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Community structure and activity of sulfate-reducing bacteria in an intertidal surface sediment: a multi-method approach

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ABSTRACT: The community structure of sulfate-reducing bacteria (SRB) in an intertidal mud flat of the German Wadden Sea (Site Dangast, Jade Bay) was studied and related to sedimentary biogeochemical gradients and processes. Below the penetration depths of oxygen (~3 mm) and nitrate (~4 mm), the presence of dissolved iron and manganese and the absence of dissolved sulfide indicated suboxic conditions within the top 10 cm of the sediment. Moderate to high bacterial sulfate reduction rates were measured with radiotracers throughout the sediment, and dissimilatory sulfate reduction was also demonstrated by the presence of acid-volatile sulfides (AVS, essentially iron monosulfide). Stable sulfur isotope discrimination between dissolved sulfate and AVS was dominated by sulfate reduction. The diversity of SRB was studied using denaturant gradient gel electrophoresis of 16S rDNA, phospholipid fatty acid analysis and counting viable cells with the most probable number technique. Phylogenetic groups of SRB identified with these techniques were almost evenly distributed throughout the top 20 cm of the sediment. Application of fluorescence *in situ* hybridization, however, demonstrated a maximum of active members of the *Desulfovibrio* and *Desulfosarcina-Desulfococcus-Desulfofrigus* groups between 2 and 3 cm depth. These 2 groups encompass acetate and lactate utilizing SRB. The coincidence of this SRB maximum with a local maximum of sulfate reduction rates and the depletion of acetate and lactate reflects the microbiological processes related to sulfate reduction.

KEY WORDS: Sulfate-reducing bacteria · Microbial diversity · Intertidal sediments · Sulfate reduction rates · Stable sulfur isotopes · Phospholipid fatty acids · Most probable number · Fluorescence *in situ* hybridization

INTRODUCTION

Continental margin sediments include shelf sediments and intertidal mud flats of the coastal ranges. These sediments are generally characterized by a high input of organic matter. Up to 30% of the oceanic primary production takes place in the shelf areas. From this, 25 to 50% sinks to the sediment (Wollast 1991). In addition, the deltaic areas of the shelf regions are receiving up to 90% of the global flux of particles and organic matter transported by rivers to the oceans (Gibbs 1981). Since most of the deposited matter is remineralized (Berner 1982), continental margin sediments play a prominent role in the marine carbon cycle, even though they encompass only 10% of the total ocean area. The oxidation of organic carbon is performed by microorganisms that employ an array of electron acceptors, each allowing a different yield of free energy. In accordance with the decreasing yields,

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a zonation of the oxidants is usually observed in the vertical sediment profiles: O_2, NO_3^-, Fe(III) and Mn(IV), SO_4^{2-}, and finally CO_2 (Froelich et al. 1979). Using the $^{35}SO_4^{2-}$ radiotracer technique (Jørgensen 1978, King 2001) it was shown that up to about 50% of the organic matter in continental margin sediments is mineralized via microbial sulfate reduction (Jørgensen 1982a). Since sulfate concentration in seawater is about 28 mM, it may still be abundant in deeper layers of marine sediments and, consequently, the dominant electron acceptor (Fenchel et al. 1998). The areal rates of dissimilatory sulfate reduction in intertidal sediments may vary considerably between about 0.2 and 104 mmol m^{-2} d^{-1} (Trudinger 1992). Sulfate reduction rates are controlled by temperature (Vosjan 1974) and the availability of reactive organic compounds (Schubert et al. 2000), and are correlated to the abundance of sulfate-reducing bacteria (SRB) (Sahm et al. 1999, Böttcher et al. 2000).

The importance of sulfate reduction for the remineralization of organic carbon in marine sediments is reflected also by the variety of metabolic capacities of SRB isolated from these environments. Pure cultures of SRB have been shown to utilize H_2 and organic substrates such as short chain fatty acids, alcohols and aromatic compounds, and to completely oxidize acetate to CO_2 (Widdel 1988, Rabus et al. 2000), all important substrates in the anoxic zones of marine sediments. Phylogenetic analysis of isolates (Devereux et al. 1990) and the introduction of 16S rRNA based molecular tools (Amann et al. 1995) now allow SRB to be identified and quantified in their natural habitat. This type of investigation was only recently applied to marine sediments (Devereux et al. 1996, Llobet-Brossa et al. 1998, Ravenschlag et al. 1999, 2000, Sahm et al. 1999, Bowman et al. 2000, Wieringa et al. 2000).

Up to now, the contribution of SRB to the remineralization of carbon in marine sediments has mainly been studied by application of selected, specialized methods. However, the complexity of the system requires multiple methods in order to obtain a comprehensive overview of the controlling factors and their interactions. Therefore, we applied a fully integrated approach by combining cultivation-dependent and -independent microbiological techniques with biogeochemical and stable isotope analyses, to advance our understanding of the relationship between the community structure of sulfate-reducers and zones of dominant usage of the different electron acceptors. For the present study, a marine sediment with high activity of SRB was chosen. Site Dangast, an intertidal sediment at the southern boundary of the North Sea, met these criteria due to high loads of organic material and abundant sulfate.

MATERIALS AND METHODS

Study site. The sampling station is located in the 'Jadebusen' (Jade Bay), a meso- to macrotidal embayment (Irion 1994), which is situated in the coastal area to the west of the Weser estuary in the northern part of Lower Saxony (Germany). The sediment is exposed to the air for about 5 h, leaving it inundated for about 7 h. The sampling station, Site Dangast, is located 2 km west of the small village of Dangast, about 25 m west of a tidal creek ('Dangast Tief'), connected to a freshwater outlet ('Dangast Siel') and about 15 m north from the shore-line (Fig. 1).

Processing of sediment samples. Sediment cores were obtained on June 28, 1999, at low tide with polycarbonate tubes (diameter 8 to 10 cm; length 50 cm).

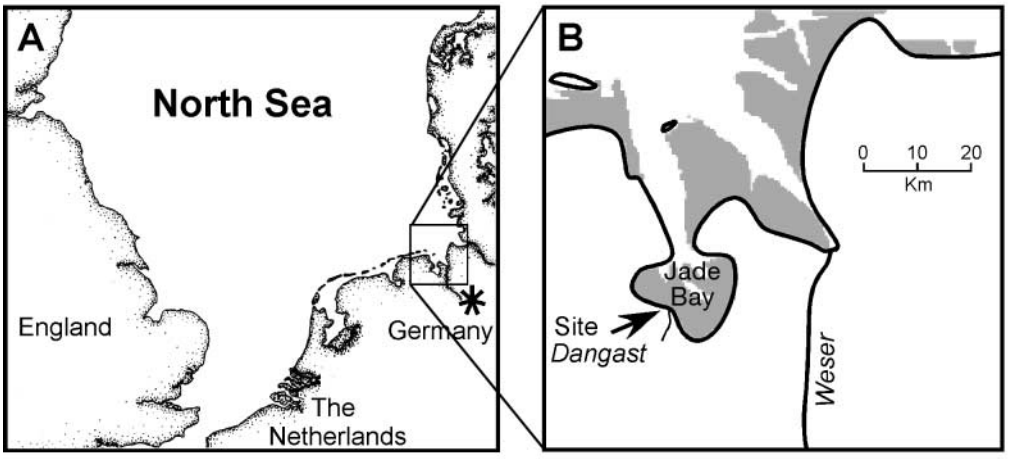

Fig. 1. Location of the sampling site 'Dangast'. (A) The southern coastal area of the North Sea. (*) Bremen. (B) Position of Site Dangast in the Jade Bay is indicated by an arrow. Shading represents areas of intertidal sediment

The tubes were closed with air-tight rubber stoppers on both ends and transported cool (approx. 4°C) and in the dark to the laboratory for further processing of the sediment cores within about 3 h.

