ENRICHMENT OF ABUNDANT SULFATE REDUCERS FROM TIDAL SEDIMENTS

Master Thesis

A thesis submitted to the International Max Planck Research School for Marine Microbiology and the University of Bremen in partial fulfillment of the requirements to receive the degree Master of Science.

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15th March 2013
This thesis has been conducted within the MarMic Program at the Max Planck Institute for Marine Microbiology in Bremen, Department of Molecular Ecology of Prof. Dr. R. Amann in the period between September 2012 and March 2013.

1st Reviewer Dr. Marc Mußmann
2nd Reviewer Dr. Katrin Knittel
Erklärung

Statement

Hiermit versichere ich, dass ich diese Arbeit selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

I herewith confirm that I have written this thesis unaided and that I used no other resources than those mentioned.

(Ort und Datum / Place and Date) (Unterschrift / Signature)
Acknowledgements
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Abstract

Up to 50% of carbon remineralization in marine sediments is mediated by the sulfate-reducing bacterial community. Bacteria from the *Desulfosarcina-Desulfococcus* group that are related to the DPB1 endosymbiont of *Olavius* spp. (Sva0081-SRB) occur worldwide at all depths and account for up to 10% of all cells in sulfidic conditions. However, the physiology of this ecologically important group remains unknown. The complexity of the microbial community in sediments challenges traditional isolation and metagenomics. No Sva0081-SRB bacteria have been cultivated, nor are there available genomes for close relatives, which makes metagenome assembly more difficult. We present an alternative approach that combines culture-dependent and independent methods to study Sva0081-SRB. We developed a low substrate concentration continuous culture that allowed us to maintain the Sva0081-SRB group for more than 69 days in the laboratory. This culturing method allows physiological experiments of metabolically active cells at defined conditions in the laboratory. We specifically sorted cells through fluorescence-activated cell sorting (FACS) of the CARD-FISH hybridized Sva0081-SRB community. Subsequently, whole genome amplification through multiple displacement amplification (MDA) allowed access to population derived metagenomic DNA. The PCR based screening for the adenosin-5'-phosphosulfate reductase subunit A indicated successful amplification of CARD-FISH treated and FACS sorted cells. Future sequence-based studies can reveal a potential core-genome of the population. We can directly verify it by testing the hypothesis obtained through sequence analysis with culture based physiological experiments. This combination of the strong characteristics of both approaches allows further investigation of the ecological relevance of this highly abundant group.
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<th>Definition</th>
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<tbody>
<tr>
<td>BLASTn</td>
<td>Basic Local Alignment Search Tool: nucleotide sequence against nucleotide sequences</td>
</tr>
<tr>
<td>BLASTx</td>
<td>Basic Local Alignment Search Tool: nucleotide sequence against amino acid sequences</td>
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<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>CARD FISH</td>
<td>catalyzed reporter deposition fluorescence <em>in situ</em> hybridization</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
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<td>DPB</td>
<td><em>Deltaproteobacteria</em></td>
</tr>
<tr>
<td>DSS</td>
<td><em>Desulfosarcina-Desulfococcus</em></td>
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<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
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<tr>
<td>HB</td>
<td>hybridization buffer</td>
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<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
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<tr>
<td>MDA</td>
<td>multiple displacement amplification</td>
</tr>
<tr>
<td>MilliQ</td>
<td>demineralized water in Millipore Quality</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PFA</td>
<td>perfluoralkoxy</td>
</tr>
<tr>
<td>psu</td>
<td>practical salinity units</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
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<tr>
<td>rRNA</td>
<td>ribosomal RNA</td>
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<tr>
<td>RT</td>
<td>room temperature</td>
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<tr>
<td>spp.</td>
<td>subspecies</td>
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<tr>
<td>SRB</td>
<td>sulfate-reducing bacteria</td>
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<tr>
<td>ss/ds/g/DNA</td>
<td>single stranded/double stranded/ genomic DNA</td>
</tr>
<tr>
<td>VFA</td>
<td>volatile fatty acids</td>
</tr>
<tr>
<td>WB</td>
<td>washing buffer</td>
</tr>
<tr>
<td>WGA</td>
<td>whole genome amplification</td>
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1. Introduction

Marine sediments play a major role in global element cycles. Although organic matter produced by photosynthesis is mainly degraded aerobically in the water column, in ocean realms with high nutrient supply and consequently high primary productivity, great amounts of organic matter escape aerobic remineralization in the water column and accumulate on the ocean floor. In example, it was reported that up to 50% of the biomass can reach the sediment surface in tidal flats (Wollast, 1991). There, the benthic microbial community mediates the subsequent remineralization. By that, the sediment and its biotic community supply nutrients to the ecosystem thus closing the food-web-cycle.

Degradation of organic matter follows a combination of microbial aerobic and anaerobic physiologies. After extracellular depolymerization, the fermenting community further degrades organic substrates. Typical products of these process are low-mass molecular compounds such as acetate, propionate, formate and lactic acid as described for the study site ‘Janssand’ (Gittel et al., 2008; Lenk, 2011). Complete oxidation of these substances is mediated by the anaerobic chemotrophic community, since sediments with high input of organic matter experience a depletion of oxygen within milli- to centimeters.. Consequently, all respiration processes are dependent on remaining terminal electron acceptors such as nitrate, manganese oxides, iron oxides, sulfate and carbon dioxide. The order of depletion in the sediment over depth corresponds roughly to their redox potential (Table 1). Oxygen depletion is followed by depletion of nitrate, manganese, iron and sulfate.

<table>
<thead>
<tr>
<th>Redox couple</th>
<th>Reduction potential (E0(^{'})) [V]</th>
</tr>
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<tbody>
<tr>
<td>O(_2)/H(_2)O</td>
<td>+0.820</td>
</tr>
<tr>
<td>Mn(^4+)/Mn(^2+)</td>
<td>+0.798</td>
</tr>
<tr>
<td>*Fe(^3+)/Fe(^2+)</td>
<td>+0.0770</td>
</tr>
<tr>
<td>NO(_3^{-})/NO(_2^{-})</td>
<td>+0.0480</td>
</tr>
<tr>
<td>S(_{O_4}^{2-})/HSO(_3^{-})</td>
<td>-0.520</td>
</tr>
</tbody>
</table>

The reduction of sulfate is the most important anaerobic form of respiration in marine sediments, since the high concentration of sulfate in sea water (28 mM) exceeds oxygen,
nitrate, manganese and iron concentrations by at least two orders of magnitude. In the study site "Janssand" it is reported to occur up to 2-3 m depth (Gittel et al., 2008). Calculations described that dissimilatory sulfate reduction accounts for up to 55% of total remineralization of organic carbon in marine sediments (Canfield et al., 2005).

Sulfur species have a wide spectrum of oxidation states. Sulfur in form of sulfate has the oxidation state +VI and is the most oxidized sulfur species. Hydrogen sulfide (H₂S), the most reduced species, has an oxidation state of −II. Several sulfur species exist in between that can serve either as electron acceptor or as a donor. Highly reduced species such as H₂S additionally interact abiotically with metals such as iron (Canfield et al., 2005). Therefore, sulfate reduction directly affects not only the biology but also the geochemistry of sediments.

Sulfate reduction is a widely distributed form of anaerobic respiration which is performed by a total of six prokaryotic lineages (Muyzer & Stams, 2008) – *Deltaproteobacteria* being the most relevant for marine sediments. Cultivation-independent methods revealed the dominant presence of distinct groups of *Deltaproteobacteria* in marine sediments. Clone libraries of marine sediment samples often contain 16S rRNA gene sequences affiliated with the families *Desulfobulbaceae* and *Desulfobacteraceae* (Llobet-Brossa et al., 2002; Musat et al., 2006; Mußmann et al., 2005; Ravenschlag et al., 2000). However, the in situ quantification via fluorescence in situ hybridization (FISH) and catalyzed reporter deposition-fluorescence in situ hybridization (CARD-FISH) showed the numerically abundance of the *Desulosarcina-Desulfococcus*-Group (DSS-group), a subgroup of the *Desulfobacteracea* (Llobet-Brossa et al., 2002; Musat et al., 2006; Mußmann et al., 2005). The DSS-group comprises related taxonomic groups of the genera *Desulosarcina* and *Desulfococcus* that depict a sequence divergence on the 16S rRNA gene level of up to 18% (Aeckersberg et al., 1991; Harms et al., 1999; Widdel & Bak, 1992).

Members of the DSS-group also participate in a symbiosis discovered a decade ago (Dubilier et al., 2001). This symbiosis includes the mouth and gutless oligochaete *Olavius* spp., two aerobic *Gammaproteobacteria* (γ), two anaerobic sulfate reducing *Deltaproteobacteria* (δ) and a spirochete of unknown involvement. Comparative phylogenetic analysis of the 16S rRNA gene revealed a distinct cluster of DSS-members that share high identities with the δ1 symbiont of the *Olavius* spp. Sequences obtained from the study site Janssand are particular closely related with identity values >98% (Figure 1). The group, named Sva0081 after a
sequence obtained from the Svalbard area (Ravenschlag et al., 2000), is highly abundant in sulfidic sediments of the German Bight. Ovanesov (2012) quantified the in situ abundance and accounted up to 10% of all inspected cells (DAPI total DNA stain) to the sulfate reducing bacteria of the Sva0081-group (Sva0081-SRB). The 16S rRNA gene amplification 454-pyrotag data from a >4000 km transect along the European Atlantic coast further supports its dominating abundance of SRB. The group accounted for 20-50% of all sequences that affiliated with SRB (Mußmann, personal communication).

Figure 1: 16S rRNA gene tree (Maximum Parsimony). Red sequences: obtained single cell genomes from study site Janssand. Blue: 16S rRNA gene sequence of the deltaproteobacterial endosymbiont 1 of Olavius spp. Grey shaded area: The Sva0081-SRB group in Janssand sediments. Scale bar represents 10% sequence difference. Figure courtesy: Mußmann, unpublished.
Most data regarding the Sva0081-SRB is derived from culture independent methods, as no member was cultured yet. In fact, only approximately 2.5% of all benthic marine bacteria can be cultured using standard media and conditions (Bruns et al., 2002). Alternatively to isolation is the enrichment, that supplies conditions and substrates to selectively favor the growth of a distinct physiology (Overmann et al., 2013).

Generally, isolation and enrichments of sulfate reducers are accomplished through batch cultures with high substrate concentrations as this allows high cultivation throughout and requires little maintenance (Gittel et al., 2008; Köpke et al., 2005; Llobet-Brossa et al., 2002; Müßmann et al., 2005). Unfortunately, this enrichments rarely represent the in situ key players, as these approaches usually enrich the ‘r-strategist’ that have high growth rates and low substrate affinity (Overmann et al., 2013). However, in situ conditions are mostly oligotrophic and therefore represent a community of ‘K-strategists’ with high substrate affinity and low growth rates (Watve et al., 2000).

To enrich the ‘K-strategist’, culture conditions should mimic in situ conditions. Studies that applied solely filtrated sea water have increased cultivability of marine microorganisms to 20-30% (Button et al., 1993). Low substrate concentrations of amended sea water are usually achieved through applications of continuous cultures (Girgis et al., 2005). A continuous culture is a flow system in which the same volume of medium is added, which is then removed through the effluent. Consequently, the volume in the culture vessels remains stable. Additionally to low substrate concentrations, a continuous culture allows the removal of metabolic waste products and motile organisms.

However, enrichment still requires selective growth conditions. An enrichment culture is defined as “a technique (…) to enable one particular type of microorganism to grow faster than all others” (Overmann et al., 2013). During the enrichment process that targets a specific group of organisms within a complex microbial community, the sample is “ (…) kept under conditions which favor the growth of a particular physiological type (…)”(Overmann et al., 2013). Additionally to specific physiology promoting substrates, such approaches usually require homogenous conditions in the culture conditions to prevent formations of niches with deviating substrate conditions.

