Community structure, activity and ecophysiology of sulfate-reducing bacteria in deep tidal flat sediments

Struktur, Aktivität und Ökophysiologie sulfatreduzierender Bakteriengemeinschaften in tiefen Sedimenten des Deutschen Wattenmeeres

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Ich will euch nur sagen, dass es gefährlich ist, zu lange zu schweigen.
Die Zunge verwelkt, wenn man sie nicht gebraucht.

Astrid Lindgren

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Noch kenne ich Dich gar nicht richtig, und dabei bist Du mir so wichtig.
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Zusammenfassung

Sandige Sedimente wurde lange Zeit als „mikrobielle Wüsten“ bezeichnet, konnten jedoch in jüngsten Untersuchungen an Oberflächensedimenten sandiger Wattflächen durch hohe Zelldichten und signifikante Remineralisierungsraten charakterisiert werden. Zum einen wirken sandige Sedimente als „Biofilter“ – aufgrund ihrer hohen Permeabilität kann Meerwasser in die obersten Schichten eindringen, in denen dann organisches Material gefiltert und angereichert wird. Eine weitere Besonderheit sandiger Wattgebiete ist der advektive Transport von Porenwasser in permeablen Sedimentschichten, der durch die Ausbildung eines Druckgradienten zwischen Wasserlinie und Platenoberfläche angetrieben wird. Dieser Porenwassertransport kann bis in tief gelegene Sedimentschichten erfolgen und ermöglicht gegebenenfalls die Versorgung der dortigen mikrobiellen Gemeinschaft mit verwertbaren Substraten.

Im Rahmen der vorliegenden Arbeit wurde die Abundanz, Aktivität und Ökophysiologie sulfatreduzierender Bakterien untersucht, die als Endglied anaerober Abbauprozesse fungieren und damit repräsentative Mitglieder der mikrobiellen Gemeinschaft darstellen. Als Untersuchungsgebiet diente ein typisches Sandwatt im Deutschen Wattenmeer, das bis in eine Tiefe von fünf Metern untersucht wurde.

Es konnte gezeigt werden, dass Sulfatreduzierer auch in mehreren Metern Tiefe noch außerordentlich abundant und physiologisch aktiv sind, obwohl sie dort sowohl variablen Sulfatkonzentrationen als auch temporärer Sulfatlimitierung ausgesetzt sind. Ein Großteil der kultivierten und *in situ* quantifizierten Sulfatreduzierer konnte zwei weit verbreiteten phylogenetischen Gruppen innerhalb der *Deltaproteobacteria* zugeordnet werden. Es handelt sich hierbei um die große Gruppe der *Desulfobulbaceae* sowie um Verwandte der Gattungen *Desulfobacula* und *Desulfobacter*, die zur Familie der *Desulfobacteraceae*

gehören. Viele Vertreter dieser beiden Gruppen sowie die im Rahmen dieser Arbeit gewonnenen Isolate sind in der Lage, ein breites Spektrum organischer Substrate als Energie- und Kohlenstoffquellen zu nutzen als auch in Abwesenheit von Sulfat Energie durch Gärprozesse zu gewinnen. In tiefen Sedimenten des Janssands konnten mehr als zwei Drittel aller detektierten Sulfatreduzierer diesen beiden Gruppen zugeordnet werden. Ihre hohe Abundanz und ihr metabolisches Potenzial deuten zum einen auf die mögliche Versorgung der mikrobiellen Gemeinschaft mit Nährstoffen und gelösten organischen Komponenten hin. Es erscheint außerdem möglich, dass die identifizierten Organismen neben der dissimilatorischen Reduktion von Sulfat auch alternative, energieliefernde Prozesse wie Gärung und die Syntrophie mit anderen Anaerobiern nutzen und so die Erhaltung einer stabilen Gemeinschaft von Sulfatreduzieren in tiefen Sedimenten gewährleistet wird.

Neben der hohen Abundanz sulfatreduzierender Bakterien deuteten zeitliche Variabilitäten in Porenwasserprofilen sowie erhöhte Gesamtzellzahlen und Sulfatreduktionsraten darauf hin, dass Porenwassertransport und damit der Transport metabolischer Produkte vom Plateninneren an die Platenränder stattfindet und dieses Phänomen zur Stimulation der indigenen Gemeinschaft führt. Ein wichtiges Ziel weiterer Studien ist es daher, Porenwasserflüsse zu quantifizieren und die Qualität des organischen Materials genauer zu charakterisieren.