Sediment cores were sliced into the following layers: 0–0.5, 0.5–1, 1–2, 2–3, 3–4, 4–5, 5–10, 10–15 and 15–20 cm (top-down). Sediment samples for enumeration of viable cells (most probable number, MPN) and molecular analysis (denaturing gradient gel electrophoresis, DGGE and fluorescence *in situ* hybridization, FISH) were taken from the same sediment layers of a single core. For geochemical measurements, parallel cores were used and sliced in 1 cm layers under inert gas (N_2) in a temperature-controlled room (4°C). All cores were taken in close proximity from an area of 1 by 2 m.

Characterization of organic material and sulfur speciation. Pore water contents in the sediments, total carbon (TC) and total inorganic carbon (TIC) were determined as described earlier (Böttcher et al. 2000). Total organic carbon (TOC) contents were obtained from the difference of TC and TIC. Phospholipid fatty acids (PLFA) were extracted essentially as described previously (Bligh & Dyer 1959). The mild alkaline methanolysis procedure was used to transmethylate the ester linked fatty acids of phospholipids to methyl esters (Palojärvi & Albers 1998). A sample volume of 2.0 µl was injected in a splitless mode onto a fused silica capillary column (Optima-5-MS, Macherey und Nagel). The fatty acid methyl esters (FAME) were identified by the use of a gas chromatography-mass spectrometry (GC-MS) system and quantified with a gas chromatography-flame ionisation detection (GC-FID) (GC-Q, Finnigan, and GC-Autosystem, Perkin Elmer). The GC-temperature-program is described elsewhere (Palojärvi & Albers 1998). Quantification was based on nonadecanoic acid methyl ester as internal standard and identification on authentic FAME standards (Supelco; Biotrend). Biomass was determined by lipid-bound phosphate analysis (Findlay et al. 1989).

The fraction of acid volatile sulfide (AVS) was separated from the wet, Zn-acetate preserved sediment by the reaction with cold 6 N HCl containing $SnCl_2$ (Duan et al. 1997) in a stream of nitrogen. The addition of $SnCl_2$ increased the recovery of the AVS fraction within the first 5 cm, but no further influence was observed at greater depths (Böttcher unpubl. data). Sulfur isotope ratios (see below) of the AVS fraction recovered by both methods agreed within 1‰ (Böttcher unpubl. data). The sum of (essentially) pyrite and (minor) elemental sulfur (fraction 'Cr-II') was obtained by the distillation with hot acidic Cr(II)chloride solution (Fossing & Jørgensen 1989). H_2S was trapped as Ag_2S in an $AgNO_3$ solution, carefully washed with 10% NH_3 solution and distilled water, dried, and quantified gravimetrically.

For stable sulfur isotope analysis ($^{34}S/^{32}S$), sulfate was precipitated from filtered (membrane filter with 0.45 µm pore width) Zn-acetate preserved pore water samples as $BaSO_4$, carefully washed and dried. Sulfur isotope ratios of the AVS, Cr-II, and pore water sulfate fractions were measured by combustion isotope-ratio monitoring mass spectrometry (C-irmMS) (Pichlmayer & Blochberger 1988). Samples were converted to SO_2 using a Eurovector elemental analyzer which was connected to a Finnigan MAT Delta$^+$ gas mass spectrometer via a Finnigan Conflo II split interface. Isotope ratios are given in the δ-notation versus the SF_6-based Vienna-Canyon Diablo Troilite (V-CDT) standard (Ding et al. 2001). International standards IAEA-S-1, IAEA-S-2 and IAEA-S-3 were used to calibrate the mass spectrometer. A $\delta^{34}S$ value of 21.0‰ was measured for the international barium sulfate standard NBS 127.

Pore waters. Air and pore water temperatures were measured with a digital sensor (GTH 1150 digital thermometer) at the beginning and the end of the sampling session. Pore waters were separated from the sediment sections by centrifugation in closed vessels under inert gas (N_2). Prior to analyses, pore waters were filtered through membrane filters (0.45 µm; Sartorius) and acidified with nitric acid (reagent grade quality) into precleaned polyethylene (PE) bottles. Concentrations of dissolved iron, manganese and sulfate were analyzed after appropriate dilution by means of inductively coupled plasma optical emission spectroscopy (ICP-OES) (Perkin Elmer Optima 3000 XL). Results from a gravimetric quantification of dissolved sulfate as $BaSO_4$ in a parallel core agreed well (Fig. 2). H_2S was measured in selected samples preserved with 2% $ZnCl_2$ solution according to Cline (1969). Salinity of filtered samples was measured with a hand refractometer.

The porewater volatile fatty acid (VFA) concentrations were measured by HPLC as 2-nitrophenyl hydrazine derivates as described by Albert & Martens (1997). The concentration of butanol was reduced to 1.25%, and the concentration of tetrabutylammonium hydroxide to 1 mM in Solvent A and the concentration of tetradecyltrimethylammonium bromide was reduced to 25 mM in Solvent B, as compared to the original method. The detection limit for glycolate and lactate was 0.2 µM, for acetate and propionate 0.3 µM and for formate 0.6 µM.

Microsensor measurements. Profiles of oxygen, hydrogen sulfide, and nitrate concentrations at the sediment-water interface were measured in the laboratory with microelectrodes. The determination of oxygen profiles was done with Clark-type O_2 sensors with guard cathodes (Revsbech 1989) and calibration was

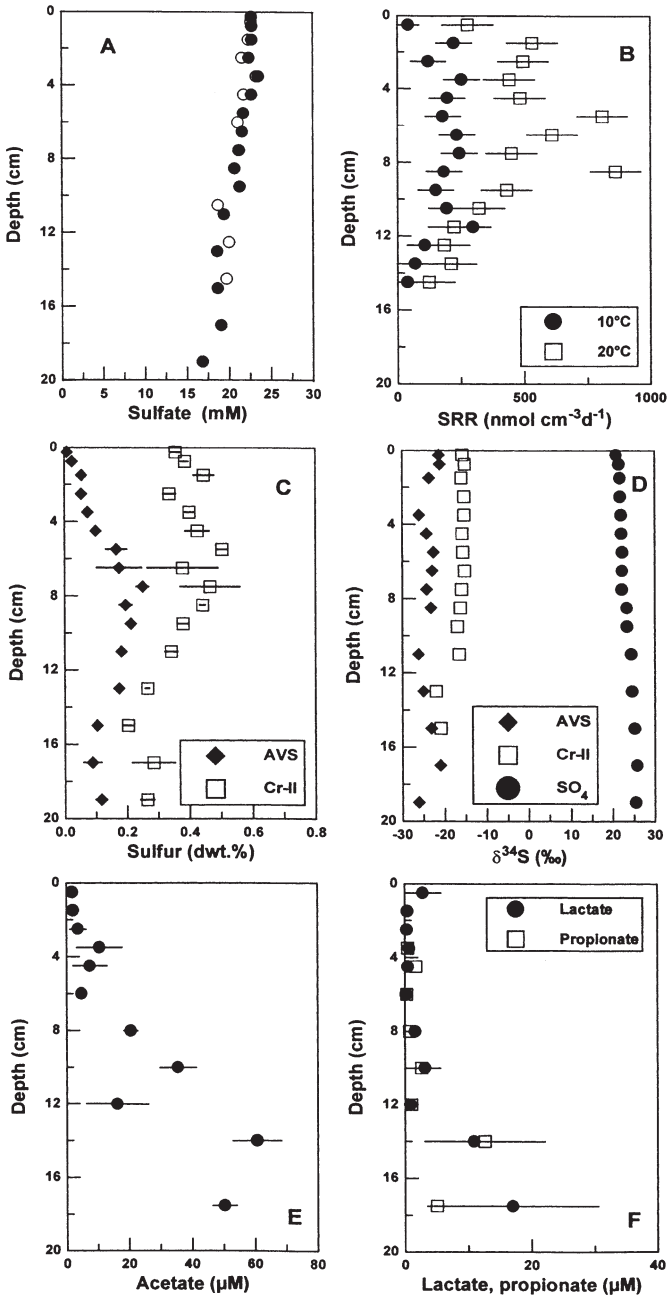

Fig. 2. Biogeochemical analysis of the sediment at Site Dangast. In all cases, the uncertainty of the analytical measurements is within the size of the symbols. (A) Dissolved sulfate (open and solid symbols are from 2 parallel cores). (B) Microbial (SRR) at 2 incubation temperatures approximating the temperate shift during day-night cycles within the sampling period (variance bars result from a comparison with extensive SRR measurements at different temperatures in surface sediments from Site Dangast; Böttcher et al. unpubl.; Llobet-Brossa et al. unpubl.). (C) Cr(II)-reducible sulfur (Cr-II) and acid volatile sulfides (AVS). Variance bars result from duplicate distillations. (D) Isotopic composition of sulfur species. (E) Acetate concentrations (variance bars result from 2 parallel cores). (F) Lactate and propionate (variance bars result from 2 parallel cores)

based on calculations described elsewhere (Garcia & Gordon 1992). Amperometric H_2S microsensors were constructed, calibrated and applied as described by Kühl et al. (1998). Nitrate was measured with a microbiosensor in which nitrate and nitrite are reduced to N_2O by bacteria and N_2O is detected electrochemically (Larsen et al. 1997).