Despite that no member of the Sva0081 is cultured, assumptions on physiological capabilities can be draw from omics-data of the closely related δ1 symbiont of Olavius spp.
Recalculations of sequence data (Acosta-Gonzalez et al., 2013; Martiny et al., 2011) revealed, that the relative proportions of Sva0081-SRB sequences to total SRB sequences increased in oxidized layers (Mußmann, personal communication). Metaproteomic data of the symbiosis supports this possible trait. High abundances of oxygen-detoxification enzymes in the δ1 symbiont were found in the metaproteome (Kleiner et al., 2012). The metagenome (Woyke et al., 2006) and metaproteome (Kleiner et al., 2012) further revealed carbon monoxide, hydrogen, propionate and acetate as potential electron donors and high abundance of high affinity uptake systems. This implies a high substrate affinity, and thus adaptation to low substrate concentrations. Fed-batch cultures indicate such ecophysiology for the Sva0081-SRB too. Only additions of 100 µM acetate per day, which corresponds to 800 µM electrons, resulted in a re-activation (Ovanesov, 2012). Acetate, and potentially propionate, are likely important substrates of the Sva0081-SRB. This shows how cultivations-independent obtained data can help to improve culture conditions.

However, for most members of complex microbial communities, omcis-data does not provide such comprehensive information. The most challenging process remains the assembly of raw data due to lack of suitable reference genomes. In fact, for 75% of the metagenomic reads of the Global Ocean Sampling survey (GOS), no reference genome is available with a nucleotide identity >50 % (Lasken, 2012). In contrary to omics-data of the simple community described above, most culture-independent methods cannot establish a link of phylogenetic affiliation and metabolic capability (Yilmaz & Singh, 2012).

To reduce the complexity of the community, single cells can be isolated. The taxon specific physical separation of microbial cells with subsequent whole genome amplification (WGA), allows the targeted access to the genomic potential of a defined microbial community from environmental samples (Lasken, 2012; Miyauchi et al., 2007; Stepanauskas & Sieracki, 2007; Zhang et al., 2006). Several cell-isolation techniques are available, of which the fluorescence-activated cell sorting (FACS) has become the preferred method (Yilmaz & Singh, 2012). FACS combines the specific hybridization of cells e.g. with a FISH probe and the selective sorting of positively hybridized cells (Figure 2). The hybridized sample is injected into the carrier fluid (sheath) of the flow cytometer. The sheath fluid is forced through a fine hydrodynamic nozzle that breaks the water stream into single 1 nL droplets. If a droplet contains a single cell that is hybridized, it emits light upon excitation and will subsequently be sorted. Depending on the investigators initial questions, only single cells or populations of the
same taxonomic group are sorted. Clean sorting requires a strong signal that is usually exhibited through CARD-FISH. However, although CARD-FISH has a great signal to background ratio, it is not particularly suitable for WGA, as the protocol requires harsh treatment which can damage the cells DNA. Recent advances in increased signal intensity of multiple labelled FISH probes could potentially substitute the CARD-FISH procedure in the near future (Stoecker et al., 2010).

As sorted cells do not contain enough gDNA for further sequence based analysis, it requires whole genome amplification. The most widely applied method is multiple displacement amplification (MDA), that is mediated by the Phi29 DNA polymerase (Dean et al., 2001). Upon binding to random hexamers, its high processivity allows amplification from minute amounts of DNA (Dean et al., 2001). Its strand replacement capability circumvents the need for denaturation and makes amplified dsDNA available for further processing, such as PCR based screening or the construction of a (meta)genome.

**Figure 2: Schematic diagram of FACS.** An environmental sample is hybridized with a taxon specific probe. Subsequently, cells are sorted through FACS by detection of positively hybridized cells; all remaining unhybridized cells are discarded.
Due to the environmental importance of the Sva0081-SRB in marine sediment and little knowledge of their ecophysiology, more research has to be conducted. By combining culture-independent and culture-dependent approaches, we expected to shed more light into the yet unknown role of the Sva0081-SRB.

The two aims of this study were:

(A) The establishment of a set-up and culture strategy to enrich the Sva0081-SRB with substrates of assumed spectrum and \textit{in situ} importance (acetate, propionate, formate, lactate) at \textit{in situ} conditions of low substrate concentrations.

(B) The targeted access to the genomic potential of the Sva081-SRB from an environmental sediment sample by FACS. To reveal the genomic potential of the Sva0081-community, WGA is performed on a population of sorted cells rather than on single cells.
2. Methods

2.1. Sampling site and sample retrieval

Sediment and sea water samples were retrieved twice from tidal sand flat Janssand (Figure 3) located between the Frisian mainland and the Island of Spiekeroog in the German Wadden Sea. This sand flat has been a subject of numerous biogeochemical and microbiological studies (Billerbeck et al., 2006; Ishii et al., 2004; Jansen et al., 2009; Mußmann et al., 2005). Sediment samples were obtained on September 25th and October 25th 2012 from the upper sand flat during low tide. Sediment samples obtained in September were exclusively used as inoculum for the fluidized-bed continuous sediment culture. The sediment sampled in October was used as the inoculum for the stirred continuous sediment culture and the orbitally shaken continuous sediment culture and hybridized for the cell sorting. Here, approximately 200 mL of sediment were sampled from 8-10 cm depth and were shoveled into a glass beaker. Water temperatures were 14.0°C (September 25th, 2012) and 10.2°C (October 25th, 2012) respectively.

On October 25th 2012 20 L of sea water (33 psu) were collected from an inundated channel for medium preparation, and stored in 10 L plastic carboys.

The sediment and sea water were transported to the laboratory within 6 hours after sampling. For FISH analysis sediment from October 25th, 2012 was sub-sampled. To minimize the oxygen exposure, two samples were taken from the centre of the black to gray colored sediment from 2 cm beneath and 6 cm beneath the sediments surface. Sea water and sediment were stored at 4°C in the dark. Sediment for FISH analysis was fixed in formaldehyde and stored at -25°C.
2.2. Continuous cultivation of sulfate-reducing bacteria in sediment culture vessels

2.2.1. Principle set-up of the continuous sediment cultures

The continuous sediment culture set-up comprised an anoxic medium, two duplicates of culture vessels and its respective effluent reservoirs (Figure 4). Three different set-ups of culture vessel systems were tested for its applicability. Detailed descriptions are found later under section 2.2.3. The general concept of a continuous addition of medium and removal of overflow as well as general set-up were identical among all three tested systems and is described in the following.
Medium reservoir and inflow into vessels

Sea water medium was purged with N₂ and was stored in a air-tight PLASTIGAS® gas bags (Linde, München, Germany) as reservoirs. These gas bags provided the optimal way for supplying of anoxic medium, as flexible bag prevented the establishment of an under-pressure or pressure equalization through exchange with oxic atmosphere. In each setup duplicate vessels were fed from a single anoxic medium supply by an IPN 4 peristaltic pump (IDEX Health & Science, Glattbrugg, Germany) equipped with autoclavable PharMed® 2-Stopper-tubing (Ø 0.65 mm, IDEX Health & Science). The pump performance was set to 1%, corresponding to an influent flow rate of ~50 mL/24 h. Autoclaved Viton influent-tubing (Lézaud & Co, Marpingen, Germany) was connected to needles with a diameter of 0.6 mm and 0.9 mm (Braun, Melsungen, Germany). The needles with small diameter were pinched through the septum of the PLASTIGAS® gas bag containing the medium. Small-diameter needles were used since needles with bigger diameter generally led to leakage. The more rigid needles reached into the headspace of the culture vessels and enabled inflow of medium by single drops. By this, direct contact of medium and culture sea water was avoided. The influent tubing was occasionally flushed with water to remove potential biofilms in the tubes and was subsequently autoclaved.

Pumping of medium to culture vessels led to a transient increase of pressure. To allow equalizing a gastight, stiff perfluoralkoxy (PFA) tube (1/4”, Bohlender, Grünsfeld, Germany) reached into the sea water in the culture-vessel that facilitated overflow of medium into effluent reservoirs. The volume of sea water in culture vessel was thus determined through lower level of the effluent PFA tube. Both effluent reservoirs of culture-vessels were placed at a lower level than culture-vessels. Inflow tube in effluent reservoirs was constantly submerged, which decreased oxygen contamination of the culture-vessels through effluent tubing.

Tubing and connections

Viton lab tubing with low O₂ permeability (Lézaud & Co, Marpingen, Germany) was used for all set-ups to prevent leakage of atmospheric O₂ into the system. Only tubing of peristaltic pumps was made of air-permeable PharMed® material. All connections of single components such as tubes, butyl rubber stopper and PFA tubes were covered with high vacuum grease. Viton and PharMed® tubes were additionally fitted with cable ties to reduce atmospheric O₂ to be sucked into the tubes. The holes for PFA tubes in butyl rubber stoppers proved to be particularly clean and straight, when stoppers were placed in liquid nitrogen for 10 seconds.
and drilled with a 5 mm driller (Gunter Wegener, personal communication). To check gas tightness of tubing and vessels, respective modules were set under pressure and checked after 18 h for potential decrease of pressure.

![Diagram of continuous sediment culture system]

**Figure 4: Concept of continuous sediment culture.** Anoxic medium was stored in flexible gas bag and added to culture vessel A and B by a peristaltic pump. Over pressure in culture-vessels drove overflow into effluent reservoir.

### 2.2.2. Preparation of medium

The medium consisted of sea water (33 psu) amended with a carbon-, nitrogen- and phosphorus-source. Sea water was filtered through a 0.2 µm cellulose acetate filter (Sartorius, Göttingen, Germany) into an autoclaved bottle containing a stir bar. Sterile sea water was subsequently heated to 50°C in a water bath to reduce oxygen solubility to 4.6 mg/L,
corresponding to 148 µM (Weiss, 1970). The headspace was purged aseptically with N2 while sea water was stirred on a magnetic stirrer until sea water cooled to room temperature (RT). A PLASTIGAS® gas sample bag (Linde, München, Germany) was flushed aseptically with N2 and the top cut open with a sterile scalpel. Subsequently, anoxic sea water was transferred under the clean bench into gas bag, top bit folded, closed with gastight tape and several times inverted to check for potential leaks. Remaining headspace in gas bag was exchanged with argon gas (99.999%, Air liquide, Bremen, Germany) or crystal mix of CO2 and N2 (10:90, Air liquide) for at least five times. The volume of sterile sea water added was determined gravimetrically. Depending on desired substrate concentration in medium reservoir, respective volumes of anoxic carbon stock containing 271 mM sodium acetate (Fluka, Buchs, Switzerland), 77.6 mM sodium propionate (Fluka), 271 mM HCOONa (Sigma-Aldrich, Steinheim, Germany) and 45.2 mM sodium lactate (Fluka), pH 5.25 and nitrogen/phosphorus stock containing 600 mM KH2PO4 (Fluka) and 600 mM NH4Cl (Merck, Darmstadt, Germany), pH 7.10 were added. Of each medium prepared or adjusted, 1 mL sample was sterile filtrated and stored at -25°C as a back up for VFA analysis.

Addition of stock solutions and exchange of headspace was done aseptically under the flame. Syringes were always flushed with 0.22 µm filtered N2 three times. For addition of stock solutions, butyl rubber stoppers of stock solutions were flamed with 96% (v/v) ethanol (Merck). Septum of medium reservoir was cleaned with ethanol (96%). Henceforth, the concentration of carbon sources in the medium and culture vessels are described as total pool of electrons to simplify calculation of theoretical sulfate reduction rates (8 moles of electrons per mol sulfate). Table 2 shows the number of electrons that released when substrates are fully oxidized. Based on available electrons and concentrations in the medium acetate, propionate, lactate and formate contributed in a 4:2:1:1 ratio to the total electron pool.