Eine ausgeprägte Sulfat-Methan-Übergangszone sowie die Stimulation endogener Sulfatreduktion durch die Zugabe von Methan deuteten außerdem darauf hin, dass die anaerobe Oxidation von Methan (AOM) von bedeutender Relevanz in den untersuchten Sedimenten ist. Tatsächlich konnte ein Vertreter der potenziell AOM-involvierten *Desulfosarcina-Desulfococcus*-Gruppe angereichert werden, was die Anwesenheit als auch Kultivierbarkeit der entsprechenden Organismen zeigt. Allerdings konnte der Organismus nicht in Reinkultur gebracht werden, so dass offen bleibt, ob die im Rahmen dieser Studie identifizierten Sulfatreduzierer oder ein bisher unbekannter Teil der Gemeinschaft in den Prozess der AOM eingebunden ist.

Die Ergebnisse dieser Arbeit zeigen, dass tiefe Sedimente sandiger Wattflächen potenzielle *Hot Spots* mikrobieller Aktivität darstellen und daher signifikant zum Abbau organischen Materials und damit den Nährstoffkreisläufen in tidalen Ökosystemen beitragen können.

Summary

Surface sediments of tidal sand flats were recently recognized to be densely populated by microorganisms and characterized by high remineralization rates supporting the opinion that sandy sediments should no longer be generally described as "microbial deserts". Due to their high permeability, seawater might drain into the sediment and organic matter is filtered and enriched in the sediment surface. Beside acting as a "biofilter", tidal sand flats are characterized by enhanced pore water exchange driven by a hydraulic pressure gradient. This phenomenon facilitates the transport of pore water to deep sediment layers and potentially provides microbial communities with usable substrates.

The present thesis focused on the abundance, activity and ecophysiology of sulfate-reducing bacteria (SRB) as they are the terminal instance in anaerobic degradation and therefore representative members of the microbial community. It was shown that SRB form highly abundant and more remarkably, active populations in several meter deep sediments of a tidal sand flat in the German Wadden Sea. The majority of the isolated and *in situ* quantified SRB were members of two widely distributed phylogenetic groups within the *Deltaproteobacteria*, in particular related to the families of the *Desulfobulbaceae* and the *Desulfobacteraceae*. Many cultured representatives of these two groups as well as the isolates obtained in this thesis share the capacity to utilize a broad spectrum of organic substrates as electron and carbon sources. Furthermore, they likely possess the capability to grow fermentatively in the absence of sulfate and therefore probably gain sufficient energy to survive under these uncomfortable conditions. In deep sediments at site "Janssand", at least two-thirds of all detected SRB were affiliated with these to phylogenetic groups. Their

high abundance as well as their metabolic potential are indicative for a supply with nutrients and dissolved organic compounds to the microbial community. Additionally, it appeared feasible that beside dissimilatory sulfate reduction, fermentation and more likely, syntrophic relationships with other anaerobes are potential alternatives for the identified SRB to sustain a considerable community size.

Beside the high abundance of SRB in deep sediment layers, seasonal variability in pore water profiles as well as elevated numbers of microorganisms and enhanced activity of SRB supported the hypothesis that together with pore water metabolic products are transported from upper to lower flat sites stimulating the activity of the indigenous communities. The detailed analysis of pore water fluxes and composition remains an important goal for future studies.

Both the presence of a pronounced sulfate-methane transition zone as well as the significantly enhanced activity of sulfate reducers after methane addition suggested that anaerobic methane oxidation (AOM) is of considerable relevance to anaerobic remineralization processes *in situ*. Indeed, a member of the AOM-associated *Desulfosarcina-Desulfococcus* group was successfully enriched, thus proving its presence and culturability. As the organisms could not be brought into pure culture, it still remains to be investigated whether and to which extend the here identified SRB or a so far unidentified fraction of SRB are involved in AOM.

In conclusion, deep sediments of tidal sand flats were shown to be potential "hot spots" of microbial activity and have a remarkable impact on organic matter degradation as well as on the transfer and cycling of nutrients within this ecosystem.

List of publications

This thesis includes three manuscripts that are submitted for publication or are prepared to be submitted within the next months.