Sulfate reduction rates (SRR). Bacterial SRR were measured using the whole-core incubation technique with the injection of a carrier-free $^{35}SO_4^{2-}$ tracer (Jørgensen 1978, Fossing & Jørgensen 1989). After sampling, sediment cores were equilibrated in the laboratory at 10 and 20°C for several hours and subsequently incubated with the radiotracer (~200 kBq per injection at 1 cm intervals) for 4.5 h in the dark. Activities counted with a Packard liquid scintillation counter were corrected for blank contributions derived from the counting and the distillation procedures. The apparent activation energy was calculated from an Arrhenius equation using the depth-integrated SRR for 10 and 20°C.

Nucleic acid extraction and DGGE amplification. Parallel sediment cores were processed to avoid changes in the community composition caused by handling and containment as described before (Rochelle et al. 1994). DNA and RNA of each sediment horizon were extracted from 1.5 ml wet sediment by bead-beating, phenol extraction and isopropanol precipitation as described previously (Sahm & Berninger 1998). PCR amplification specific for SRB of the δ-subclass of *Proteobacteria* was carried out with the forward primer SRB385 carrying a GC-clamp and the reverse primer 907 (Sass et al. 1998). DGGE, excision of bands, reamplification, and sequencing were performed as previously described (Muyzer et al. 1996).

DGGE partial sequences were added to an alignment of about 30 000 homologous bacterial 16S rRNA genes (Maidak et al. 2002) by using the aligning tool of the ARB program package[1]. Aligned sequences were inserted within a stable tree by using the ARB parsimony tool (Ludwig et al. 1998). Accession numbers of the partial sequences are AF538272 through AF538278, and deposited at GenBank.

FISH and cell counts. Hybridizations, microscopic examination and counting of hybridized cells and total cell counts were performed as previously described (Snaidr et al. 1997, Llobet-Brossa et al. 1998). For each sample, between 700 and 1000 DAPI-stained cells were analyzed and counted; duplicates were carried out for each sample. The oligonucleotide probes used

[1]Strunk O, Gross O, Reichel B, May M and 10 others (1998–2002) ARB: a software environment for sequence data. Available at: www.mikro.biologie.tu-muenchen.de

in this study were purchased from Interactiva with Cy3 fluorochrome at the 5′ end. The probes used are listed in Table 1. For FISH of sediment samples, 2 replicate sediment cores were cut and processed as described before (Llobet-Brossa et al. 1998). From MPN tubes that showed growth, aliquots of 1 to 2 ml were withdrawn with N_2-flushed syringes. Cells were sedimentated by centrifugation and washed once with 1 × PBS. Subsequent fixation and hybridization were performed as previously described (Snaidr et al. 1997).

Media and enumeration of viable cells by MPN. A defined, bicarbonate-buffered, sulfide-reduced (1 mM) seawater-like mineral medium was used for cultivation experiments (Widdel & Bak 1992). Na-dithionate at a final concentration of 10 µg ml^{-1} was applied as an additional reductant. Organic substrates were added from concentrated stock solutions. Gaseous substrates (H_2/CO_2) were supplied by applying an overpressure of 1 atm to the headspace of the culture tubes.

Viable SRB were enumerated using an MPN technique with liquid media and with agar shakes. Samples from each layer of the sediment core were transferred via a funnel to glass bottles (250 ml volume) and mixed 1:1 with substrate-free, anoxic media under a steady stream of N_2/CO_2 (90:10 [v/v]), yielding approximately 100 ml of homogenate. The bottles were then anoxically sealed with butyl-rubber stoppers and screw caps. These sediment slurries were diluted in steps of 1:10 by transferring aliquots to substrate-free, anoxic media under N_2/CO_2 (90:10 [v/v]) in

Table 1. Oligonucleotide probes

Probe	Target	Sequence (5′–3′) of probe	[%] FA[a] *in situ*	Source
EUB338	Bacteria 16S rRNA, position[b] 338–355	GCT GCC TCC CGT AGG AGT	0–35	Amann et al. (1990)
Arch915	*Archaea* 16S rRNA, position 915–935	AG GAA TTG GCG GGG GAG CAC	35	Amann et al. (1990)
DNMA657	*Desulfonema sp.* 16S rRNA, position 657–676	TTC CGY TTC CCT CTC CCA TA	35	Fukui et al. (1999)
DSV698	*Desulfovibrio* (16 species) 16S rRNA, position 698–717	GTT CCT CCA GAT ATC TAC GG	35	Manz et al. (1998)
DSV1292	*Desulfovibrio* (13 species) 16S rRNA, position 1292–1310	CAA TCC GGA CTG GGA CGC	35	Manz et al. (1998)
DSV407	*Desulfovibrio* (3 species) 16S rRNA, position 407–424	CCG AAG GCC TTC TTC CCT	50	Manz et al. (1998)
DSD131	*Desulfovibrio* (1 species) 16S rRNA, position 131–148	CCC GAT CGT CTG GGC AGG	20	Manz et al. (1998)
DSV214	*Desulfovibrio/D'microbium* 16S rRNA, position 214–230	CAT CCT CGG ACG AA TGC	10	Manz et al. (1998)
DSS658	*Desulfosarcina/D'coccus/D'frigus* 16S rRNA, position 658–675	TCC ACT TCC CTC TCC CAT	60	Manz et al. (1998)
DSB985	*Desulfobacter/D'bacula* 16S rRNA, position 985–1003	CAC AGG ATG TCA AAC CCA G	20	Manz et al. (1998)
SRB221	*Desulfobacterium* 16S rRNA, position 221–238	TGC GCG GAC TCA TTC AAA	35	Devereux et al. (1992)
SRB660	*Desulfobulbus* 16S rRNA, position 660–679	GAA TTC CAC TTT CCC CTC TG	60	Devereux et al. (1992)
DSBO224	*Desulfobotulus* 16S rRNA, position 224–242	GGG ACG CGG ACT CAT CCT C	60	Manz et al. (1998)
DSMA488	*Desulfovibrio/D'monile* 16S rRNA, position 488–507	GCC GGT GCT TCC TTT GGC GG	60	Manz et al. (1998)
DSR651	*Desulforhopalus* 16S rRNA, position 651–668	CCC CCT CCA GTA CTC AAG	35	Manz et al. (1998)
DTM229	*Desulfotomaculum* 16S rRNA, position 229–246	AAT GGG ACG CGG AXC CAT	15	Manz et al. (1998)
SVAL428	*Desulfotalea/D'fustis* 16S rRNA, position 428–446	CCA TCT GAC AGG ATT TTA C	25	Sahm et al. (1999)
NON338	None (negative control)	ACT CCT ACG GGA GGC AGC	0–35	Wallner et al. (1993)

[a]Percentage of formamide (FA) in hybridization buffer. [b]*Escherichia coli* numbering

butyl-rubber sealed glass bottles (volume of bottle was 250 ml, total volume of dilution was 100 ml). The transfer of sediment suspensions between the glass-bottles was carried out with N_2-flushed syringes. Prior to transfer, the sediment suspensions were shaken vigorously to achieve optimal mixing. These 'master' dilutions were used to inoculate culture tubes for MPN counts in liquid media. Similarily, 'master' dilutions were prepared for the inoculation of MPN counts in agar shakes. However, in this case, not every single sediment layer was diluted but rather a homogenous mix of the top 5 cm of the sediment core.