**Table 2: Potentially available electrons for metabolic needs through complete oxidation of supplied carbon substrates.**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Complete Oxidation</th>
</tr>
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<tbody>
<tr>
<td><strong>Acetate</strong></td>
<td>CH₃COO⁻ + 2H₂O → 2CO₂ + 7H⁺ + 8e⁻</td>
</tr>
<tr>
<td><strong>Propionate</strong></td>
<td>CH₃CH₂COO⁻ 4H₂O → 3CO₂ + 13H⁺ + 14e⁻</td>
</tr>
<tr>
<td><strong>Lactate</strong></td>
<td>CH₃CHOHCOO⁻ + 3H₂O → 3CO₂ + 11H⁺ + 12e⁻</td>
</tr>
<tr>
<td><strong>Formate</strong></td>
<td>CHO⁻ → CO₂ + H⁺ + 2e⁻</td>
</tr>
</tbody>
</table>
2.2.3. Testing and operation of culture-vessel systems

Sediment used for inoculation of the first sediment culture set-up was sampled on September 25th, 2012 and stored at 4°C for 1-3 weeks until inoculation. Sediment used for inoculation of the two other set-ups were retrieved on 25.10.2012 and stored in the dark at 4°C for three weeks and then at RT for one week. Since no other sediment stored at 4°C was available anymore this sediment stored at RT was used for subsequent inoculations. Sediment was homogenized under normal air exposure by stirring sediment with a spatula for at least 3 min in a beaker rinsed with sea water. Sub-samples were taken for DNA extraction, porosity determination in case of one sample and fixed for CARD-FISH analysis. Sea water added to culture vessel was sterile-filtered and made anoxic as described for medium. All sediment cultures were operated at RT.

Set-up 1: Adapted fluidized bed-culture-system

Fluidized bed-reactor-culture systems guarantee homogenous substrate conditions in the culture vessel through constant sparging of gas or high flow rates of reaction liquids. Such an application minimizes the presence of gradients in the culture vessel. Figure 6A shows the set-up of this system, which comprised two culture vessels and two additional peristaltic pumps (620S, Watson-Marlow, Rommerskirchen, Germany) connected to each culture-vessel independently from the continuous culture set-up. The culture-vessel, a double-threaded glass cylinder, 45 mm in diameter (Glasgerätebau Ochs, Bovenden, Germany) were mounted vertically and fitted with butyl rubber stoppers at the top and bottom (Figure 5). Additionally to the effluent tube, a constantly submerged tube reached from the top into the sea water 2 cm further down then effluent tube. The tube was connected through a high performance peristaltic pump with PharMed® tubing (Ø 16 mm) to three inflow tubes at the bottom of the glass cylinder (Figure 5). The closed circle allowed a transport of liquid supernatant from the top, which was then fed back through inflow tubes into the bottom. Such a recycling of medium with high pumping speed guaranteed constantly re-suspended sediment. Before inoculation all tubes were flushed with N₂. Then anoxic sea water was directly fed through the recycle pump and inflow tubes into the glass cylinder at low flow rates. In parallel the cylinder was degassed with N₂ through a needle pinched through the bottom butyl rubber stopper and 60 mL wet sediment added from the top into open glass cylinder. Before the glass
cylinder was closed and the recycle flow connected, N₂ addition was stopped to prevent N₂ or sediment particles to be sucked into the recycle flow. The effluent tube was adjusted to a maximum volume of 250 mL in the vessel. The final volume of sea water comprised pore water of sediment (~21 mL), sea water in vessel (~190 mL) and sea water in recycle tubing (~50 mL). Regarding the inflow rates (~50 mL/24 h) the mean residence time of sea water was ~5 days.

After addition of sediment and overflow of sea water surplus, the recycle flow was stopped and the vessel was immediately flushed with N₂ for 20 min. It was of particular importance that sediment did not start to settle before flushing began, as otherwise N₂ accumulated below, pushing sediment up, which eventually resulted in system malfunctioning. The recycle flow was set to 220 mL/min. Both culture vessels were individually covered with black neoprene to prevent growth of phototrophic organisms.

---

**Figure 5: Schematic drawing of one culture vessel of fluidized bed-culture system.**

Depicted are: Double threaded glass cylinder with recycle flow. Brown-beige gradient corresponds to suspended sediment and sediment free supernatant. Medium was added through headspace, avoiding direct contact. Anoxic medium reservoir and effluent reservoir are not depicted.
**Set-up 2: Stirred culture-system**

This continuous sediment culture applied a lower sediment:sea water ratio. To reduce the formation of stable gradients, it was constantly mechanically mixed with a stir bar (Figure 6B). This continuous sediment culture consisted of two 250 mL flasks (Schott, Stafford, UK), in which sediment was not resuspended but stirred with a bar to minimize gradient formation. Both flasks contained a 6 cm silicone coated stir bar and were fitted with butyl rubber stoppers with an effluent tube as described above. 50 mL wet sediment and 80 mL sea water were added, corresponding to ~100 mL sea water and mean residence time of ~2 days. Both flasks were stirred at the lowest rate possible and degassed in parallel with N₂ for 20 min before being connected to medium. Subsequently the set-up was covered to keep flasks in darkness.

**Set-up 3: Orbitally shaken culture-system**

The third continuous sediment culture did not re-suspend the sediment. This final set-up was somewhat similar to set-up #2, but consisted of two 2 L flasks (Schott), in which sediment was allowed to settle on the bottom and medium in the supernatant was exchanged continuously (Figure 6C). In contrast to set up #2 the sediment was not stirred but only shaken to reduce gradient formation. Both flasks were fitted with butyl rubber stoppers and two PFA tubes. The effluent PFA tube reached far down into the flask, determining the maximum volume to approximately 100 mL. The second PFA tube reached into the headspace of 2 L flasks and was used for pressure release when degassed. Volumes of sediment and sea water added and corresponding operational parameters similar to those in the previous set-up. Both flasks were flushed with N₂ before, during and after inoculation. Flasks were then placed on an orbital shaker at 79 rpm and covered with a box. Medium was connected only after 12 hours, as sediment had been stored for >1 month. The flow rate of the medium remained unchanged, while the substrate concentration was increased throughout the culturing of 69 days. Table 3 depicts different phases of the culturing period with corresponding electron concentrations in the medium and calculated concentrations of electrons added. One needs to recapture, that at maximum, for most of the time less, 50 mL / day of the medium was added to 100 mL sea water in the culture vessels. Each drop added, was subsequently diluted and potentially partly removed through effluent tube.
Table 3: Electron concentrations in medium and supplied to continuous sediment cultures per culturing phase. Values are based on calculations from added carbon stock and determined added volumes of medium through gravimetical analysis of effluent.

<table>
<thead>
<tr>
<th>Phase</th>
<th>Parameters of culturing phase</th>
<th>Electron concentration in medium [mM]</th>
<th>Accumulated electrons [mM]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Days</td>
<td>Vessel A</td>
</tr>
<tr>
<td>1</td>
<td>Inoculation, extremely little addition of medium</td>
<td>4.3</td>
<td>0-11</td>
</tr>
<tr>
<td>2</td>
<td>Low medium addition</td>
<td>4.3</td>
<td>11-29</td>
</tr>
<tr>
<td>3</td>
<td>Increased medium addition</td>
<td>9.4</td>
<td>29-46</td>
</tr>
<tr>
<td>4</td>
<td>Regular medium addition</td>
<td>13.8</td>
<td>46-56</td>
</tr>
<tr>
<td>5</td>
<td>Regular medium addition</td>
<td>63.0</td>
<td>56-69</td>
</tr>
<tr>
<td>∑</td>
<td></td>
<td>69</td>
<td>452</td>
</tr>
</tbody>
</table>

Figure 6: Overview of tested culture vessel set-ups (A) Adapted fluidized bed-culture-system with yellow high performance pump and vertical mounted glass tubes (covered with black neoprene). (B) Stirred culture-system. (C) Orbital shaken culture-system, applied in this study.
2.3. Sediment sampling for catalyzed reporter deposition-fluorescence in situ hybridization (CARD-FISH) and other parameters

To monitor the continuous sediment culture in the orbital shaken culture-system, samples were taken on a regular basis. Samples for CARD-FISH, DNA extraction, sulfide and volatile fatty acid (VFA) analysis, and pH measurement were taken every two weeks in order to follow the development of the microbial community and as a back-up to potentially trace physiochemical processes. For CARD-FISH and DNA extraction 2x400 μL of sediment from each vessel were transferred with a 5 mL cut-off glass pipette into 2 ml plastic vials for duplicates of DNA extraction and FISH fixation samples. The pH was measured for the sea water in the sediment after flasks were shaken (761 Calimatic, Knick Elektronische Messgeräte, Berlin, Germany). Both vessels were degassed after sampling procedure with N₂ for at least 15 min.

Sediment for CARD-FISH analysis was fixed as followed. Cells were fixed for 60 to 80 minutes at RT. Then, samples were centrifuged at 7000xg for five minutes and supernatant replaced with PBS (130mM NaCl, 10mM Na₂HPO₄, pH 7.4). This washing step was repeated twice. Subsequently PBS supernatant was replaced with PBS:ethanol (99% [vol/vol]) at 1:1 ratio and samples stored at -25°C for further processing.

Gravimetrically determination of added substrate to culture-vessels

To document the substrate supplied, volumes of medium added were documented. The volume of medium added, corresponds to the increase of volume in the effluent reservoirs. By measuring the weight of the effluent reservoirs we could determine how much substrate was added to individual culture-vessels.
2.3.1. Catalyzed reporter deposition-fluorescence in situ hybridization (CARD-FISH)

To document changes of the microbial community in the cultured sediment, CARD-FISH was performed on membrane filters (Ishii et al., 2004). Cells were fixed as described. For Detachment of cells from sediment particles, fixed samples were sonicated five times for 30 seconds with one pulse per second at 20% intensity with an UltraSound Sonication Probe (Bandelin, Berlin, Germany). After each step, 500 µL of supernatant were transferred into a separate vial and replaced with PBS:ethanol. For the final step, 1000 µL of sonicated supernatant were transferred leading to a total volume of 3 mL sonication product. Sonication product was shaken an allowed to settle for 10 seconds, after which 100-200 µL of product were transferred into 10 mL 0.22 µm filtered PBS. The diluted product was filtered at 200 mbar on a 47 mm 0.2 µm cyclopore filter (Whatman) supported with 0.2 µm cellulose acetate filter (Sartorius). Filters were dried at 46°C and coated in 0.1% low melting agarose (50°C) solution (Metaphor, Southborough, USA) to prevent cell loss during handling. The filter were fully dried at 46°C for 20 min, sectioned, labeled with a pencil and stored at -25°C.

All subsequent steps were done directly prior to hybridization. Endogenous peroxidases were inactivated by incubating filter pieces in 99% methanol (Fluka) containing 0.15% hydrogen peroxide (H₂O₂) for 30 minutes at RT and washed in excess volumes of MilliQ. Subsequently the filter were directly transferred into lysozyme (Fluka) solution (10 mg ml⁻¹ in 0.05 M EDTA, pH 8.0; 0.1 M Tris-HCL, pH 8.0) and incubated for 45 minutes at 37°C for cell wall permeabilization. The filters were then washed in excess PBS for >five min to remove lysozyme, dipped in MilliQ and ethanol (96% v/v) and allowed to air dry.

Hybridization was conducted in airtight glass chambers at 46°C supplemented with a wetted tissue of appropriate hybridization buffer (HB). Filter pieces were placed in petri dishes and covered with ~50 µL hybridization buffer (Table 4) containing horseradish peroxidase labeled (HRP) specific oligonucleotide (0.08 µM). In case of probe DSS1431, an equimolar mixture of helper-, competitor- and HRP-labeled probe, was prepared and added. Petri dishes were transferred into the glass chambers and allowed probe hybridization for 2 h. Probe DSS658 was only hybridized for 1.5 h according to Ovanesov (2012).
For stringent washing, filters were transferred to freshly prepared washing buffer with the appropriate NaCl concentration (Table 5) for 15-20 minutes at 46°C and washed in PBS for 10 min at RT. Subsequently filter pieces were soaked in amplification buffer (Table 7), containing 1.3 μL Alexa Fluor®488 labelled tyramides and 10 μL 0.015 H2O2/mL amplification buffer, and placed in petri dish. Remaining amplification buffer was poured over filter pieces in petri dish that was placed in airtight glass chambers and left at 37°C for 40 minutes.