A. Gittel, M. Mußmann, H. Sass, H. Cypionka and M. Könneke.
Directed isolation of *in situ* abundant representatives of the sulfate-reducing community in deep tidal sediments.
Submitted to *Environmental Microbiology*

Concept and sampling: A.G., M. K.; Porewater analyses: A.G., H.S.; Isolation: A.G., M.K.; Physiological tests: A.G.; Phylogenetic analyses: A.G., M.M.; *In situ* quantification: A.G.; First draft of the manuscript: A.G.; Revision by M.M., H.S., H.C., M.K.

A. Gittel, M. Seidel, H. Cypionka and M. Könneke.
Description of *Desulfocucumis infernus* gen. nov., sp. nov., an abundant sulfate reducer in deep tidal flat sediment.
Prepared for submission to *International Journal of Systematic and Evolutionary Microbiology*

Concept, sampling and isolation: A.G., M. K.; Phospholipide analysis: M.S.; Physiological and phylogenetic analyses: A.G.; First draft of the manuscript: A.G.; Revision by H.C., M.K.

A. Gittel, S. Reischke, H. Cypionka and M. Könneke.
Activity and stimulation of sulfate-reducing prokaryotes in deep tidal flat sediments. Prepared for submission to *Microbial Ecology*

Concept and sampling: A.G., M. K.; Chemical analyses: A.G., M.K.; Determination of microbial activities: A.G., Cultivation and monitoring: S.R.; First draft of the manuscript: A.G.; Revision by H.C., M.K.

Further publication not included in this thesis:

B. Engelen, K. Ziegelmüller, L. Wolf, B. Köpke, **A. Gittel**, T. Treude, S. Nakagawa, F. Inagaki, M. A. Lever, B. O. Steinsbu and H. Cypionka. Fluids from the ocean crust support microbial activities within the deep biosphere. Submitted to *Geomicrobiology Journal*

Presentations in national and international meetings:

M. Seidel, **A. Gittel**, K. Bischof, J. Köster, H. Sass, J. Rullkötter. Lipid biomolecules in the subsurface of tidal flats: Indicators for microbial diversity. International Meeting on Organic Geochemistry (IMOG), 2007. **Poster**

M. Seidel, **A. Gittel**, K. Bischof, J. Köster, H. Sass, J. Rullkötter. Diverse active microbial communities in a tidal flat sediment as deciphered by a multidisciplinary approach. Goldschmidt Conference, 2007. **Poster**

A. Gittel, S. Reischke, M. Mußmann, H. Sass, H. Cypionka and M. Könneke. Sulfate-reducing bacteria form highly active and abundant populations in deep tidal flat sediments. Annual Meeting of the German Society of General and Applied Microbiology (VAAM) 2007.
Oral presentation

A. Gittel, H. Sass, M. Mußmann, H. Cypionka and M. Könneke. Subsurface Wadden Sea sediments harbour an active and diverse community of sulfate-reducing bacteria. Annual Meeting of the International Society of Microbial Ecology (ISME) 2006. **Poster**

A. Gittel, H. Sass, M. Mußmann, H. Cypionka and M. Könneke. Does the vertical distribution of cultured sulfate-reducing bacteria reflect elevated activities in deep sediments of an intertidal sand flat? Annual Meeting of the German Society of General and Applied Microbiology (VAAM) 2006. **Poster**

A. Gittel, Y. C. Hilker, B. Engelen, H. Cypionka and H. Sass. Detecting Sulfate-Reducing Bacteria in Coastal Subsurface Sediments by Geochemical and Molecular Approaches. Summer Meeting of the American Society of Limnology and Oceanography (ASLO) 2005. **Poster**

Y. C. Hilker, **A. Gittel**, H. Cypionka and B. Engelen. Vertical distribution of bacterial communities involved in the methane cycle of tidal flat sediments. Summer Meeting of the American Society of Limnology and Oceanography (ASLO) 2005. **Poster**

Abbreviations

AOM	Anaerobic Oxidation of Methane
DAPI	4',6-DiAmidino-2-Phenyl-Indol
DNA	DesoxyriboNucleic Acid
dsrAB	Gene encoding for subunits A and B of the Dissimilatory (Bi)-Sulfite Reductase
CARD	Catalyzed Reporter Deposition
FISH	Fluorescence *In Situ* Hybridization
HRP	Horse Radish Peroxidase
MAR	MicroAutoRadiography
mRNA	Messenger RiboNucleic Acid
rRNA	Ribosomal RiboNucleic Acid
SRB	Sulfate-Reducing Bacteria
SRP	Sulfate-Reducing Prokaryotes
SRR	Sulfate Reduction Rate(s)