MPN counts in liquid media were carried out in glass tubes (160 × 16 mm) sealed with butyl-rubber stoppers and screw caps that contained anoxic media with or without substrate under an N_2/CO_2-atmosphere (90:10 [v/v]). Substrates used were as follows: H_2/CO_2 (90:10 [v/v], applied with 1 atm to the gas head space); H_2/CO_2 + 2 mM acetate; 15 mM formate; 7.5 mM acetate; 5 mM propionate; 5 mM lactate. Each tube contained 9 ml medium and was inoculated with 1 ml from the corresponding 'master' dilution. Three replicates were prepared for each substrate condition. To sustain reduced conditions, 0.5 mM Na_2S were added after about 4 wk of incubation. The tubes were incubated at 23°C for 10 mo. Growth of SRB was determined by measurement of the optical density (660 nm) and sulfide using the semiquantitative method described by Cord-Ruwisch (1985) and by macroscopic and microscopic examination.

MPN counts in agar shakes were conducted to obtain single colonies. Glass tubes sealed with butyl-rubber stoppers containing approximately 9 ml of anoxic media with molten agar and substrates as described above were prepared as summarized previously for the isolation of SRB (Widdel & Bak 1992). The tubes were inoculated with 1 ml from the corresponding 'master' dilution. Replicates and incubation conditions were as described above for MPN counts in liquid media. Agar shakes were incubated at 23°C. Growth was determined by microscopically observing formation of brownish colonies which are typical for SRB. Such colonies were picked by means of finely drawn Pasteur pipettes (Widdel & Bak 1992) and transferred to liquid medium containing the corresponding substrate.

RESULTS

Sediment

Downcore temperatures in the sediments increased from 15.8°C at the surface to 17.5°C at depth during sampling. The sediments displayed 2 distinct color changes: the oxic upper part was brown, which turned into a darker olive green with some greyish diffuse streaks within the first 1 to 2 cm. At about 9 to 12 cmbsf (cm below surface), the sediment turned black. These vertical changes were also reflected by geochemical and biological parameters. The sediment was characterized by bioturbation, and living polychaetes were found down to about 16 cmbsf. The first 10 cm almost completely consisted of mud (clay and silt grain size fraction < 63 µm; essentially phyllosilicates; Böttcher et al. 2000). An increase in the sand grain fraction was observed below that depth (Böttcher et al. 2000). Correspondingly, the pore water contents decreased continuously with depth. During sampling in June 1999, for instance, a maximum water content of 67% was observed near the surface (0.5 cm depth), which decreased to 52 and 29% at 10 and 29 cm depth, respectively. The TOC contents decreased with depth from 3% dry weight (surface to 8 cmbsf) to 1% (16 cmbsf) in parallel with the pore water contents. This covariation is also known from other tidal sediments of the German Wadden Sea (Delafontaine et al. 1996, Böttcher et al. 1998a, 2000, Volkman et al. 2000).

AVS (essentially iron monosulfide) were found in all sections and showed a maximum at around 8 cm depth (Fig. 2C). The stable sulfur isotopic signature of AVS essentially mirrors that of H_2S which is derived from the overall metabolic processes in the sulfur cycle (Böttcher et al. 1998b). The AVS fraction ($\delta^{34}S$ values between –21.1 and –26.2‰) was significantly enriched in the lighter sulfur isotope compared to coexisting pore water sulfate ($\delta^{34}S$ values between +20.8 and +25.8‰; Fig. 2D). Apparent sulfur isotope enrichment factors between –42 and –53‰ are calculated from the data given in Fig. 2D which are at the upper end or even exceed the results obtained in experiments with pure cultures of SRB (Kaplan & Rittenberg 1964, Chambers et al. 1975, Bollinger et al. 2001). The isotopic composition of the Cr-II fraction, which consists essentially of pyrite, was more or less constant in the top 11 cm ($\delta^{34}S$ of –16 ± 1‰) but decreased at greater depths (Fig. 2D). Sulfur isotope data are within the range reported previously for iron sulfides in intertidal sediments (Chambers 1982, Böttcher et al. 1998a, 2000).

Pore waters

The salinities of the pore waters during sampling in June 1999 were rather constant with 28‰. This corresponds well to earlier mean data (26‰) obtained during sampling on a seasonal base over a 2 yr period (Böttcher unpubl.).

The composition of interstitial waters sensitively mirrors the biogeochemical processes taking place in the sediment. Microsensor measurements revealed oxygen

and nitrate penetration depths during sampling of 3.1 and 3.6 mm, respectively. No hydrogen sulfide was detected by microsensors in the sediment cores within the first 20 mm. Sub-oxic, non-sulfidic conditions between about 5 mm down to at least 12 cm depth were indicated by the presence of dissolved Fe(II) (12 to 120 µM) and Mn(II) (36 to 63 µM) and sulfide concentrations below 5 µM. Sulfate concentrations at the sediment-water interface correspond well to the measured salinities, indicating that dilution of seawater with sulfate-poor freshwaters took place in the surface waters of the coastal region. Sulfate remained essentially constant within the first 4 to 5 cm and decreased further downcore (Fig. 2A), associated with an enrichment in ^{34}S (Fig. 2D). This is expected for a system in which the consumption of sulfate by bacteria exceeds the supply of sulfate by transport from the sediment-water interface or by reoxidation of sulfide (Hartman & Nielsen 1969). Despite minor changes in dissolved sulfate, moderate to high microbial SRR were measured with radiotracers through the whole sediment core and showed a maximum in the suboxic zone at around 7 cmbsf (Fig. 2B). An apparent activation energy of 66 kJ mol^{-1} is obtained from the depth-integrated SRRs at 10 and 20°C. The measured SRR were within the range reported previously for fine-grained tidal sediments of the North Sea (Oenema 1990, Böttcher et al. 2000, Kristensen et al. 2000), and a temperature-shift laboratory experiment with surface sediments from Site Dangast (Llobet-Brossa et al. unpubl.).

The concentrations of VFA (acetate, propionate, and lactate; Fig. 2E,F) were determined in the pore waters of 2 sediment cores. In both cases, concentrations of VFA except for acetate were below 5 µM in the top 5 cm of the sediment. Acetate concentration increased with depth to concentrations of about 50 to 60 µM. Concentrations of glycolate and formate on the other hand did not exceed 5 µM throughout the entire core. Elevated concentrations of lactate and propionate ranging between 10 and 30 µM were detected in the deepest horizon of 15 to 20 cm. This accumulation of organic acids occurred only below the maximum of the SRR (Fig. 2B,E,F).

PLFA analysis

In the sediment samples at Site Dangast, 34 PLFA were identified. The profiles of 6 selected PLFA, which were previously described as marker fatty acids for SRB and other anaerobic bacteria (Findlay & Dobbs 1993), are presented in their relative and absolute abundance (Fig. 3). Within the upper 3 horizons (top 2 cm of the sediment) relative and absolute values of all 6 selected PLFA increased significantly. In a sediment depth of 2 to 5 cm, only minor variations of the absolute values were observed, whereas in deeper layers a strong decrease occurred. Below 2 cm depth, the profiles of the absolute values coincided with those of the total biomass, as estimated by the lipid-bound phosphate concentration (data not shown). Therefore, with the exception of i17:1 and cyc17:0, the relative amounts of the selected PLFA varied only slightly with depth below 2 cm.