Afterwards the filter pieces were washed in 37°C prewarmed PBS for 10 minutes at RT, briefly dipped in MQ, ethanol and air dried. For verification of FISH signals, filter pieces were counterstained with 4,6-diamidino-2-phenylindole (DAPI, Sigma) solution (1μg/mL)) for 10 minutes at RT, washed in MilliQ and ethanol (96%) and embedded in mounting medium 4:1 mixture of Citifluor (Citiflour Ltd., London, UK) and Vectashield (Cevtor Laboratories, Burlingame, USA).

Additionally, the probe NON338 was applied as a negative control. To prevent underestimation due to quenching of DAPI to CARD-FISH signals, total cell counts were determined by DAPI staining of a filter pieces without hybridization. Cells were counted with the epifluorescence microscope Nikon 50i at a magnification of 1000x. A total of 900-1700 DAPI stained cells were counted for each sample. For CARD-FISH signal quantification the filter area, namely the number of grids counted, was compared to the abundance of DAPI counts/area unit without prior hybridization.

**Table 4: Composition of hybridization buffer (HB).** Formamide volume depended on stringency conditions indicated in probe list (Table 7).

<table>
<thead>
<tr>
<th>Compound</th>
<th>mL or g/ 20 mL HB buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 M NaCl</td>
<td>3.60</td>
</tr>
<tr>
<td>Tris HCl (pH 8.0)</td>
<td>0.40</td>
</tr>
<tr>
<td>Blocking Reagent</td>
<td>2.00</td>
</tr>
<tr>
<td>10% Sodium dodecyl sulfate</td>
<td>0.04</td>
</tr>
<tr>
<td>Dextran sulfate</td>
<td>2.00</td>
</tr>
<tr>
<td>Formamide</td>
<td>See table 8</td>
</tr>
<tr>
<td>MilliQ water</td>
<td>Fill up to 20 mL</td>
</tr>
</tbody>
</table>
### Table 5: Composition of washing buffer

<table>
<thead>
<tr>
<th>Compound</th>
<th>mL or g/ 20 mL HB buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 M NaCl</td>
<td>See Table 6</td>
</tr>
<tr>
<td>1 M Tris HCl (pH 8.0)</td>
<td>1.00</td>
</tr>
<tr>
<td>0.5 M EDTA</td>
<td>0.50</td>
</tr>
<tr>
<td>MilliQ</td>
<td>Fill to 50 mL</td>
</tr>
<tr>
<td>0% Sodium dodecyl sulfate</td>
<td>0.05</td>
</tr>
</tbody>
</table>

### Table 6: Volumes of 5 M NaCl depend on hybridization stringency.

<table>
<thead>
<tr>
<th>Concentration of formamide in hybridization buffer</th>
<th>Volumes of 5M NaCl to be added to washing buffer [mL]</th>
</tr>
</thead>
<tbody>
<tr>
<td>30%</td>
<td>1.02</td>
</tr>
<tr>
<td>45%</td>
<td>0.30</td>
</tr>
<tr>
<td>50%</td>
<td>0.18</td>
</tr>
</tbody>
</table>

### Table 7: Composition of amplification (AMP) buffer f

<table>
<thead>
<tr>
<th>Compound</th>
<th>mL or g/ 40 mL AMP buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 × PBS, pH 7.6</td>
<td>2.00</td>
</tr>
<tr>
<td>Blocking Reagent</td>
<td>0.40</td>
</tr>
<tr>
<td>5 M NaCl</td>
<td>16.00</td>
</tr>
<tr>
<td>MilliQ</td>
<td>Add to 40 mL</td>
</tr>
<tr>
<td>Dextran sulfate</td>
<td>4.00</td>
</tr>
</tbody>
</table>
Table 8: HRP-labeled probes and unlabeled oligonucleotides used in this study. 1: unlabeled competitor oligonucleotides hybridized together with probe DSS1431. 2: Unlabeled helper probes targeting regions up- and downstream of DSS1431 site to improve accessibility. Formamide concentrations applied in this study.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Specificity</th>
<th>Sequence (5’-3’)</th>
<th>Formamide</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EUB338 I-III</td>
<td>almost all bacteria</td>
<td>GCWGCCCWCCCGTAGGWGT</td>
<td>30%</td>
<td>(Amann et al., 1990)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Daims et al., 1999)</td>
</tr>
<tr>
<td>DSS658</td>
<td>most members of the <em>Desulfosarcina-Desulfococcus</em>-group (<em>Desulfobacteraceae</em>)</td>
<td>TCCACTTCTCTCTCCCAT</td>
<td>50%</td>
<td>(Manz et al., 1998)</td>
</tr>
<tr>
<td>DSB706</td>
<td>most <em>Desulfobulbaceae</em></td>
<td>ACCGGTATTCCTCCCGAT</td>
<td>45%</td>
<td>(Loy et al., 2002)</td>
</tr>
<tr>
<td>NON338</td>
<td>nonsense probe (negative control)</td>
<td>ACTCCTACGGGAGGCAGC</td>
<td>30%</td>
<td>(Wallner et al., 1993)</td>
</tr>
<tr>
<td>DSS1431</td>
<td>Sva001 sediment group (subgroup of the DSS group) including δ1-symbionts of <em>Olavius</em> spp.</td>
<td>GGTTTGCCCAACGACTTC</td>
<td>30%</td>
<td>Mußmann, unpubl.</td>
</tr>
<tr>
<td>¹cDSS1431a-e</td>
<td>certain <em>Deltaproteobacteria</em> Competitor to probe DSS1431</td>
<td>GGTTTGCCCAACAACCTTCGGTTAGCCCAACAACCTTCGGTTCCGCCCAACCAACCTTCAGTTTGCCCAACAACCTTCGGTTGGCCCAACAACCTTC</td>
<td>30%</td>
<td>Mußmann, unpubl.</td>
</tr>
<tr>
<td>²DSS1431a_us3</td>
<td><em>Olavius</em> spp. δ1-endosymbiont related <em>Desulfo bacteraceae</em>, helper probe</td>
<td>TGGTACAACCAACTCTCATGG</td>
<td>30%</td>
<td>Mußmann, unpubl.</td>
</tr>
<tr>
<td>²DSS1431a_ds3</td>
<td><em>Olavius</em> spp. δ1-endosymbiont related <em>Desulfo bacteraceae</em>, helper probe</td>
<td>TTAGGGCGCCTGCATCCCCGAA</td>
<td>30%</td>
<td>Mußmann, unpubl.</td>
</tr>
<tr>
<td>²DSS1431b_us3</td>
<td><em>Olavius</em> spp. δ1-endosymbiont related <em>Desulfo bacteraceae</em>, helper probe</td>
<td>TTAGGGCGCCTGCATCTCTGTAA</td>
<td>30%</td>
<td>Mußmann, unpubl.</td>
</tr>
</tbody>
</table>
2.4. Flow-sorting of Sva0081-SRB bacteria and whole genome amplification

2.4.1. Fluorescence in situ hybridization with multiply labeled probes (CLICK-FISH)

The probe DSS1431 labeled with four ATTO 488 molecules was tested for its applicability for fluorescence-activated cell sorting (FACS). Additionally, EUB338 I probes from different batches labeled with two to four fluorophores through were also tested to compare signal intensity. In Table 9 the applied probes, fluorophores and the base the fluorophores were linked to are listed. In case of 4x ATTO 488 labeled EUB338 I, no information on labeled bases was available. The fluorophores are most likely labeled to same bases as stated for 4x FLUOS labeled probe. All fluorophores were covalently attached through the same CLICK-based chemistry (Becer et al., 2009). Detailed mechanisms, which CLICK-process was applied are classified (Biomers.net, personal communication). All probes were obtained from Biomers.net (Ulm, Germany) and dissolved in PCR-grade water three months (ATTO 488 labeled probe DSS1431), nine months (FLUOS labeled probes EUB338 I), or at least five months (ATTO 488 labeled probe EUB338 I) prior to this study.

All hybridizations with probes were done on sediment samples that had been shown to contain a probe DSS1431-visualized population by CARD-FISH prior. FISH was performed as described in (2.3.1) with following modifications. All hybridizations were conducted with hybridization buffer of 35% formamide that was aliquoted directly onto the filter pieces. 1 µL of respective EUBI338 probe working solution (8.3 µM) was added to 10 µL hybridization buffer on filter and well mixed by pipetting up and down. The exact final formamide and probe concentrations were 32% and 0.75 µM respectively. For DSS1431 hybridizations, 1 µL of probe working solution (8.3 µM), 1 µL of equimolar mixture of helper probes working solution (each 8.3 µM) and 1 µL of competitor working solution (each 8.3 µM) were added to 10 µL hybridization buffer and mixed. Due to dilution by helper and competitor probes the final formamide and probe concentrations were 27% and 0.64 µM each respectively.

Several hybridizations were conducted with times that ranged from 0.5 to 66 h. After hybridization, filters were washed in stringent washing buffer but not in PBS. All samples were first analysed without DAPI stain and subsequently counterstained with DAPI. Hybridization results were evaluated on a Nikon 50i epifluorescence microscope with DAPI.
Long pass F36-499 Filter (AHF) and FITC filter F11-001(AHF) or Zeiss Axioskop2 mot plus (Carl Zeiss, Jena, Germany) with DAPI Filter set 2 Long pass 420 (Carl Zeiss) and Alexa 488 F36-525 filter. Pictures were taken with the Nikon 50i microscope equipped with an AxioCam MRc camera (Carl Zeiss) at same exposure times. Post modification was done using Zen Version 7.0.0.285 (Carl Zeiss).

Table 9: Specifications of multiply labeled probes applied in this study. Bold marked bases correspond to bases labeled with respective fluorophore. N.a.: Not available, no information on labeled bases could be retrieved.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Number of fluorophores</th>
<th>Fluorophore</th>
<th>Position of fluorophore attached to (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EUB338 I</td>
<td>2</td>
<td>FLUOS</td>
<td>GCTGCCTCCCGTAGGAAT</td>
</tr>
<tr>
<td>EUB338 I</td>
<td>4</td>
<td>FLUOS</td>
<td>GCTGCCTCCCGTAGGAAT</td>
</tr>
<tr>
<td>EUB338 I</td>
<td>4</td>
<td>ATTO 488</td>
<td>n.a.</td>
</tr>
<tr>
<td>DSS1431</td>
<td>4</td>
<td>ATTO 488</td>
<td>GGTTTGCCCAACGACTTC</td>
</tr>
</tbody>
</table>

2.4.2. Fluorescence-activated sorting of Sva0081 SRB cells after CARD-FISH

Fixation of freshly collected sediment

Sediment hybridized for subsequent cell sorting was sampled on 25.10.2012 and kept at 4°C and at RT. At November 30th, 2012 a subsample of 20 mL was fixed with 20 mL ethanol 99% (v/v) (Roth) for 10 min at RT on a rotary shaker. The ethanol-fixed sediment was sonicated as described. Larger sediment particles in the supernatant were allowed to settle for ca. 60 seconds. From the supernatant 2 mL of 3 mL were filtered on 25 mm filter. Filters were air dried at 46°C.

