planctomycetes* | EF075416 | *Planctomycetacia* bacterium WSF3-27 | 92 |
| 16S-SIP-2.36 | 1 | SII_2_86 | *Spirochaeta* | AB234282 | *Spirochaeta* sp. TM3 | 97 |
| 16S-SIP-5.13 | 1 | SII_5_60 | *Spirochaeta* | AB234282 | *Spirochaeta* sp. TM3 | 97 |
| 16S-SIP-5.30 | 1 | SII_5_95 | TG1 | AB280391 | *Actinobacterium* YJF1-30 | 80 |
| 16S-SIP-5.35 | 1 | SII_5_25 | uncultured | DQ223200 | *Gloeobacter violaceus* PCC 7421 | 84 |
| 16S-SIP-2.24 | 2 | SII_2_39,SII_2_72 | uncultured | EF018887 | *Thermoanaerobacter tengcongensis* | 85 |

Table 18. All *dsrAB* sequences obtained out of the 2 months incubation clustered into OTUs of 90% amino acid sequence similarity. Clones used for calculations of phylogenetic affiliations are in bold print. Affiliations are based on neighbour joining trees calculated in ARB. dsrS_II_2 refers to the sulphate amended incubation, dsrS_II_5 refers to the control incubation.

| OTU name | # of clones | Clones | Next relative | Habitat | Next Blastn Hit |
|---|---|---|---|---|---|
| dsrSIP 1 | 2 | dsrSII_2_6,**dsrSII_2_15** | Uncultured sulfate-reducing bacterium clone OTU-20 | anoxic paddy soil | FJ472883 |
| dsrSIP 2 | 3 | dsrSII_2_9,**dsrSII_2_52**,dsrSII_2_58 | Uncultured sulfate-reducing bacterium clone W17 | metalliferous organic soil | DQ855255 |
| dsrSIP 3 | 8 | dsrSII_2_27,dsrSII_2_28,dsrSII_5_9,dsrSII_2_38**dsrSII_2_91**,dsrSII_5_4,dsrSII_5_25,dsrSII_5_15 | Uncultured prokaryote clone dsrSbII-3 | Schlöppnerbrunnen fen soil | AY167467 |
| dsrSIP 4 | 11 | **dsrSII_2_35**,dsrSII_2_45,dsrSII_5_13, dsrSII_2_47,dsrSII_2_61,dsrSII_2_93, dsrSII_5_45,dsrSII_2_89,dsrSII_5_21, dsrSII_5_53,dsrSII_5_10 | Uncultured sulfate-reducing bacterium clone W3 | metalliferous organic soil | DQ855249 |
| dsrSIP 5 | 2 | **dsrSII_2_49**,dsrSII_5_17 | Uncultured prokaryote clone dsrSbII-3 | Schlöppnerbrunnen fen soil | AY167467 |
| dsrSIP 6 | 2 | dsrSII_2_50,**dsrSII_2_101** | Uncultured sulfate-reducing bacterium clone W3 | metalliferous organic soil | DQ855249 |
| dsrSIP 7 | 2 | **dsrSII_2_54**,dsrSII_5_16 | Uncultured sulfate-reducing bacterium clone W3 | metalliferous organic soil | DQ855249 |
| dsrSIP 8 | 1 | **dsrSII_2_57** | Uncultured sulfate-reducing bacterium clone W3 | metalliferous organic soil | DQ855249 |
| dsrSIP 9 | 2 | **dsrSII_2_71**,dsrSII_5_30 | Uncultured prokaryote clone dsrSbII-3 | Schlöppnerbrunnen fen soil | AY167467 |
| dsrSIP 10 | 1 | **dsrSII_2_73** | Uncultured sulfate-reducing bacterium clone W6 | metalliferous organic soil | DQ855250 |
| dsrSIP 11 | 2 | **dsrSII_2_79**,dsrSII_2_80 | Uncultured sulfate-reducing bacterium clone W3 | metalliferous organic soil | DQ855249 |
| dsrSIP 12 | 1 | **dsrSII_2_88** | Uncultured sulfate-reducing bacterium clone DSRIV-4 | Aarhus bay sediment | FM179973 |
| dsrSIP 13 | 6 | **dsrSII_2_92**,dsrSII_5_8,dsrSII_5_28,dsrSII_5_40dsrSII_5_46,dsrSII_5_26 | Uncultured sulfate-reducing bacterium clone W3 | metalliferous organic soil | DQ855249 |
| dsrSIP 14 | 1 | **dsrSII_5_3** | Uncultured prokaryote clone dsrSbII-34 | Schlöppnerbrunnen fen soil | AY167468 |
| dsrSIP 15 | 1 | **dsrSII_5_14** | Uncultured sulfate-reducing bacterium clone OTU-20 | anoxic paddy soil | FJ472883 |
| dsrSIP 16 | 1 | **dsrSII_5_47** | Uncultured sulfate-reducing bacterium W3 | metalliferous organic soil | DQ855249 |

# 9 Curriculum Vitae

| | |
|---|---|
| May 10, 1982 | Geboren in Hainburg/Donau, Österreich |
| Juni 2000 | Matura am Bundesrealgymnasium Bruck/Leitha, Österreich |
| Oktober, 2000 – Oktober 2001 | Studium der Lebensmittel- und Biotechnologie an der Universität für Bodenkultur Wien, Österreich |
| Oktober 2001 – Februar 2010 | Studium der Molekularen Biologie an der Universität Wien, Österreich |

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My family for supporting me in many aspects throughout my whole university time.

And of course all my friends for listening to my scientific stories, although not understanding a lot of the vocabulary I used.

# Abgrenzung der eigenen Arbeitsleistung

Sämtliche Arbeiten dieser Diplomarbeit wurden eigenständig erbracht, mit Ausnahme der Probenvorbereitung, Alignment der 16S rRNA und *dsrAB* Sequenzen, Mini- bzw. Kochpräp der *E. coli* Klone, sowie der Berechnung der phylogenetischen Stammbäume. Diese Arbeitsschritte wurden unter Mithilfe von Dr. Michael Pester (Department of Microbial Ecology, Universität Wien) durchgeführt.

Wien, Dezember 2009 (Bittner Norbert)