DGGE analysis of sediment

Changes in the diversity of the SRB population with depth were analyzed by DGGE of PCR-amplified 16S rDNA fragments and of the reversely transcribed 16S rRNA fragments. In the same sediment sample, the

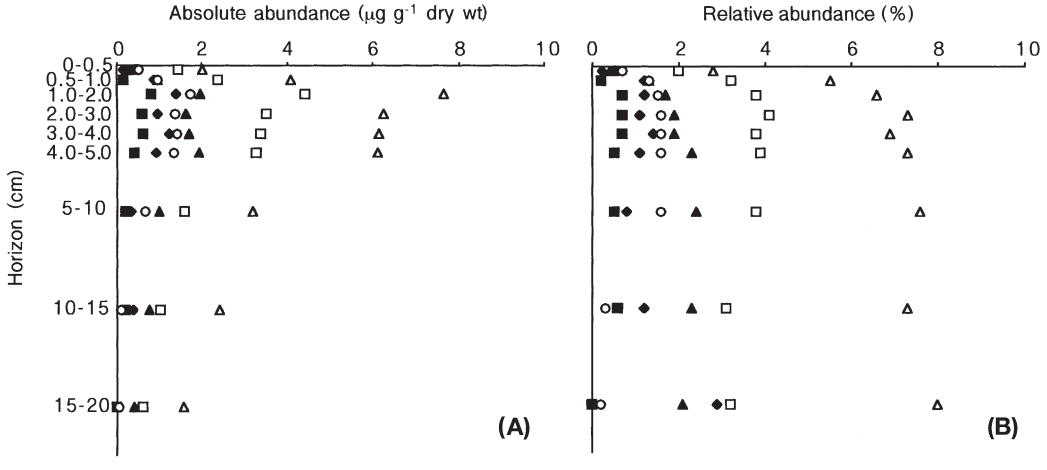

Fig. 3. Depth profiles of selected phospholipid fatty acids (PLFA) of sediment at Site Dangast. Specific PLFAs of sulfate-reducing (◆: i17:1, ■: a17:1 ▲: 10Me16:0) and other anaerobic bacteria (◇: i15:0; □: a15:0; △: cyc17:0) from different sediment horizons are plotted. (A) µg values (indicating absolute abundance). (B) Weight percent of total PLFA (indicating relative abundance)

electrophoretic profiles of the 16S rDNA fragments (Fig. 4A) were more complex than the ones observed after reverse transcription of the 16S rRNA (Fig. 4B).

DGGE of 16S rDNA showed between 6 and 8 bands of different intensities. There were no major changes between different sediment layers, except for the deepest layer at 15 to 20 cm depth.

DGGE of 16S rRNA showed only 2 to 4 bands in all sediment layers. In contrast to the 16S rDNA-based DGGE, these were not evenly distributed but appeared as distinct patterns. It was not possible to get any PCR product from the deepest layer. As rRNA-based DGGE is influenced by the 'activity-regulated' ribosome contents, this pattern might better indicate the identity and distribution of the active SRB in the sample.

All DGGE bands analyzed by sequencing were shown to originate from members of the δ-subclass of *Proteobacteria*, the taxonomic group that encompasses most Gram-negative SRB (Fig. 4C). DGGE bands 1 and 2 were affiliated with a sequence similarity of 99% to *Desulfobulbus* sp. (accession numbers L40786 and L40785, respectively). DGGE band 3 was found to be closely related to *Desulfovibrio caledoniensis* (accession number U53465), sharing similarities of 97%. DGGE band 5 was identified as *Desulfobacter* sp. with 96% similarity (accession number L40787). DGGE bands 6 and 7 were related with 99% similarity to *Desulfonema limicola* (accession number U45990), and to *Desulfonema ishimotoei* (accession number U45991), respectively. DGGE band 8 had 98% sequence similarity with *Desulfuromonas palmitatis* (accession number U28172).

MPN counts of sulfate-reducing bacteria

A variety of defined substrates was used to meet specific substrate preferences of different SRBs possibly occurring in the studied sediment. The bacterial numbers from MPN counts with liquid media are shown in Table 2.

After about 6 wk of incubation only low cell numbers ranging between 1.1×10^3 and 4.6×10^5 cells cm^{-3} could be observed, with the highest cell numbers

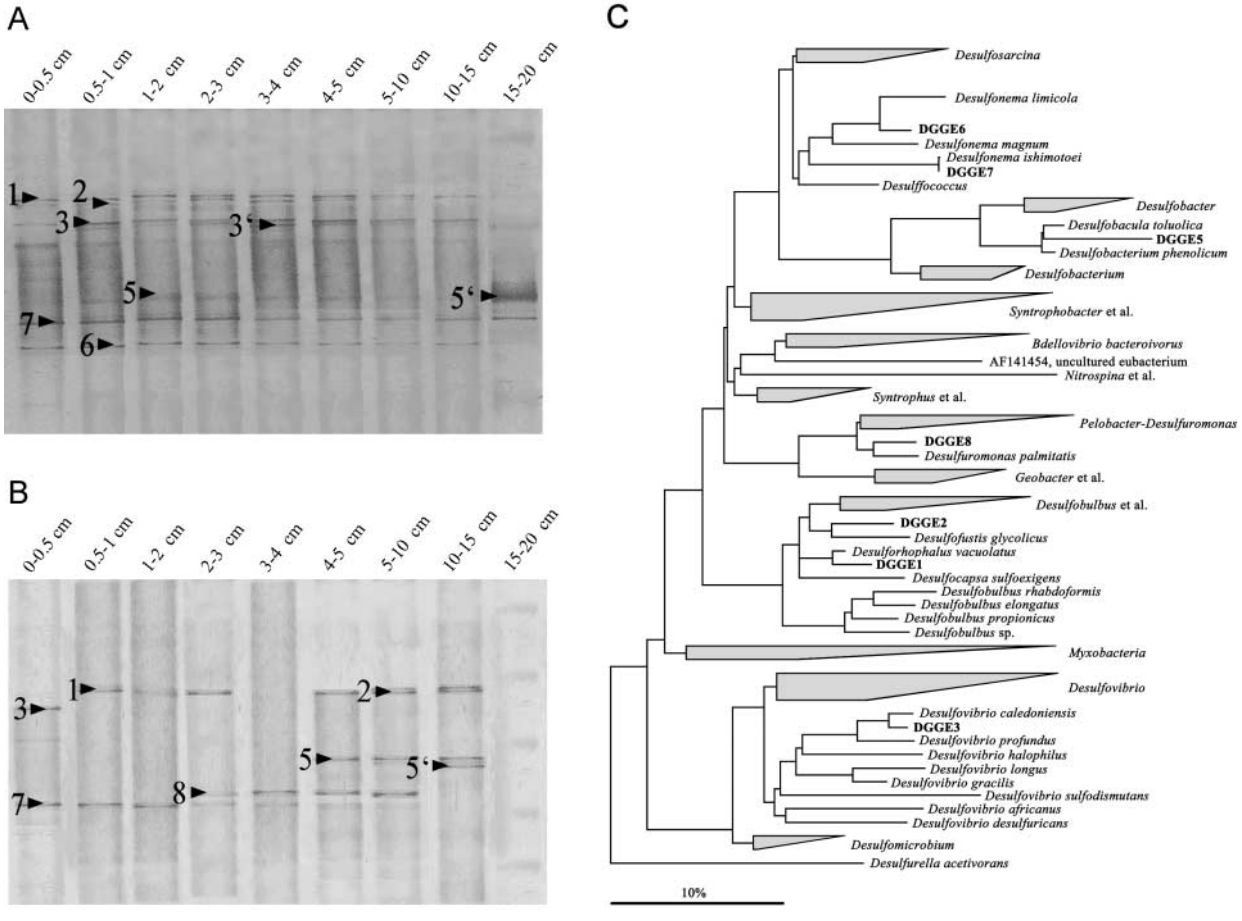

Fig. 4. DGGE analysis of sediment horizons at Site Dangast. (A) DGGE profiles of 16S rDNA. (B) DGGE profiles of reversely transcribed 16S rRNA. (C) Affiliation of sequenced DGGE fragments

determined in MPN cultures with H_2/CO_2/acetate as substrates. In general, cells grew homogeneously in the medium. These fast-growing SRB appeared to be evenly distributed across a depth profile down to 15 cm. However, cell numbers decreased by about 1 order of magnitude in the lowest horizon of 15 to 20 cm. With all substrates tested, rather similar cell numbers were obtained for all horizons analyzed.