Fluorescence-activated flow sorting (FACS)

The hybridization of CLICK-FISH probe DSS1431 did not result in detectable signals. Consequently, we applied CARD-FISH to sort the Sva0081 SRB community via FACS. In order to avoid possible DNA and rRNA degradation sediment that was fixed one day before sorting and hybridizing was used. Cells were hybridized by CARD-FSIH a few hours
before sorting. CARD-FISH for FACS deviated from the one previously described. Prepared filter was not embedded in agarose, nor was it subjected to harsh lysozyme or methanol treatments. For negative control, ¼ of the filter was cut out and hybridized separately with a HRP-labeled probe NON338, the remaining filter was hybridized with HRP labeled probe DSS1431 and its helpers and competitors. The filter was handled with extreme care to minimize biomass loss during the CARD-FISH protocol. Hybridization conditions were identical to the ones described for multiple labeled FISH, although shortened to 1.5 h, with subsequent washing and amplification of CARD-FISH protocol. After the final washing step in MilliQ, filters were directly transferred in 2.5 mL (DSS1431) and 1 mL (NON338) 150 mM NaCl with 0.05% Tween® 80 (Fluka) and vortexed on a Vortex Genie 2 (Scientific industries, Bohemia, NY, USA) for 1 h at full power (Sekar et al., 2004). Then the vortexed product was filtered through 8 µm filter to prevent clogging of the flow cytometer nozzle. 70 µL of filtered product were spotted onto a filter to confirm the successful hybridization of target cells before sorting.

Cell sorting was conducted with a MoFlow flow cytometer (Cytomation Incorporation, Fort Collins, USA) equipped with an argon laser tuned to an excitation wavelength of 488 nm (400 W). Alexa Fluor®488 emitted light was detected via 530/10-nm band-pass filter, the side angle light scatter (SSC) through a 488/10-nm band-pass filter. Before sorting the flow cytometer was washed with autoclaved MilliQ for half a day to remove residual contaminating DNA. The sheath fluid was autoclaved 0.075% (w/v) NaCl solution that was additionally 0.1 µm filtered with an inbuilt filtering system before fed to the sorting apparatus. The low salt concentration was of particular importance for salt-sensitive applications on sorted cells. Calibration of the instrument was done with yellow-green fluorescing 0.2 µm polystyrene beads (Polysciences, Warrington, USA). Twenty fluorescent beads were sorted 12 times to align the instrument and control the sorting mode. Sorting mode was set to “Single 1 droplet”. This mode targets a maximum of purity: Single droplets of 1.0 nL are not sorted if the FISH-signal within is not centred or previous or following droplet contains a signal as well.

As CARD-FISH signals were strong, the NON338 nonsense probe hybridized cells were not necessary to be sorted as negative control. Subsequently, only cells hybridized with DSS1431 were further processed. Before sorting, a few thousand events were recorded online with summit software version 4.10 (Cytomation Inc) and plotted in a bivariant dot plot diagram of logarithmic amplitude values of side light scatter (SSC) and 530 nm fluorescence. Events of a
particularly strong fluorescence were selected as populations of interest. Subsequently, ~800 events of each defined population were sorted, counterstained with DAPI and microscopically evaluated (epifluorescence microscope Nikon 50i).

Two populations of fluorescent events were defined through graphical interface. The Population A was defined through a greater region and included events of lower fluorescence. The population B was defined as a subgroup of population A and therefore included only events of high fluorescence. After evaluation, several batches of 500 events of two defined populations were sorted directly into PCR reaction vials (Biozym Scientific Hess. Oldendorf, Germany) at an amplitude of 9.95 and delay of 42 9/16 droplets leading to a total volume of 0.5 µL. PCR vials were closed directly after sorting and stored at -25°C. Sorting and handling conditions were not sterile. To verify the aseptic nature of the sheath fluid and ubiquitous polystyrene beads, 6x 500 beads were sorted as a negative control for following applications.

2.4.3. Whole genome amplification (WGA) and PCR-based diversity analysis

In order to gain access to genomic DNA of the sorted Sva0081-SRB, whole genome amplification was performed on all batches of sorted cells. The reactions were performed with the Illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare, Waukesha, USA), which is based on the multiple displacement amplification (MDA) capabilities of Phi29 DNA polymerase. Preparation of all MDA related work was done under a UV hood that is exclusively used for preparation of PCR and MDA reactions. Prior to use it was cleaned with 70% (v/v) ethanol and UV-sterilized for 20 min.

All pipetting steps were conducted with freshly opened, filtered RNase and DNase free tips (Biozym Scientific Hess) on ice. The sorted cells were defined by population A that included a less specific sorting gating that population B that only sorted cells with particular strong fluorescence. Ten batches of sorted cells of population A, four batches of population B and three batches of sorted beads were re-suspended in 2.5 µL sample buffer (GE healthcare). Sample buffer was added to two PCR vials that served as positive and negative controls for the MDA reaction. Closed vials were transferred into a Mastercycler (Eppendorf, Hamburg, Germany), incubated at 95°C for 10 min and directly transferred on ice. Subsequently, 2.5 µL of MDA reaction master mix were added to lysed cells and controls. Final reaction volume of
5.5 µL contained 0.5 µL DNA template, 2.5 µL sample buffer (GE Healthcare), 1.95 µL reaction buffer (GE Healthcare), 0.25 µL GenomiPhi V2 enzyme mix (GE Healthcare), 0.1 µL 10x SYBR green (Quiagen, Hilden, Germany) and 0.2 µL 0.5 µM fluorescein (final concentration 0.025 µM). The DNA template in samples derived from 500 sorted cells that were lysed through heat treatment. For the MDA controls, sterile PCR grade water for negative control and GenomiPhi-supplied positive control were added instead. The positive control was diluted corresponding to a final concentration in reaction of 10 ng/21 µL, as suggested by the manufacturer’s protocol.

The MDA reactions were conducted on an iQ5® Thermal cycler (BioRad, Hercules, USA). The reaction was performed at 35°C with a subsequent final enzyme inactivation step at 65°C for 10 min. The relative increase in fluorescence was followed in real-time and amplification process was aborted manually after 104 min and followed by the inactivation. For quantification and a following PCR based screening series, 1 µL MDA product was added to 9 µL PCR grade water.

Quantification of MDA V2 product was performed with Qubit® dsDNA high sensitivity Assay (Invitrogen, San Diego, USA) according to manufacturer’s instructions with the following deviation. Mixed reagents were incubated for 20 min to yield more reliable results (Nicole Rödinger, personal communication).

Polymerase chain reactions (PCR) conditions

All polymerase chain reaction (PCR) amplifications, described in the next section, were performed according to following conditions. Total volume of PCR reactions was 19 µL that contained 2.0 µL Taq buffer 10x (Eppendorf), 1.6 µL dNTPs solution containing 2.5 mM of each nucleotide (Roche, Mannheim, Germany), 2.0 µL Bovine Serum Albumin (BSA) solution 3 mg/mL (Sigma), 0.04 µL Taq DNA polymerase 5U/µL (Eppendorf), 0.4 µL forward primer solution 50 pmol/µL, 0.4 µL reverse primer solution 50 pmol/µL (both: Biomers.net, Ulm, Germany) and 12.6 µL sterile PCR grade water (Sigma-Aldrich). To each reaction 1 µL of (diluted) DNA template was added which was substituted with PCR grade water for negative control. 1 µL of heat treated (10 min at 65°C) cell pellet of Desulfotalea psychrophila (Knoblauch et al., 1999) culture, that served as a positive control.

All reactions were carried out in a Mastercycler, Mastercycler personal or Mastercycler gradient (Eppendorf). Amplified PCR products were mixed with 10% 10x loading dye (aprA
products) or 20% 5x loading dye (16S rRNA gene products) and visualized by electrophoresis on a 1-1.5% agarose gel in TAE buffer.

**Table 10: Specifications of primers used for the amplification of the 16S rRNA and apr gene and for vector insert screening** applied in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Target molecule</th>
<th>Annealing temp.*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
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<td>APS1F</td>
<td>TGGCAGATCATGATMAYGG</td>
<td>Bacterial adenosine-5’-phosphosulfate reductase subunit A gene</td>
<td>48°C</td>
<td>(Meyer &amp; Kuever, 2007)</td>
</tr>
<tr>
<td>APS4R</td>
<td>GCGCCAACYGGRCCRTA</td>
<td>Bacterial adenosine-5’-phosphosulfate reductase subunit A gene</td>
<td>48°C</td>
<td>(Meyer &amp; Kuever, 2007)</td>
</tr>
<tr>
<td>GM3F</td>
<td>AGAGTTTGATCMTGGC</td>
<td>Bacterial 16S rRNA gene, bp 8-27</td>
<td>48°C</td>
<td>Muyzer et al., 1996</td>
</tr>
<tr>
<td>GM4R</td>
<td>TACCTTGTTACGACTT</td>
<td>Bacterial 16S rRNA gene, bp 1491-1509</td>
<td>48°C</td>
<td>Muyzer et al., 1996</td>
</tr>
<tr>
<td>M13F</td>
<td>GTAAAACGACGGCCAG</td>
<td>pCR®4-TOPO® and pGem T Easy Vector</td>
<td>55°C</td>
<td>(Yanisch-Perron et al., 1985)</td>
</tr>
<tr>
<td>M13R</td>
<td>CAGGAAACAGCTATGAC</td>
<td>pCR®4-TOPO® and pGem T Easy Vector</td>
<td>55°C</td>
<td>(Yanisch-Perron et al., 1985)</td>
</tr>
</tbody>
</table>

**Screening PCR for presence of adenosine-5’-phosphosulfate (APS) reductase**

All amplification products from MDA reactions were screened by PCR targeting the gene apr encoding adenosine-5’-phosphosulfate (APS) reductase subunit A. In dissimilatory sulfate reducers the APS reductase catalyzes the reduction of adenyl-sulfate to sulfite. Although homologues are also found in sulfur-oxidizing prokaryotes, a positive PCR product gave first indication of a successful amplification of gDNA from potentially sulfate-reducing bacteria.

The apr gene was amplified with the primer pair APS1F and APS4R (Table 10) performing a touch-down PCR (Meyer & Kuever, 2007). The touch-down PCR program consisted of an initial denaturation step at 96 °C of 5 min, followed by 10 cycles at 95°C for 45 seconds, annealing temperature at touch-down conditions for 45 seconds and a elongation at 72°C for 45 seconds. Touch-down PCR started at an annealing temperature of 53°C, which was decreased by 0.5°C per cycle until the final annealing temperature reached 48°C. The
following 30 cycles were performed as described above with an annealing temperature of 48°C. The last step was a final elongation step at 72°C for 10 min.

**DNA-free conditions for cell sorting and whole genome amplification**

To test for potential DNA contaminations during the sorting and in the MDA reagents, the sorted fluorescent beads, the positive and the negative control MDA reactions were also subjected to PCR amplification of the 16S rRNA and aprA genes. The aprA gene was amplified in triplicates. The amplification of the 16S rRNA gene with the primer pair GM3F and GM4R (Table 10) was done in four parallels. The cycling conditions were: 1 cycle at 95°C for 5 min., followed by 36 cycles of 95°C for 1 min, annealing at 48°C for 1 min, extension at 72°C for 2 min and a final extension at 72°C for 10 min. PCR reactions were checked by gel-electrophoresis.

**Clone libraries of 16S rRNA and aprA genes**

From two MDA-generated gDNA products that were shown to be contain amplifiable aprA-genes 16S rRNA gene and aprA clone libraries were established. Three and four parallel PCR reactions targeting the aprA and 16S rRNA gene respectively were pooled and gel-purified by running a gel-electrophoresis for 40 min at 75 V. The agarose gel was stained for 30 min in freshly prepared ethidium-bromide bath. Bands were visualized with UV-transilluminator and manually cut out. Cut bands of replicate PCR products were dissolved and combined. Combined replicates were purified through QIAquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Four µL of pooled purified 16S PCR product were ligated into pCR®4-TOPO® Vector (Invitrogen, San Diego, USA) according to manufacturers instructions. Transformations were performed with high efficiency competent cells of *Escherichia coli* (OneShot®TOP10). Two µL of ligation mix were added to competent cells and incubated on ice for 10 min. Cells were then exposed to 42°C for 30 sec and immediately transferred onto ice. Transformed cells were incubated for one hour at 37°C, after addition of 250 µL SOC medium (Invitrogen). Subsequently, all cells were plated on pre-warmed (37°C) selective Luria-Bertani (LB) plates containing ampicillin (100 µg/mL), IPTG (0.15 mM) and X-gal (0.0004%). Plates were incubated overnight at 37°C. 48 white clones of each 16S rRNA clone
library were picked into liquid LB medium containing ampicillin (100 µg/mL) and 10% glycerol. Liquid cultures were incubated overnight and transferred to -80°C. The *E. coli* clones were screened for the correct insert size by PCR. 1 µL of overnight liquid culture was transferred to 19 µL PCR reaction with the primer pair M13F/M13R. Screening PCR for 16S rRNA gene insert was performed as described for the 16SrRNA gene, but annealing temperature was adjusted to 55°C and number of cycles was set to 40. The *aprA* products were cloned, sequenced and kindly provided by Jörg Wulf (MPI Bremen).