Chapter 1

Introduction

1.1 Degradation processes in marine sediments

From outer space, the Earth appears as a "blue planet" since world's oceans cover about 70 % of its surface. At the bottom of these huge water masses, sediments form the transition zone to Earth's crust. In the photic zone of the water column, inorganic carbon is converted to organic biomass via photosynthetic primary production. The amount of organic compounds that reaches the sediment surface strongly varies with respect to the water depth and the remineralization activity of heterotrophic organisms within the water column. Large parts of the open ocean are nutrient-depleted and therefore show only low primary productivity. Thus, at these sites, that may reach water depths of up to several thousand meters, less than 1 % of the produced organic matter reaches the sediment surface (Hedges, 1992). In contrast, coastal areas of high primary productivity (like tidal flats, off-coast upwelling areas) are characterized by a high input of organic matter as up to 50 % of the primary production may settle down to the sediment surface (Wollast, 1992).

The organic matter is remineralized by microorganisms utilizing a variety of electron acceptors. Molecular oxygen is the energetically most favorable electron acceptor, but possesses only a low solubility in seawater. Its diffusion into the sediment is mostly restricted to the upper few centimetres where it is rapidly consumed by aerobic microorganisms. In areas of high productivity, the interface between oxygen-penetrated and oxygen-free, sulfidic zones can extend

close to the sediment surface (Jørgensen and Revsbech, 1989). In the anoxic part of the sediment, organic matter is remineralized via anaerobic respiration processes (like denitrification, manganese and iron reduction, sulfate reduction and methanogenesis; Fig. 1) and fermentative pathways (Canfield et al., 1993). The vertical succession of various electron acceptors and degradation processes reflects the corresponding redox potentials and therefore the amount of energy that is gained through the redox reactions.

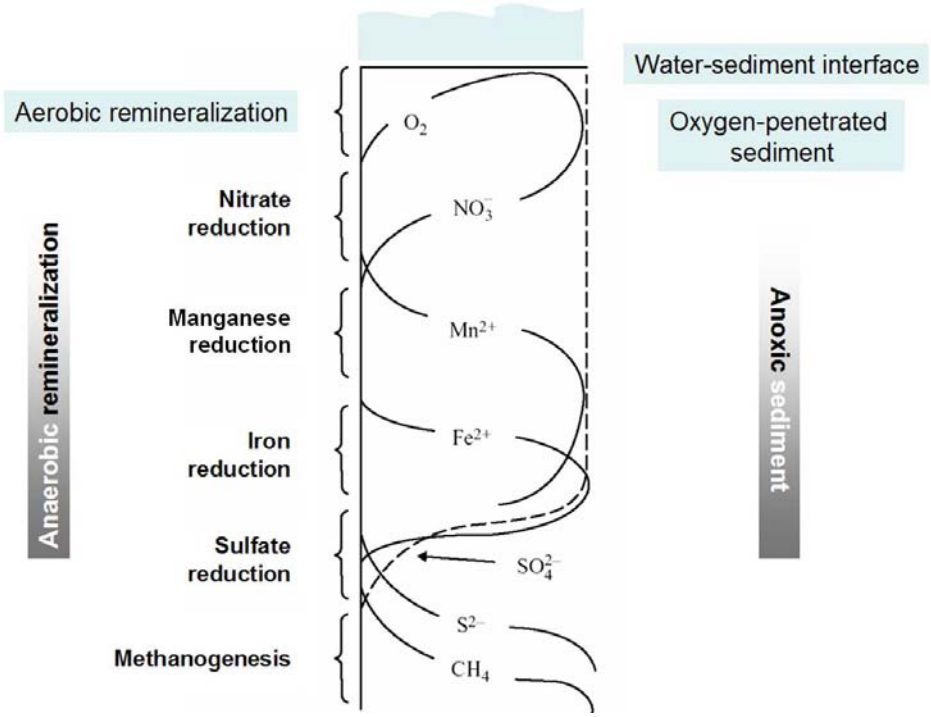

Figure 1: Simplified scheme of the vertical succession of anaerobic respiration processes in marine sediments (modified after Megonigal et al., 2004).