Prolonged incubation (10 mo) of the MPN cultures allowed the detection of slowly growing but more abundant SRB. Counts increased up to 1.1×10^7 cells cm^{-3}. In contrast to the faster growing SRB, here cells did not grow homogeneously distributed in the medium, but rather formed flocs and aggregates, sometimes similar of the cell packages known from *Desulfosarcina* spp. As with the fast-growing SRBs,

Table 2. Total cell counts (cells cm^{-3}, by MPN[a]) and identification (by FISH, probes as described in Table 1) of sulfate-reducing bacteria (SRB) from different sediment horizons from Site Dangast after 1.5 and 10 mo of incubation at 23°C with various substrates typical for SRB. For each sediment horizon analyzed and each substrate used for growth, 2 lines of investigations were followed. First, viable cell numbers were determined by MPN. Second, the phylogenetic affiliation of bacteria grown in the highest MPN dilution was determined by FISH. Results for both investigations are presented in the table. nd: not detected

		Horizon (cm)								
		0–0.5	0.5–1	1–2	2–3	3–4	4–5	5–10	10–15	15–20
Hydrogen (CO_2)[b]										
1.5 mo	MPN	1.1×10^3	4.6×10^3	4.6×10^3	1.1×10^3	1.1×10^3	1.1×10^3	nd	1.1×10^3	nd
	FISH	DSB985	nd	SRB221	SRB221	SRB221	SRB221	nd	SRB221	nd
10 mo	MPN	4.6×10^6	1.1×10^7	1.1×10^6	4.6×10^6	1.1×10^6	4.6×10^6	4.6×10^6	4.6×10^6	1.1×10^6
	FISH	DSB985/[c] SRB221	DSB985	SRB221	SRB221	SRB221	nd	SRB221	SRB221	SRB221
Hydrogen (CO_2 + acetate)[b]										
1.5 mo	MPN	4.6×10^4	1.1×10^5	1.1×10^5	1.1×10^4	1.1×10^5	4.6×10^5	4.6×10^5	1.1×10^5	1.1×10^3
	FISH	DSV698	DSV698	DSV698	DSV698	DSV698	DSV698	DSV698	DSV698	DSV698
10 mo	MPN	1.1×10^7	1.1×10^7	1.1×10^7	1.1×10^6	1.1×10^6	1.1×10^6	4.6×10^6	4.6×10^6	4.6×10^5
	FISH	DSV698[d]	DSV698[d]	DSV698[d]	DSV698[d]	DSV698[d]	DSV698[d]	nd	nd	nd
Formate										
1.5 mo	MPN	nd	nd	1.1×10^4	1.1×10^4	4.6×10^4	4.6×10^3	1.1×10^4	1.1×10^4	4.6×10^3
	FISH	nd	nd	nd	nd	DSV698/ DSV214	DSV698/ DSV214	DSV698	nd	DSV698/ DSV214
10 mo	MPN	1.1×10^6	nd	4.6×10^5	1.1×10^6	4.6×10^6	4.6×10^6	1.1×10^7	1.1×10^6	4.6×10^5
	FISH	nd	nd	DSV698	DSV698	DSV698	DSV698	DSV698	DSV698	nd
Acetate										
1.5 mo	MPN	4.6×10^3	1.1×10^4	1.1×10^4	1.1×10^4	1.1×10^4	1.1×10^4	4.6×10^4	1.1×10^4	1.1×10^3
	FISH	DSB985	DSB985	DSB985	DSB985	nd	DSB985	DSB985	DSB985	DSB985
10 mo	MPN	1.1×10^5	4.6×10^5	1.1×10^4	1.1×10^6	1.1×10^4	4.6×10^5	1.1×10^7	3.9×10^6	4.6×10^3
	FISH	DSB985/ DSS658	DSB985/ DSS658	DSB985	DSB985	DSB985	DSB985	DSB985	DSB985	DSB985
Lactate										
1.5 mo	MPN	1.1×10^4	1.1×10^4	4.6×10^5	4.6×10^3	4.6×10^4	4.6×10^4	1.1×10^4	4.6×10^4	4.6×10^3
	FISH	DSV698	DSV698	DSV698	DSV698	DSV698	DSV698	DSV698	DSV698	DSV698
10 mo	MPN	1.1×10^4	3.9×10^5	4.6×10^5	4.6×10^6	4.6×10^6	4.6×10^6	1.1×10^4	4.6×10^6	4.6×10^5
	FISH	DSV698	DSV698	DSV698	DSV698	DSV698	DSV698	DSV698	DSV698	nd
Propionate										
1.5 mo	MPN	1.1×10^4	1.1×10^4	1.1×10^4	1.1×10^4	1.1×10^4	1.1×10^4	1.1×10^4	1.1×10^4	4.6×10^3
	FISH	SRB660	SRB660	SRB660	SRB660	nd	nd	SRB660	SRB660	SRB660
10 mo	MPN	1.1×10^5	1.1×10^5	4.6×10^5	1.1×10^6	1.1×10^6	4.6×10^6	4.6×10^5	1.1×10^6	4.6×10^3
	FISH	nd	nd	nd	SRB660	nd	nd	SRB660	SRB660	SRB660

[a]To confirm the low numbers of bacterial cells observed after the short incubation period a control experiment was performed. A comparative MPN study was carried out with sediment (2 to 5 cm depth) from the same location at Dangast and from sediment at Horumersiel, north of Jade Bay in September 1999. The latter site is characterized by a locally high organic input and a high sulfidogenic activity. The MPN tubes were incubated for 2.5 mo at 23°C. MPN counts with all substrates tested ($H_2 + CO_2$, $H_2 + CO_2$ + 2 mM acetate, 7.5 mM acetate, 5 mM lactate and 5 mM propionate) were between 1.1×10^4 an 1.1×10^5 cm^{-3} for the Dangast sediment. In contrast, MPN counts for the Horumersiel site were between 1.1×10^6 and 1.1×10^8 cells cm^{-3}

[b]Carbon source

[c]Positive hybridization with more than 1 probe

[d]Reduced brightness of the signal after 8.5 mo incubation

MPN counts indicated that cells were evenly distributed throughout the upper 15 cm of the sediment profile, with a decrease in cell numbers only in the deepest horizon.

From the highest dilution steps that still showed growth, samples were recovered for FISH analysis, the results of which are summarized in Table 2.

MPN in agar shakes yielded numbers of colony forming units somewhat higher than those observed with MPNs in liquid media (data not shown). Colonies from the highest dilution were transferred to fresh liquid media containing the same substrates as used in the corresponding agar shakes. Subsequent analysis of growth cultures by FISH revealed no other phylogenetic groups as already identified in MPNs with liquid media.

FISH of sediment samples

Total cell counts of the upper 20 cm of the sediment were determined microscopically (Table 3). From top to bottom, DAPI counts strongly decreased from 53.9×10^8 in the first cm to 1.0×10^8 cells cm^{-3} of sediment in the zone below 15 cm.

The microbial community dwelling in Dangast sediments was dominated by Bacteria. In the top 0.5 cm of the sediment, up to 82.3% of the total microorganisms hybridized with the probe EUB338 (Table 3). Archaeal and eucaryal counts remained below the detection limit of 0.1% of the DAPI-stained cells.

Recently, the bacterial probe EUB338 was shown not to detect all members of the domain Bacteria (Daims et al. 1999). Some bacterial phyla, most notably the Planctomycetales and Verrucomicrobia, are missed by this probe. Consequently, total bacterial numbers monitored in this study were most probably underestimated. Initial experiments with the newly designed additional probes EUB338-II and -III (Daims et al. 1999) indicated that this underestimation may amount to 4–10% of the total microbial community.