To test whether the 16S rRNA gene could be amplified only from sorted cells without prior WGA, batches of sorted cells were directly subjected to PCR reactions. Two batches of sorted cells of population B, and three batches of sorted beads were diluted (1:50 in PCR grade water). This dilution was equivalent to the number of cells that served as template for PCR reactions from WGA reactions. The PCR reactions were performed in triplicates

**Sequencing, sequence processing and phylogenetic identification**

Clones containing the 16S rRNA gene were transferred onto a LB agar plate containing ampicillin and were sent for sequencing by a company (GATC, Konstanz, Germany) for partial Sanger-sequencing with primer M13R.

All sequences were quality checked using Sequencer version 4.6 (Gene Codes Corporation, Ann Arbor, USA). Remaining primer and vector sequences were removed automatically. Sequences of 16S rRNA were aligned with SILVA online aligner (Pruesse *et al.*, 2012) to check identity among them. If sequences showed high identity, representative sequences were submitted to classifier of the ribosomal database project II.

The *aprA* sequences were aligned with ClustalW in BioEdit version 7.0.5.2. To test for micro diversity among retrieved sequences, an identity matrix was constructed and visualized through cluster analysis (Euclidian distance measure). Following, representative *aprA* sequences were submitted as nucleotide sequences to BLASTx 2.2.27. The identity, query coverage and E-value for the first seven hits and closest cultured representative were noted.
3. Results

3.1. Continuous sediment cultures for enrichment of Sva0081-SRB from a complex microbial community

Three different culture vessel set-ups that mainly differed in their way of sediment and medium mixing were tested for their applicability. The aim was to identify a suitable continuous sediment culture set-up providing anoxic, sulfate-reducing conditions at low substrate concentrations. The set-up presumed to be most appropriate for enrichment of Sva0081-SRB was chosen for extended culturing period and for molecular analysis.

Set-up 1: Adapted fluidized bed-culture-system

The continuous suspension of sediment particles with recycling medium was chosen to reduce formation of substrate-gradients and to minimize workload of producing fresh medium. The continuous suspension of sediment particles with recycling medium was only suitable with a low sediment:sea water ratio of <0.15 to avoid clogging of tubings. Furthermore, in this set-up the initially reduced sediment (dark grey to black in color Figure 7A) always turned into a oxidized state after 2-3 days in both vessels (Figure 7B). Oxygen concentration measurements (data not shown) indicated presence of oxygen in the vessels. This was reproducible for several fresh inoculations.

Usually such systems with high pumping rates provide anaerobic growth conditions through the supply of substrate concentrations in the mM range, that reduce trace amounts of O₂. Consequently, we added pulses of up to 6 mM electrons parallel to inoculation and after increase of O₂ was detected. Even though these added substrate concentrations corresponded to 10 times the desired initial concentration supplied per day, the change of color of the sediment and the reconvention of increasing oxygen content in the vessel could not be prevented. Accordingly, this set-up did not meet the requirements to provide anoxic conditions at low substrate concentrations and was therefore disregarded.
Figure 7: Fluidized bed-culture-system. (A) Vessel after inoculation with reduced sediment. (B) Vessel after 3 days of operation with oxidized sediment.

Set-up 2: Stirred culture-system

The second tested system could maintain the sediment at reduced conditions as preliminary tests indicated (data not shown), but the sediment could not be fully mixed. Repeated efforts could not prevent the stir bar from being pushed to the sediment surface and therefore required regular manual mixing of flasks. To verify conditions that support cell growth, total cell counts were analyzed for the first five days and are reflected in figure 8. Initial values of the common inoculum were $1.12 \times 10^8$ cells/g sediment, whereas cell concentrations after three days of culturing were $8.98 \times 10^7$ cells/g sediment in culture vessel A and $1.06 \times 10^8$ cells/g sediment in culture vessel B. Two additional days of culturing revealed cell concentrations of $3.20 \times 10^7$ cells/g sediment and $7.13 \times 10^7$ cells/g sediment respectively. Microscopic evaluation furthermore revealed high proportions of filaments after three and five days.
Figure 8: Change in cell numbers in stirred continuous sediment culture vessels during five days of culturing. Cell concentration determined in single counts.

Set-up 3: Orbitally shaken culture-system

In this set-up sediment was allowed to settle at the bottom of the vessels and only supernatant was exchanged in a continuous way. Mixing with sediment was enhanced by orbitally shaking and regular manual mixing of sediment and sea water. Here, sediments in vessels also remained reduced after extended culturing. Despite the presence of probably (vertical) gradients, this set-up met the major requirements of anoxic conditions at low substrate concentrations and was selected for further sediment cultivation for a total of 69 days.

The operation of both vessels varied in regular addition and removal of medium. This was particular the case in the first two weeks. Only little medium was added to culture vessels, which corresponded to a lack of a sulfidic smell. However, after approx. 25 days the operation of both vessels was stable and correlated with a dark black sediment color and a strong sulfidic odor. This was particularly the case after 29 days, throughout phase four and especially throughout the whole last phase. After 57 days (phase 5) the medium in the culture vessels turned turbid. Moreover, shortly after increase to final substrate concentration of 63 mM electrons in the medium, white crystal-like structures appeared in center of flask and on the inner walls. Furthermore, both effluents showed the recurring formation of a floating
object with a veil-like-appearance and few sediment particles flushed in effluent reservoirs were covered with a flocculent, fluffy lawn.

3.2. SRB community composition in continuous sediment cultures

The SRB community composition in original sediment samples used as inoculum and in sediments of both culture vessels after 45 and 69 days of culturing were investigated by CARD-FISH. The mean cell number in the inoculum was $1.75 \times 10^8$ cells/g sediment, while cell numbers in the original sediment were in average $3.3 \times 10^8$ cells/g of sediment. Figure 9 shows the development of total cell counts throughout the culturing period. In the first 45 days cell numbers increased by 1.2 to 1.9-fold, followed by a decrease after 69 days. Cell numbers were 1.3 to 3.4-fold lower than after 45 days.

Figure 9: Total cell counts in sediment cultures during culturing period of 69 days. Values presented are biological duplicates.
Figure 10 reflects the relative share of total bacteria, targeted by the probes EUB338 I-III, members of the Desulfosarcina-Desulfococcus-group, targeted by the probe DSS658 and member of the Sva0081-SRB, targeted by probe DSS1431, of total cell counts (DAPI) of the continuous sediment cultures.

The Sva0081-SRB were present throughout the entire culturing period with relative abundances of 5-8% in the inoculum as well as after 45 days and of 3% to 4% after 69 days. Thus, relative abundances of the target group did not increase significantly. The entire DSS-group, targeted by probe DSS658, contributed 9-25% to total cell counts. The variation of counts with probe DSS658 between biological duplicates was similarly high as between culture vessels.

In the original sediment sample from 25th October 2013, the Sva0081 and DSS-group accounted for in average 7 and 17% of total cell counts and thus did not significantly differ throughout the experimental period of in total 110 days.

The Desulfobulbaceae, targeted by the probe DSB706, made up generally less than 2% of cell counts, and were not included in Fig. 8. The FISH counts obtained for probe NON338 were neglectable (<0.2% of all CELL) for all samples.

![Figure 10: Relative proportions of microbial community of cultivated sediment samples of entire period of 69 days.](image)

Seven pH measurements were conducted in vessels A and B. Measurements revealed values between 7.42 and 8.45 with greatest variance of 0.25 pH values among culture vessels (Appendix figure 1). Samples for analysis of chemical parameters were not analyzed as set-ups were not stable and process parameters changed during culturing period.
3.3. Flow-sorting and whole genome amplification of Sva0081-SRB cells from sediment

Evaluation of the 4x ATTO488-labelled probe DSS1431

The 4x ATTO488-labelled probe DSS1431 was tested as a potential substitute for HRP-labeled probes for subsequent fluorescence-activated cell sorting to avoid CARD-inherent problems. Probe DSS1431 labeled with 4x ATTO 488 was compared to differently-labeled EUB338 I probes (Table 9). All EUB338 I probes gave signals, although signal intensity for FLUOS-labeled probes were bleached. Signals of 4x ATTO488-labeled EUB probe were strong and clearly distinguishable from the background figure 11. Probe signals were particularly strong for cells that also depicted a strong signal for DAPI stain. Despite the presence of a population targeted by DSS1431 as shown with CARD-FISH, only very faint signals were obtained for the 4x ATTO488-labeled probe DSS1431 (Fig. 9). Consequently the HRP-labeled probe DSS1431 was used for FACS.

![Micrographs showing FISH/CARD-FISH signals](image)

**Figure 11:** Micrographs showing FISH/CARD-FISH signals of (A) EUB338 labeled with 4x ATTO 488 and (B) DSS1431 labeled with 4x ATTO 488 and (C) DSS1431 labeled with HRP, amplified with Alexa 488 tyramides. All images were acquired with the identical exposure time. Scale bar corresponds to 2 µm.
Flow-sorting of Sva0081-SRB cells

To selectively separate Sva0081-SRB cells from remaining members of the microbial communities, FACS was performed. Ethanol-fixed cells were hybridized with DSS1431 (incl. helpers and competitors) and with NON338, 8 µm filtrated and microscopically evaluated. The NON338 hybridization showed background fluorescence but no signals representing a cell morphology, which indicated the absence of significant endogenous peroxidases, autofluorescence or unspecific probe binding.

Figure 12: Flow cytometric detection of DSS1431 hybridized cells. (A) Sorting gate of population A. (B) Sorting gate of population B. Dashed line indicates polystyrene beads. The color-code represents abundance of detected events of respective SSC and probe fluorescence. Diagram B therefore reflects overall more sorted events than diagram B. Events of $<10^3$ probe fluorescence, here green to dark blue, reflects autofluorescent sediment particles.

As signals for DSS1431 hybridization were strong and unspecific fluorescence was neglectable, background discrimination was considered sufficient. Before the sorting, a few thousands events were detected and plotted as a dot blot (Figure 12). Events of fluorescence $>10^3$ and side light scatter values between $10^1$ and $10^3$ formed a distinct cloud separated from the background fluorescence ($<10^1$ fluorescence) and polystyrene beads. Two populations within the cloud were gated: Population A included events with lower fluorescence compared to population B. By redefining population B as a subpopulation of
population A, a cleaner sorting was expected. Microscopic evaluation of sorted cells showed positively hybridized single cells of coccoid morphology for both populations (Figure 13). No DAPI signals depicting a cell morphology was detected. Additionally to coccoid morphology, deviating morphologies with Alexa 488 signals were apparent (Figure 13). Due to low sample volume, no comparative quantification of different morphologies or gated populations was conducted. From population A 10x 500 events and from population B 8x 500 events were sorted.

![Figure 13: Epifluorescence microphotographs of sorted cells of population B. Scale bar corresponds to 2 µm. Only Alexa 488 signals are depicted. All samples were acquired at the same exposure time.](image)

**Whole genome amplification of sorted populations**

To access further analyse the gDNA of sorted cells, the gDNA of the sorted population was amplified applying MDA. Ten replicates of 500 sorted events of population A, four replicates of population B and three replicates of 500 sorted polystyrene beads were amplified by MDA. As no calibration was conducted prior to MDA, fluorescence does not reflect DNA concentration but a relative increase of double stranded DNA in each reaction. All MDA reactions of sorted cells of population A and population B depicted an increase of relative fluorescence after 104 min. Fluorescence initially increased steadily and then sharply after ca. 80 min for sorted cells of both populations (Figure 14A and B). The MDA reaction 11, sorted cells of population B, did not result in final sharp increase.
The MDA reactions of two batches of sorted polystyrene beads (reaction 14 and 15) depicted similar developments of fluorescence (Figure 14C) like sorted cells, with a delay of 10-15 min. The fluorescence in the DNA-free negative control (Figure 14C) instead, depicted a slight upward trend but did not reach a considerably high increase of fluorescence. The fluorescence of the positive control increased initially sharp and eventually reached a saturation-like appearance.