1.1.1 Dissimilatory sulfate reduction

Dissimilatory sulfate reduction is a very ancient process that evolved more than 3.4 billion years ago (Shen et al., 2001). The ability to use sulfate as terminal electron acceptor is unique to prokaryotes, which therefore represent an essential biotic component of the global sulfur cycle. Taxonomically they

form a rather heterogenous group as their members belong to four different bacterial phyla (*Deltaproteobacteria*, *Firmicutes*, *Thermodesulfobacteria* and *Nitrospira*) and one archaeal phylum, the *Euryarchaeota* (Rabus et al., 2006). They oxidize a variety of organic and inorganic compounds including fermentation products like acetate, lactate and hydrogen, but also hardly degradable compounds like alkanes and aromatic substances (Widdel and Bak, 1992; Rabus et al., 2006). The ability to completely oxidize organic carbon to CO_2 makes them a terminal instance in anaerobic degradation processes providing CO_2 for primary production.

Oxidation of organic matter via sulfate reduction yields less energy than other anaerobic respiration processes, e. g. nitrate reduction. However, sulfate reaches high concentrations in seawater (an average of 28 mM) and diffuses into sediment layers where other electron acceptors like nitrate, ferric iron, and manganese oxides have already been consumed (Fenchel et al., 1998). Therefore, sulfate reduction is regarded as the major terminal anaerobic degradation process in marine sediments accounting for up to 50 % of the total carbon remineralization (Jørgensen, 1982). The highest turnover of organic matter via sulfate reduction was reported for an upwelling area off the coast of Chile, where 80 % of the organic matter were remineralized via sulfate reduction (Thamdrup and Canfield, 1996).

1.2 Tidal flat sediments

Tidal flat sediments cover large areas of the continental shelf in e. g. Southeast Asia, Australia or at the Southern North Sea coast and are characterized by a high variability in time and space (Dittmann, 1999). They are tide-influenced and therefore exposed to permanently changing environmental conditions like temperature and light intensity, bioturbation, air exposure and hydrodynamic features, like currents. Due to their unique position at the boundary between land and sea, these complex, dynamic ecosystems are essential for the transfer and recycling of nutrients between these two compartments.

With respect to the dominating grain sizes, tidal sediments are generally classified as sand flats (>0.1 mm), mixed flats (0.06 mm to 0.1 mm) and mud flats (<0.06 mm). The variation in grain sizes results in flat-typical sediment

structures. Large grains produce a large interstitial space and therefore transport of solutes is promoted in sand flats in comparison to mud flats.

The Wadden Sea along the Southern North Sea coast is the world's largest coherent tidal flat system (Fig. 2). Due to a high input of organic matter from both pelagic and benthic primary production to the sediment surface and rapid microbial remineralization processes, sediments within this area exhibit steep geochemical gradients compared to open ocean sites (Parkes et al., 2005; Wilms et al., 2007). Microbial communities and activities in surface and surface-near sediments have been extensively studied (Llobbet-Brossa et al., 2002; Ishii et al., 2004; de Beer et al., 2005; Mußmann et al., 2005; Billerbeck et al., 2006b; Musat et al., 2006). Recently, studies were extended to several meters deep sediments at mud flat sites in the backbarrier of Spiekeroog Island. It was shown that these sediments harbour typical "subsurface communities" including so far uncultivated representatives of the "*Chloroflexi*" and the candidate subdivision JS1 (Köpke et al., 2005; Wilms et al., 2006a; Webster et al., 2007). However, studying sand flats was restricted to the upper 50 cm the deepest in the past and was just recently extended to deeper layers (Beck et al., 2007). Thus, on little information is available on the microbial communities and the environmental settings they are forced to thrive in deeper layers.

1.3 Sandy sediments as microbial "hot spots"

Sandy sediments prevail in coastal areas and they were generally assumed to harbour less active microbial communities than mud-dominated sediments. They were shown to be poor in organic matter due to larger grain sizes, their lower specific surface area and adsorption capacity (Bergamaschi et al., 1997; Rusch et al., 2003). Additionally, sandy sediments were described to harbour less microbial cells (Llobet-Brossa et al., 1998). Just recently microbial activities in sand flat sediments comparable to those reported for organic-rich mud flats were measured at several sampling sites, e. g. in the German Wadden Sea (Cammen, 1991; de Beer et al., 2005; Billerbeck et al., 2006b). It is argued that the high permeability of sandy sediments allows for enhanced advective pore water transport in permeable sediment layers. The trapping of dissolved