Detection rates with the probe EUB338 decreased exponentially over the vertical profile. Thus, not only the absolute cell numbers decreased with depth, but also the percentage of detectable cells with our FISH protocol.

A set of 15 different probes specific for SRB of the δ-subclass of Proteobacteria was tested (Table 1) with sediment samples from Site Dangast. Only 5 of these probes (i.e. SRB221, DSB985, DSR6512, DSS658, and DSV698), gave counts above the detection limit set at 0.1% of total DAPI counts (Table 3). The target groups of the other probes were either not present in high abundance (>0.1% of the total cell counts) or not detectable by FISH, e.g. due to a low ribosomal content per cell.

The most abundant SRB present in the sediments were members of the *Desulfosarcina-Desulfococcus-Desulfofrigus* group (probe DSS658) and *Desulfovibrio* spp. (probe DSV698) with maximum values of $2.9 \pm 0.5 \times 10^8$ and $2.8 \pm 0.6 \times 10^8$ cells cm^{-3} of sediment, respectively. The counts of these 2 groups decreased strongly with depth to values of $1.0 \pm 0.6 \times 10^6$ and $0.6 \pm 0.4 \times 10^6$ cells cm^{-3} of sediment at 20 cm depth, respectively.

SRB detected with the probe DSR651 specific for *Desulforhopalus* were also counted with decreasing numbers from top to bottom from $1.1 \pm 0.3 \times 10^8$ cells cm^{-3} of sediment in the first 0.5 cm to $2.7 \pm 0.3 \times 10^6$ cells cm^{-3} sediment between 10 and 15 cm depth. Cell numbers between 15 and 20 cm depth were below the detection limit.

Desulfobacter spp. (probe DSB985) were found across the entire sediment profile in relatively low abundance. The cell numbers decreased from $1.1 \pm 0.3 \times 10^7$ to $0.1 \pm 0.06 \times 10^5$ cells cm^{-3} throughout the sediment. *Desulfobacterium* spp. targeted with the probe

Table 3. Microbial community structure in different horizons from sediments at Site Dangast studied by FISH. nd: not detected

Horizon (cm)	Cell numbers ($\times 10^8$ cm^{-3})		Percentage of hybridized cells[a] (mean ± SD)					
	DAPI stain	Probe EUB 338	Probe SRB221	Probe DSB985	Probe DSR651	Probe DSS658	Probe DSV698	Sum of SRB probes
0–0.5	53.9 ± 2.2	44.4 ± 7.5	nd	0.21 ± 0.07	2.0 ± 0.7	2.5 ± 1	4.3 ± 0.9	8.6
0.5–1	53.7 ± 0.9	37.4 ± 7.0	0.22 ± 0.04	0.18 ± 0.1	1.9 ± 0.7	4.7 ± 1.3	5.3 ± 1.1	12.3
1–2	56.5 ± 1.3	39.4 ± 2.8	nd	nd	2.1 ± 0.7	5.3 ± 0.9	3.9 ± 0.8	11.3
2–3	53.5 ± 2.3	28.4 ± 1.6	0.15 ± 0.03	nd	1.7 ± 0.6	3.8 ± 0.8	4.4 ± 0.4	10.0
3–4	33.1 ± 1.5	15.1 ± 1.3	nd	0.1 ± 0.08	2.4 ± 0.9	2.4 ± 0.7	4.2 ± 0.7	9.1
4–5	25.5 ± 2.3	9.5 ± 1.5	nd	0.1 ± 0.09	1.7 ± 0.8	3.5 ± 0.9	6.2 ± 1.2	11.5
5–10	17.0 ± 2.1	4.3 ± 0.5	nd	nd	0.7 ± 0.3	2.2 ± 0.4	3.3 ± 0.3	6.3
10–15	9.0 ± 3.3	1.5 ± 0.2	nd	nd	0.3 ± 0.4	1.7 ± 0.5	1.0 ± 0.7	3.0
15–20	1.0 ± 4.6	0.1 ± 0.06	nd	0.1 ± 0.04	nd	1.0 ± 0.6	0.6 ± 0.4	1.7

[a]Percent detection compared to DAPI. Numbers have been corrected by subtracting NON338 counts. Means and SD were calculated from the counts of 2 parallel cores

SRB221 were only detected in the layers between 1 and 2 cm depth ($1.1 \pm 0.1 \times 10^7$ and $8.0 \pm 0.4 \times 10^6$ cells cm^{-3}).

Maximal numbers of detectable SRB were found within the upper 3 cm of the sediment (up to $6.6 \pm 1.0 \times 10^8$ cells cm^{-3}, accounting for 8.6 to 12.3% of total cells). Below 3 cm depth, the total number of SRB decreased continuously to a minimal value of $1.7 \pm 0.8 \times 10^6$ cells cm^{-3} at 15 to 20 cm depth (corresponding to 1.7% of total cells, Table 3).

DISCUSSION

Biogeochemistry of the sediment

The accumulation of AVS and the sulfur isotopic discrimination between sulfate and the AVS fractions throughout the investigated sediment layers (Fig. 2) are clear indications for the dissimilatory activity of SRB under anaerobic conditions. The isotope enrichment factors observed in the Dangast sediment partly exceed the maximum found in pure cultures (e.g. Bollinger et al. 2001) or experiments with sediment incubations using natural mixed populations (Canfield 2001). This might be due to the activity of sulfate reducers at this site, which have so far not been used in experiments on isotope discrimination. In addition, it is known that H_2S is, to a significant portion, reoxidized, so that sulfur species with intermediate oxidation states may be formed (Jørgensen 1982b), and such that bacterial disproportionation of the latter may lead to the formation of ^{32}S-enriched H_2S (Canfield et al. 1998, Cypionka et al. 1998, Böttcher et al. 2001). Indeed, bacteria which are able to disproportionate elemental sulfur, for instance, have been enriched from the sediments at Site Dangast and shown experimentally to discriminate sulfur isotopes (Canfield et al. 1998). Therefore, the oxidative part of the sulfur cycle may contribute to the observed overall sulfur isotope effect. Solid phase sulfur with higher oxidation states (essentially pyrite with minor elemental sulfur) were found through the sediment core with depth variations essentially decoupled from the AVS trend (Fig. 2C). Compared to the AVS fraction, the physicochemically more stable phase pyrite from the top 11 cm was generally enriched in ^{34}S (Fig. 2D). In the deeper more sandy sediment layers the isotope data of both fractions are close to each other. This may indicate the influence of particle mixing by bioturbation on the pyrite pool in the upper sediment section. Additionally, the pyrite fractions may have preserved a change in the depositional environment and the corresponding near surface sulfur cycle of the Jade Bay sediments due to dyke building activities at the end of the last century (Böttcher et al. unpubl.).

In the sediment depth of 2 to 10 cm, maximal bacterial sulfate reduction occurred, even though its product, dissolved sulfide, accumulated only in limited amounts. Re-oxidation of hydrogen sulfide may be coupled to the reduction of Fe(III) and Mn(IV) compounds, resulting in the formation of sulfur species or sulfate and dissolved Fe(II) and Mn(II) (Burdige 1993, Moeslund et al. 1994, Thamdrup et al. 1994, Böttcher & Thamdrup 2001). Therefore, dissolved Fe(II) and Mn(II) may originate from both microbial and chemical reduction of the metal oxyhydroxides. Hydrogen sulfide can also react with iron compounds to form iron sulfides. In this suboxic part of the sediment a maximum in SRR coincided with highest TOC contents; only at greater depth did a parallel decrease of both parameters occur. The observed decrease in pore water content with sediment depth is due to changes in the sedimentological and mineralogical properties of the sediment caused by a mixing of minerals of the mud fraction with increasing amounts of the sand grain fraction (essentially quartz). In contrast to quartz, phyllosilicate minerals, and especially smectite, are able to adsorb significant amounts of organic matter (Keil et al. 1994, Mayer 1994). In addition, stable carbon isotope measurements on TOC at Site Dangast have shown that the labile fraction of organic matter, which is of marine origin, decreases with depth due to the preferential biodegradation of marine organic matter (Böttcher et al. 2000).