**Figure 14: Relative increase in fluorescence during MDA reactions.** (A) Ten batches of sorted cells of population A. (B) Four batches of sorted cells of population 2. (C) Three batches of sorted polystyrene beads and negative and positive control of MDA reaction. Values: Individual background fluorescence (fluorescein) subtracted from relative change in fluorescence of dsDNA through intercalation of SYBR green.

In order to test for potential contamination sources during flow sorting and of the MDA reagents, all MDA products from sorted beads and from MDA controls were screened for the presence of the 16S rRNA and *aprA* encoding genes. To increase the sensitivity, 16S rRNA gene targeting amplification were repeated in four reactions, the amplification of the *aprA* gene in triplicates. No PCR product could be obtained for either of the two genes from the
MDA negative and positive control for any of the reactions (data not shown). All PCR reactions of the MDA reactions 16 and 17 of sorted polystyrene beads were negative for amplified products (Data not shown).

Figure 15: 16S rRNA gene PCR products from sorted cells, beads and MDA controls. Template DNA from MDA reaction of sorted cells of population B (11-14), sorted beads (15-17), negative (18) and positive (19) MDA controls and positive control Desulfotalea psychrophila.

Figure 15 shows the result of three replicate 16S rRNA gene targeted PCR reactions of MDA products of sorted cells from population A and B (1-14), sorted beads (15-17) and MDA controls (18-19). Additionally to sorted cells, and PCR control, also the MDA product 15 of sorted beads resulted in a PCR product.

In Figure 16 one can see one replicate PCR reaction targeting the aprA on PCR controls, of initially aprA positive MDA products (13 and 14), MDA products of sorted beads (15-17) and MDA products of MDA controls. The same MDA product (15) of sorted cells, did also exhibit a PCR product. The remaining two and three replicates of PCR reactions targeting the aprA and 16S rRNA encoding gene, were all negative. Amplifiable products indicate the presence of bacterial DNA in MDA products of sorted beads.
3.4. *aprA* gene and 16S rRNA gene diversity in MDA product

To verify the necessity of MDA for PCR based screening, triplicates of 16S rRNA gene targeting PCR reactions were performed on solely sorted cells. No PCR amplicon was obtained for any of the reactions (data not shown).

*aprA* gene diversity in MDA products

All MDA reactions were screened for the adenyl-sulfate reductase (APS) subunit A encoding gene *aprA* in the first place to reveal potential amplification of genomic DNA of sorted cells.

**Figure 16:** *aprA* PCR products from sorted cells, beads and MDA controls. 13 and 14: The only two MDA products that were identified as positive for *aprA*. 15-17: MDA product of sorted beads. No. 15 shows a faint band. 18-19: Controls (PCR-grade water and *Desulfotalea psychrophila*) of MDA reaction

**Figure 17:** *aprA* PCR products amplified from MDA reactions. Numbers correspond to MDA reactions 1-10 in figure 14: MDA reactions of each 500 sorted cells of population A. 11-14: MDA reactions of each 500 sorted cells of population B. 15-17: MDA reaction of 500 sorted beads. 18 and 19: Controls of MDA reaction. PCR positive control: *Desulfotalea psychrophila*.
As Figure 17 shows, only MDA products 13 and 14 sorted from of population B did yield a successful amplification of *aprA* (14% of all reactions). After cloning, sequencing and manual trimming, 35 sequences and 7 sequences were available for MDA reaction 13 and 14. For a first impression BLASTx Analysis was performed: thirty three and six of these sequences depicted high identities with the gene *aprA*.

**Figure 18: Nucleotide micro diversity among all *aprA* retrieved sequences.** Each combination of a letter and number represents one sequence ID. Sequences of a sequence identity >99.5% (dashed line) are shaded. Bold sequence ID: representative sequence for colored areas displayed in Italic sequence ID: Sequences obtained for MDA product 14. Remaining sequences obtained from MDA product 13. Distance measure: Euclidian.

To identify micro diversity, all as *aprA* classified sequences were added to an identity matrix and subjected to cluster analysis. Figure 18 reflects seven defined groups that share a nucleotide identity of >99.5%. The *aprA* sequences obtained from MDA reaction 14 fall all in the same cluster of 100% identity. Representative sequences displayed in bold letters were submitted to BLASTx. Table 11 displays the amino acid identity of submitted sequence to first seven BLAST hits and the most identical cultured relative (*Desulfonema magnum* str. Montpellier). The sequences obtained from both clone libraries showed highest similarities with Sva0081 *aprA* sequences obtained by Lenk from Janssand study site (Lenk, 2011) and the *aprA* of the *Olavius* spp. δ symbiont. The query coverage of depicted representative was 99% in all cases, with E-values ranging from $1 \times 10^{-76}$ in case of alignments with the endosymbiont and $6 \times 10^{-79}$ of alignments with of uncultured Janssand sediment clones.

Interestingly, one sequence obtained from *aprA* clone library of MDA product 13 was identified as 3-octaprenyl-4-hydroxybenzoate decarboxylase of *Escherichia coli*. The sequence identity was 99-100% at 99% sequence coverage and an E-value < $3 \times 10^{-89}$. 

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Table 11: Amino acid sequence identity of representative *aprA* sequences to Sva0081-SRB *aprA* sequences and *Olavius* spp. δ1 symbiont *aprA* sequences. Selection of sequences according to cluster diagram. Sequences depicted below reflected a divergence of >0.5% of nucleotide sequence identity. Sequence identity obtained through BLASTx. Accession number to entry in GenBank.

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16S rRNA gene diversity in MDA products

16S rRNA gene libraries were constructed only from MDA reactions 13 and 14 that showed amplifiable aprA genes in the first place. For each library 46 16S rRNA gene sequences with a length from 421 bp to 903 bp were recovered. Multiple alignment revealed >99.56% sequence identity among all sequences of both clone libraries. BLASTn analysis showed that all sequences were related to the 16S rRNA gene of enterobacterial strains *Escherichia/Shigella* (>99% sequence identity). No Sva0081-SRB-related sequence was recovered.
4. Discussion and conclusion

Cultivation of Sva0081-SRB in continuous sediment cultures

The aim of the first project was to develop a continuous sediment culture that allows the culturing of the yet-uncultured Sva0081-SRB. Since most attempts to isolate environmentally important sulfate reducing bacteria using traditional batch approaches with high substrate concentrations were largely unsuccessful (Colin et al., 2013; Gittel et al., 2008; Köpke et al., 2005; Mußmann et al., 2005), the approach was designed to mimic in situ conditions with low concentrations of fatty acids. To minimize potential gradients and growth of highly motile SRB, a low ratio of sediment:sea water medium was maintained during culturing.

Among the three different continuous sediment culture set-ups tested, only the orbitally shaken continuous sediment culture provided suitable conditions. The testing of a suitable sediment culture system revealed limitations regarding the simultaneous supply of low substrate concentrations under anoxic conditions in a well-mixed sediment without gradients. The fluidized bed-culture-system initially provided enrichment conditions per definition. The continuous suspension of sediment particles enabled only weak gradients, which theoretically minimized the development of a higher microbial diversity. However, due to the unwanted, continuous O₂ influx into the fluidized-bed-culture system, anaerobic conditions could not be sustained and enrichment was not possible. Therefore, we tested the mechanically stirred continuous culture with settled sediment. Here, a decrease of cell abundances in the sediment, which can be most probably attributed to cell disruption by stirring, prevented enrichment and this approach was subsequently disregarded.

Finally, the orbitally shaken continuous culture system allowed the maintenance of low substrate concentrations under sulfate reducing conditions with relatively weak gradients. The maximum possible concentration of electrons in the culture vessel corresponded to the concentration in the medium (Table 2). However, actual substrate concentrations in the culture vessel were lower. Respiration of steadily added low concentrated medium and the nature of a continuous culture enabled substrate concentrations that were far below in situ concentrations of fatty acids (Llobet-Brossa et al., 2002).
The CARD-FISH analysis revealed stable relative abundances of the Sva0081-SRB in sediment cultures over the entire culturing period, but growth could not be significantly stimulated to abundances above \textit{in situ} values, as already observed in earlier enrichment attempts (Ovanesov, 2012).

The dominance of the DSS-group and low proportions detected of \textit{Desulfobulbaceae} are in accordance with the community composition detected \textit{in situ} (Mussmann et al., 2005). Despite great variances among biological duplicates, the analyzed community remained stable and no community shifts within the monitored sulfate-reducing groups were observed throughout the culturing period.

No growth of the frequently cultured DSS-group and \textit{Desulfobulbaceae} (Colin et al., 2013; Gittel et al., 2008) could be promoted, even though supplied compounds are usually readily taken up by a wide range of SRB (Rabus et al., 2006). As no in-depth FISH analysis was done, the remaining community is still unknown. However, \textit{Desulfovibrio spp.} might show high abundances and account to the SRB-community, as this group is readily isolated and enriched (Colin et al., 2013; Köpke et al., 2005; Mußmann et al., 2005).

Surprisingly, the culturing conditions did not allow general significant cell growth compared to the inoculum either. The total \textit{in situ} cell counts obtained for the inoculum were comparable to numbers obtained in other studies (Gittel et al., 2008), even though concentrations of a magnitude higher were reported elsewhere (Gittel et al., 2008; Musat et al., 2006). One could speculate whether the addition of fatty acids and lactic acid had an effect at all. However, before inoculation the stored sediment did not receive any additional substrate between retrieval on 25\textsuperscript{th} October 2012 and the day of the inoculation on 30\textsuperscript{th} November 2012. As a consequence the cell concentration decreased to about 60\%. In contrast, the cell concentrations in the continuous sediment cultures supplied with VFA in the first 45 days of the culturing increased slightly, which indicates an effect of the culture conditions.

The reasons for lack of overall strong growth and enrichment cannot be ultimately explained. The increase and decrease of cell numbers appeared to be unspecific, which underlines a general growth limiting issue. The vital substrates macronutrients (H, O, K, Mg, Ca, Na, Cl, Fe), microelements (Mn, Co, Cu, Mo, Zn, Ni, V, Se, W, B), nitrogen and phosphorus source and carbon-compounds for heterotrophic growth were supplied through amended sea water. Most certainly, still vertical gradients in sediment layers could have led to communities of
different physiological state, such as actively proliferating at the top layer and viable but not proliferating at the bottom. This would also explain the high variability among biological duplicates, when sediments from vessels were not optimally mixed before sub sampling. Additionally, benthic bacteria might require additional compounds that are not sufficiently supplied through sea water medium. The continuous exchange of the sea water and over time decreasing sediment proportions might have simply diluted such compounds that were initially supplied with the sediment inoculum.

Nevertheless, we could maintain a Sva0081-SRB community under laboratory conditions for more than two months. Strong DAPI and CARD-FISH signals, the sulfidic odor, and formation of FeS let deduce the SRB community to be active. This further supports the assumption that the Sva0081-SRB physiology is adapted to low concentrations of fatty acids. With this sulfide producing sediment culture, following physiological experiments can be done.