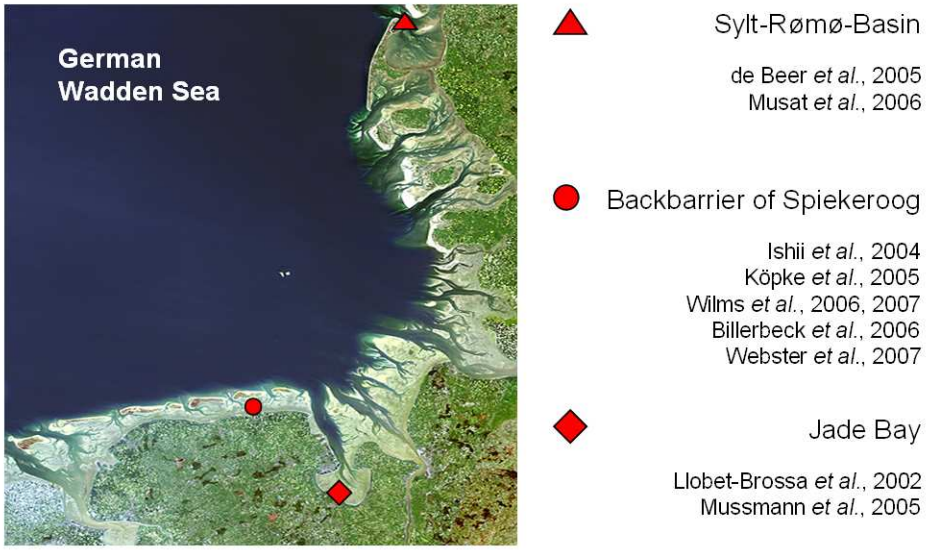

Figure 2: Frequent sampling locations of microbiological studies on sediments in the German Wadden Sea. The Sylt-Rømø-Basin represents a typical sand flat, whereas sampling locations in the Jade Bay are mud-dominated. In the backbarrier of Spiekeroog Island, all types of tidal flats are present (see text).

nutrients as well as bacteria and algae into the interstitial space is supposed to stimulate microbial activites within these sediments (Huettel and Rusch, 2000; D'Andrea et al., 2002; Billerbeck et al., 2006a). Thus, they should no longer be described as "microbial deserts", but as potential "hot spots" of microbial degradation processes (Boudreau et al., 2001).

1.3.1 Community structure and activity of sulfate-reducing bacteria in tidal sand flats

As sandy sediments are characterized by elevated oxygen penetration depths, in the following the term "surface sediments" refers to potentially oxygen-penetrated sediments. Sediments potentially not penetrated with oxygen are described as "subsurface sediments". Oxygen penetration and concentrations are affected by tides and seasons, therefore this is only an artificial, simplifying definition to distinguish between surface and subsurface microbial communities.

Surface sediments

It was commonly presumed that the abundance of microorganisms in sandy sediments is about one order of magnitude lower than in organic-rich sediments (Llobet-Brossa et al., 1998; Wieringa et al., 2000; Rusch et al., 2001). However, recent studies on tidal sand flats in the German Wadden Sea revealed cell numbers that were comparable to those of mud flats (Ishii et al., 2004; Musat et al., 2006). The strong decrease in cell numbers repeatedly detected in the top 5 - 10 cm in muddy sediments, was not shown for sandy sediments. Thus, one might argue that the microbial community benefits from the higher permeability of sandy sediments and therefore from an elevated availability of both electron donors and acceptors.

Relatives to the *Desulfosarcina-Desulfococcus* group were described to be the most abundant sulfate-reducing bacteria (SRB) within these sediments (Ishii et al., 2004; Mußmann et al., 2005; Musat et al., 2006). In contrast to minor represented phylotypes (e. g. members of the *Desulfobulbaceae*), they expressed a rather high abundance over depths and seasons. Within the scope of an integrated study on sandy sediments in the Sylt-Rømø Basin, it was shown that members of the *Desulfosarcina-Desulfococcus* group were highly abundant in oxygen-penetrated sediments as well as in periods of low sulfate reduction activity (de Beer et al., 2005; Musat et al., 2006). The authors therefore suggested that these organisms might be adapted to oxygen stress or might utilize alternative electron acceptors (Fe(III), or even oxygen).

Various other genera of SRB were detected in lower abundance (e. g. relatives to *Desulfomicrobium*, *Desulfobulbus* (Wieringa et al., 2000; Ishii et al., 2004; Musat et al., 2006). Their abundance strongly varied with depths, seasons and sampling sites.