The actual substrates of SRB are small molecules like VFA. Therefore, microbial sulfate reduction depends on the activity of fermentative bacteria which are able to degrade polymeric substances to such small molecules (e.g. Sørensen et al. 1981, Christensen 1984). Consequently, the SRR is also linked to the production rate of VFA. The concentrations of VFA in interstitial waters depend on the balance of production and consumption rates. The reactions responsible for the quantitative balance in different environments, however, are not fully understood. In the present study, high concentrations of acetate and lactate coincide with low SRR (Fig. 2). This is in agreement with observations by Novelli et al. (1988) and Holmer & Kristensen (1996). We suggest that the production rates of VFA are balanced by SRR in the top sediments, but increase relatively further downcore.

Sulfate-reducing bacteria

Specific PLFAs have often been described as biomarkers for different physiological groups of organisms (Findlay & Dobbs 1993). The iso-branched, unsaturated fatty acid i17:1 may serve as marker fatty acid for members of the genus *Desulfovibrio*, whereas the

anteiso-branched fatty acid a17:1 has only been detected in significant amounts in *Desulfosarcina variabilis* and *Desulfococcus multivorans* (Vainshtein et al. 1992, Kohring et al. 1994). The branched fatty acid 10Me16:0 has been found in members of the family *Desulfobacteriaceae* (Taylor & Parkes 1983, Dowling et al. 1986, Kohring et al. 1994, Kuever et al. 2001) and has often been used as a biomarker for SRB in marine sediments (Findlay & Dobbs 1993). The selected fatty acids i15:0, a15:0 and cyc17:0 were previously suggested as biomarkers for anaerobic bacteria, including SRB (Guckert et al. 1985, Findlay et al. 1990, Findlay & Dobbs 1993). The absolute and relative abundance of these PLFAs, with highest values in the sediment layers between 1 and 5 cm depth are in the range of those reported previously for marine sediments (Guckert et al. 1985, Findlay & Dobbs 1993, Findlay & Watling 1998). The highest absolute values of these biomarkers indicate highest cell numbers of SRB and other anaerobic bacteria in these sediment layers. The profiles of the relative amounts of these PLFAs indicate that the share of this group within the total microbial community increased within the top 2 cm and remained constant below that layer. It should be noted, however, that an absolute quantification of SRB by PLFA analysis is not possible, since the cellular fatty acid composition of SRB can be influenced by carbon sources, temperature or growth phase, as demonstrated with pure cultures (Taylor & Parkes 1983, Dowling et al. 1986, Aeckersberg et al. 1998, Könneke 2001).

DGGE analysis was carried out to define phylogenetic diversity of SRB based on 16S rDNA (Fig. 4A) and the active fraction of the SRB community based on 16S rRNA (Fig. 4B). Phylogenetic identification of the most prominent DGGE bands showed diverse affiliation within the δ-subclass of *Proteobacteria* (Fig. 4C). Interestingly, most sequences were closely related to SRB originating from marine environments, e.g. *Desulfonema ishimotoei* (isolated from Dangast, the present study area, see Fukui et al. 1999) and *Desulfobacula toluolica* (isolated from Eel Pond, Woods Hole, USA, see Rabus et al. 1993). A similar electrophoretic pattern of amplified 16S rDNA in the upper 15 cm of the sediment indicated an even distribution of SRB. However, DGGE analysis of reversely transcribed 16S rRNA showed an uneven distribution across the sediment profile. Reversely transcribed 16S rRNA indirectly reflect the ribosome content of cells, allowing active cells to be identified. Based on 16S rDNA profiling, DGGE band 5 (Fig. 4A) is evenly distributed throughout the studied sediment horizons. In contrast, this band could only be detected in the lower part (below 3 cm) of the sediment, if monitored by 16S rRNA-based DGGE profiling (Fig. 4B). This could indicate that the *Desulfobacter*/*Desulfobacula* type SRB represented by DGGE band 5 (Fig. 4C) are most active in the lower part of the sediment, even though they are also present in the upper part. The opposite was observed for DGGE band 3, representing *Desulfovibrio* type SRB. Overall, different SRB represented by 8 DGGE bands were phylogenetically identified. Except for 1 sequence (DGGE band 6), all of them were also identified as active members of the SRB community.

Viable SRB obtained with a variety of substrates were quantified and identified by combining MPN and FISH studies. Regardless of the MPN substrates used or the length of the incubation period, an even distribution of cells throughout the sediment profile was observed (Table 2). Identification of SRB in highest dilution showing growth was performed with FISH (Table 2). With respect to the individual substrates added to the MPN cultures, results were in good agreement with the nutritional properties of known SRB. For example, cultures with either lactate or H_2/CO_2/acetate known as substrates typical for *Desulfovibrio* were indeed dominated by members of this species. In addition, H_2/CO_2, requiring chemolithoautotrophic metabolism as known from *Desulfobacterium autotrophicum* (Brysch et al. 1987), yielded predominantly members of this genus. MPN cultures with acetate were dominated by members of the genera *Desulfobacter* and/or *Desulfobacula*, known to possess the capacity for complete oxidation of acetate (Rabus et al. 2000). During incubation the SRB population changed under most conditions (i.e. with different substrates used and sampling depths tested). After a short incubation time of about 6 wk a more rapidly growing population of less abundant cells developed. The more abundant population required a prolonged incubation time of about 10 mo. It should be noted that SRB of this population type did not grow homogeneously in the culture medium but formed flocs, which may result in an underestimation of the actual cell numbers determined with MPN (Table 2) as compared to FISH (Table 3).

The examination by FISH of the microbial community in the sediment showed that SRB account for a significant part of the detectable Bacteria. Up to $6.6 \pm 1.0 \times 10^8$ cells cm^{-3} were identified as SRB. Based on this number and SRR, the cellular SRR in the Dangast sediment was calculated as 1 fmol SO_4^{2-} $cell^{-1}$ d^{-1}, agreeing with recent reports from other natural settings (Sahm et al. 1999, Böttcher et al. 2000, Ravenschlag et al. 2000). Interestingly, these *in situ* values are at the lower end of cellular SRR determined for pure cultures of various SRB (Canfield et al. 2000). These low cellular rates are also in agreement with the observed sulfur isotopic discrimination of iron monosulfides and sulfate (Fig. 2D). The cell numbers determined by FISH (Table 3) were always higher than the

numbers of viable SRB observed using MPN (Table 2). A similar discrepancy between MPN counts and for instance DAPI-based cell counts have been described (Sievert et al. 1999). The absolute cell numbers might be underestimated since typically less than 1% of all bacteria in natural habitats may be accessible by current cultivation methods (Amann et al. 1995). In addition, floc formation and clumping of cells may also lead to an underestimation, since the MPN evaluation assumes that only a single cell is required to initiate growth at the highest dilution.

The 2 most abundant groups of SRB identified by FISH were the *Desulfovibrio* group and the *Desulfosarcina-Desulfococus-Desulfofrigus* group, which is in agreement with low concentrations of lactate and acetate in the upper layers of the sediment. Therefore these 2 groups—at the sampling time point in June 1999—were the key populations for the terminal mineralization step in the anaerobic zone of the temperate intertidal mud flat. The *Desulfovibrio* group was also identified by PLFA, MPN and DGGE analysis. However, the *Desulfosarcina-Desulfococus-Desulfofrigus* group could only be also identified by PLFA, but not when MPN and DGGE were applied. Possible explanations could be that the cultivation conditions used do not select for SRB affiliating with this group, and that the primers used for DGGE are not targeting this group (Ravenschlag et al. 2000). Such discrepancies caused by technical limitations are known in molecular ecology (for summary refer to Head et al. 1998). Therefore, in addition to a continuous improvement of molecular tools, fully integrated approaches are necessary to obtain a comprehensive overview of the microbial communities in complex environmental samples.

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