To my knowledge, no approach has been reported that allows the supply of such low substrate concentrations with a simple set-up. Several anaerobic benthic communities have been cultured with continuous culture systems (Girguis et al., 2003; Girguis et al., 2005; Imachi et al., 2011; Postec et al., 2005; Postec et al., 2007). However, supplied media were usually prepared as complex mixture of carbon sources such as yeast extract and sugars (Imachi et al., 2011; Postec et al., 2005; Postec et al., 2007). Girguis and colleagues cultivated anaerobic methanotrophic ANME-2c and synthrophic partner DSS-group in packed-bed sediment columns with consortium promoting CH₄, H₂S and N₂ purged sea water and could promote growth of both partners (Girguis et al., 2005). However, such packed-bed systems harbor extremely strong gradients, and great amounts of uncharacterized organic carbon substrate. Such conditions do not allow conditions that favor a certain physiological type, as this is the definition of an enrichment culture (Overmann et al., 2013).

As the Sva0081-SRB could not be enriched, the orbital shaken sediment culture set-up requires modifications for future applications. Firstly, a central requirement is the need to support general growth. However, the high throughput of medium corresponded more to in situ sulfate reduction rate than any other known approach. Nevertheless, future approaches should be supplied with substrate concentrations that correspond to the in situ concentrations of ~10 µM acetate and 5 µM of formate, lactic acid and propionate in the vessel at any time (Lenk, 2011)
To guarantee weaker gradients, I suggest the addition of glass beads or the application of artificial colonizable surfaces. As Sva0081-SRB colonize sediment particles (Marc Mußmann, personal communication), artificial substrates might enhance their growth in case of space being the limiting factor. The addition of selected fatty acid might not be sufficient, as SRB with various phylogenetic affiliations and physiological capabilities will readily oxidize such substrates (Muyzer & Stams, 2008; Rabus et al., 2006). The metaproteomic data of the closely related Olavius spp. δ1 endosymbiont indicated uptake of amino acids and peptides (Kleiner et al., 2012). Once a SRB dominated community is established, addition of such compounds might favor the growth of the Sva0081-SRB over other members of the community. An additional treatment to favor the Sva0081-SRB over other SRB is the exposure to molecular oxygen. Molecular oxygen induces the formation of superoxide radicals, which is toxic to many sulfate reducers. However, Sva0081-SRB might be oxygen-tolerant, as indicated by its presence in oxic layers and highly expressed enzymes that facilitated the detoxification of reactive oxygen species in Olavius spp. endosymbiont δ1. By alternating exposure to oxygen, Sva0081-SRB would sustain such episodes and subsequently outcompete O2 sensitive SRB.

With these minor modifications the Sva0081-SRB might not only be maintained in the laboratory but potentially enriched under defined laboratory conditions.

Isolation and genomic amplification of Sva0081-SRB from environmental sample

The second method applied, was a culture-independent method, allowing the targeted physical separation of single cells from a complex microbial community. The separation by FACS allows the enrichment of target cells in a small volume, which can be used for subsequent screening approaches.

In order to prevent the CARD-FISH procedure, 4x labeled FISH probe DSS1431 was tested for its applicability for FACS. On one hand, it is questionable whether the observed signals originated from the probes attached to cells or if it was a very weak signal of bound probes. On the other hand the high background let us deduce, that hybridization was not successful and the probe was absorbed by cells and remaining material on filter. The result from hybridization of probe DSS1431 in this study confirmed issues with hybridization of the δ1
symbiont of cross sections of *Olavius Algarvensis* with the identical probe (Schimak, personal communication). Regardless whether the observed signal was a true signal, the results would not have allowed clean sorting with such low signal intensity. The positive strong signals of parallel hybridized EUB338 I probes could confirm the potential of 4x labeled oligonucleotides for the application of FACS. To our knowledge, no experimental data is available concerning the influence of multiple CLICK chemistry attached fluorophores on the binding behavior of FISH probes. Nevertheless studies have described interactions of the fluorophores and the probe itself as well as the target DNA/RNA that influenced the hybridization efficiency and signal intensity (Moreira *et al.*, 2005; Morrison & Stols, 1993; Stoecker *et al.*, 2010). Laborious evaluation of the ATTO488 labeled probe DSS1431 could not be conducted in the scope of this study. However, it is strongly advised to further investigate the implement of such probes for FACS as I see great potential.

Despite known complications with CARD-FISH and MDA, it was applied for FACS. In order to gain access to the genomic potential of the Sva0081-SRB, single cells were sorted and its gDNA amplified through MDA. To represent the genomic potential of the Sva0081-SRB-community, MDA was performed with 500 pooled single cells. As the Sva0081-SRB-community exhibits 11% sequence divergence on the 16S rRNA gene level (Mußmann, personal communication), it was of particular importance describe the genomic potential of the community rather than of a single cell. Therefore, the amplified gDNA reflects a pan-genome of Sva0081-SRB (Medini *et al.*, 2005).

All reactions were screened for the adenosine-5’-phosphosulfate (APS) reductase subunit A encoding gene *aprA*, to verify the presence of Sva0081-SRB derived DNA in MDA products. This functional gene catalyzes the cytoplasmic reaction of APS to sulfite and AMP. Therefore it is only found in dissimilatory sulfate reducers or sulfite oxidizers (Meyer & Kuever, 2007).

The specific amplification of Sva0081-SRB-gDNA from Janssand sediment sample was possible. For two initially *aprA* positive MDA products, we constructed *aprA* clone libraries. All sequences that were identified as *aprA* sequences exhibited great identity with the Sv0081-SRB-*aprA* sequences obtained by Lenk (Lenk, 2011) (Mußmann, personal communication) and to the *aprA* sequence of the closely related *Olavius spp*.* δ1 endosymbiont. This clearly showed the successful MDA amplification of specifically sorted cells from an environmental sample.
The MDA product includes amplified contaminant DNA. To screen for the bacterial diversity, two 16s rRNA gene clone libraries of the initially aprA positive MDA products were constructed. Surprisingly, all sequences of both 16S rRNA gene libraries were exclusively classified as Enterobacteraceae, which is regarded as a contamination. The retrieval of an E. coli originating gene sequence from an aprA gene library supports these observations. Such contaminations are a common issue in MDA mediated genome amplification, have been described in the literature and are a frequent issue ‘in-house’ (Blainey & Quake, 2010; Lasken, 2012; Stepanauskas, 2012; Woyke et al., 2010). Contaminating DNA is assumed to have been introduced through unspecific cells sorting or contaminated MDA reagents (Woyke et al., 2011). The 16S rRNA gene amplicon 454-pyrotag data available for the study site did not reveal the presence of these mainly human associated Gammaproteobacteria (Mußmann, personal communication), therefore its presence was excluded in the natural sample. However, MDA of dsDNA of sorted beads as well as amplifiable bacterial genes (Figure 14) indicated the presence of contamination in sorted beads.

The presence of contamination requires improvements of the method. As E. coli is a widely applied vector in laboratory work for over expression of MDA enzymes and formation of clone libraries, such a contamination is likely to derive from up- and downstream processing or depicts a local laboratory borne contamination. The contamination of MDA kits with residue sequences of Delftia, Pseudomonas and E. coli is well known (Silke Wetzel, personal communication; Woyke et al., 2011). Furthermore, the introduction through laboratory work is a possibility, particular as the SYBR green and fluorescein solutions had been applied for two years in the department. However, two measures can be regarded as most promising to gain a pure MDA product – the reduction of the contaminant itself and the improved accessibility of the DNA template for genome amplification. Several measures to reduce contamination have been developed. These range from exchange of the sheath fluid pipes in the flow cytometer (Stepanauskas & Sieracki, 2007), repeated sorting to reduce concentration of naked DNA in sheath fluid (Chen et al., 2011), application of DNase- (Zhang et al., 2006) or UV-treatment (Woyke et al., 2011) to remove free DNA. In terms of applicability, Woyke and colleagues present a straight-forward method that breaks dsDNA & ssDNA in MDA reagents through UV treatment. This method significantly reduced contamination with no influence on genome
amplification recovery. Consequently, such precautions should be implemented for future MDA work.

Just as important as the reduction of contamination is the accessibility of the template DNA. As the MDA amplifies the product in an exponential manner, contaminants need to be present at concentrations far below the initial target DNA. Consequently, cell lysis and accessibility to gDNA is of crucial importance. Usually cells are lysed through single treatments with alkaline solution (Woyke et al., 2011), lysozyme or peptidase (Zhang et al., 2006) or a combination of such approaches. However, these cell lysis protocols can increase the likeliness of contamination. The addition of possibly contaminated solutions, lead also to an increase of reaction volume which can promote amplification of non-target DNA (Marcy et al., 2007). Consequently, the heat treatment, applied in this study, in theoretically DNA-free sample buffer was considered as superior. Nevertheless, the obtained results indicate the need for improvement.

The accessibility of the gDNA is particularly problematic in CARD-FISH treated cells. Single cell amplifications were described with 0.1 untreated cell/μL MDA reaction (Rodrigue et al., 2009; Stepanauskas & Sieracki, 2007). The MDA reaction in this study contained 900x the concentration of CARD-FISH sorted cells, of which none was detected on the 16S rRNA gene level. Accumulation of the fluorophore labeled tyramides applied in CARD-FISH could block the amplification. Tyramides are highly active phenol compounds that bind covalently to electron rich moieties, such as the amino acid residues of tyrosine and tryptophan (Bobrow et al., 1989). At concentrations added, tyramides bind to the cells endogenic ubiquitous proteins (Bobrow et al., 1989). By that, the protein-tyramide complexes might stabilize the cell and withstand lysis or genuinely impair with the accessibility of gDNA amplification. In such a case, contaminated DNA would be amplified more readily and would therefore dominate 16S rRNA gene libraries. The effect of preferentially amplification of contaminants, when template is not accessible, has been described as the so called “carrier effect”. Single compounds of template DNA, lysed cells or polystyrene beads adhere to the surface of the reaction vial, thus replacing small quantities of contaminations that are subsequently available for amplification (Handt et al., 1994). This results in an amplification of contaminations in samples, although the negative controls indicate clean conditions. Several studies reported the MDA of FACS-mediated sorted cells. However, the cells were either hybridized with single labeled FISH probes (Miyauchi et al., 2007; Podar et al., 2007)
or stained with unspecific cell or nucleic acid dyes (Fleming et al.; Woyke et al., 2009). None of these methods requires a reporter deposition, therefore keeping the gDNA available for amplification. Despite its strong signal intensity and subsequent clean sorting (Chen et al., 2011), no reports described the combination of CARD-FISH, FACS and MDA. This let us deduce, that MDA of CARD-FISH visualized cells is still a challenging process that requires more attention. For this reason, future work should approach a modified cell lysis protocol and support the substitution of tyramide independent FISH visualizations (Stoecker et al., 2010).

In spite of complications, this method clearly shows the substantial benefit of selective isolation and amplification of gDNA of key players from marine sediments. It allows for the direct link of the phylogenetic affiliation with a metabolic function. With inexpensive PCR-based functional screening (Martinez-Garcia et al., 2012) we have the ability to analyze the genomic potential of the free living Sva0081-SRB. The metagenomic and metaproteomic data of the related δ1 endosymbiont offers several starting points in regards to what genes could be screened for. The detection of acetate-CoA ligase (E.C. 6.2.1.1) and propionate-CoA ligase (E.C. 6.2.1.17) (Kleiner et al., 2012) for the Sva0081-SRB would further support the assumptions drawn from the maintenance of the Sva0081-SRB in the continuous sediment cultures. The presence of an anaerobic CO dehydrogenase and periplasmic uptake [NiFeSe] hydrogenase would not only broaden the substrate spectrum with two electron donors of highly negative reduction potential, but also possibly enable alternative ways for a successful culturing of the Sva0081-SRB. The functional screening of the free living Sva0081-SRB will furthermore shed light into the close relationship to the symbiotic δ1 Sva0081-SRB and reveal a potential core genome.

With the culture based ecophysiological verification, these two methods will substantially broaden our knowledge of the ecophysiology of this world-wide occurring group of SRB. By that, we will better understand the processes and consequences of SRB-mediated carbon re-mineralization in marine sediments.
5. References


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Silke Wetzel (personal communication).


Appendix figure 1: pH values over entire culturing period of 69 days. Depicted are values of both culture vessels A and B.