The contribution of dissimilatory sulfate reduction to total carbon remineralization might be significantly reduced due to the penetration of oxygen into the sediment. Recent studies therefore focused on the calculation of oxygen consumption and sulfate reduction rates to quantify their relative contribution to organic carbon remineralization (de Beer et al., 2005; Billerbeck et al., 2006b; Werner et al., 2006). Hence, sulfate reduction accounted for 3 - 25 % to total remineralization, depending on the season. The highest sulfate-reducing

activity usually occurred in sediment layers directly beneath the maximum depth of oxygen penetration showing a clear correlation between the activity of sulfate reducers and the presence of oxygen within the sediment.

Subsurface sediments

In contrast to the extensive study on surface sediments, there is only little information on the abundance and activity of sulfate-reducig bacteria in the subsurface of sandy tidal flats. Characterization of the microbial communities in several meters deep sediments of two contrasting mud flats in the backbarrier of Spiekeroog Island revealed that *Gamma-* and *Deltaproteobacteria* predominate the upper sediment layers (Köpke et al., 2005; Wilms et al., 2006b). Despite a pronounced discrepancy between the cultivation-based approach and the molecular screening, both studies revealed a community shift with depth. Sediment layers deeper than 200 cm were predominated by members of the *"Chloroflexi"*, *Bacteroidetes* and spore-forming *Firmicutes* with SRB represented by relatives to the *Deltaproteobacteria*, as well as *Desulfosporosinus* and *Desulfotomaculum* within the *Firmicutes*. These changes in community composition were attributed to a decreasing amount of easily degradable organic matter. However, this community shift was not observed for a 2.5 meter long sediment core recovered from a near sand flat. Detected phylotypes affiliated with previously described SRB and were members of the genera *Desulfofaba* and *Desulfobulbus* within the *Deltaproteobateria*. No SRB belonging to the *Firmicutes* were found (Wilms et al., 2006a). One might hypothesize that deep sediments at sand flat sites are also influenced by the proposed pore water transport resulting in a stimulation of the microbial community. As furthermore nothing is known about the *in situ* activity or the physiolgical state of the detected organisms within these sediments, this should be the focus of prospective studies.

1.4 Cultivation-independent detection of *in situ* abundant and active microorganisms

Cultivation-independent analysis of the diversity of microbial communities based on the detection of genetic biomarkers revealed a huge and unsuspected diversity. According to extensive phylogenetic studies, that commonly based on 16S rRNA gene sequence analyses, 52 phyla were identified so far. It turned out that the majority of these phyla contains only few cultured representatives or even exclusively consist of environmental clone gene sequences (Hugenholtz et al., 1998; Rappé and Giovannoni, 2003). It was indicated that many of these underrepresented or "uncultivated phyla" were abundant in diverse habitats pointing to their so far nearly unraveled ecological relevance. This finding, however, displays the need of cultivation-independent techniques to search for the unknown (or uncultured).

Several hybridization techniques targeting on 16S ribosomal RNA were developed to first visualize and identify and furhermore to quantify active microorganisms (i) on single-cell level (FISH, Amann et al., 1995) or (ii) by the amount of total ribosomal RNA content (rRNA slot-blot hybridization, Stahl et al., 1988). With respect to the various habitats that were studied, these techniques have been constantly modified and improved. The advancement of FISH to CARD-FISH allows for the detection of cells with low rRNA content, a typical feature for marine subsurface sediments (Pernthaler et al., 2002; Ishii et al., 2004). Here, the fluorescence signal is significantly improved by the deposition of fluorescence-labeled tyramide radicals by horse radish peroxidase (HRP) activity (Fig. 3). Each HRP conferred by a probe catalyzes the deposition of many labeled tyramides resulting in greatly enhanced sensitivity compared to probes labeled with a single fluorochrome. The fluorescence signal can therefore be easier distinguished from the sediment background. Furthermore, both FISH and CARD-FISH are not only a tool for quantifying microorganisms, but facilitate the resolution of spatial structures – one is able to detect single cells in complex environments on small scales, e.g. biofilms (Gieseke et al., 2001) or symbiotic earthworms (Schramm et al., 2003).

To correlate the abundance of microorganisms to their actual activity within the habitat, *in situ* hybridization techniques were coupled to microsensor

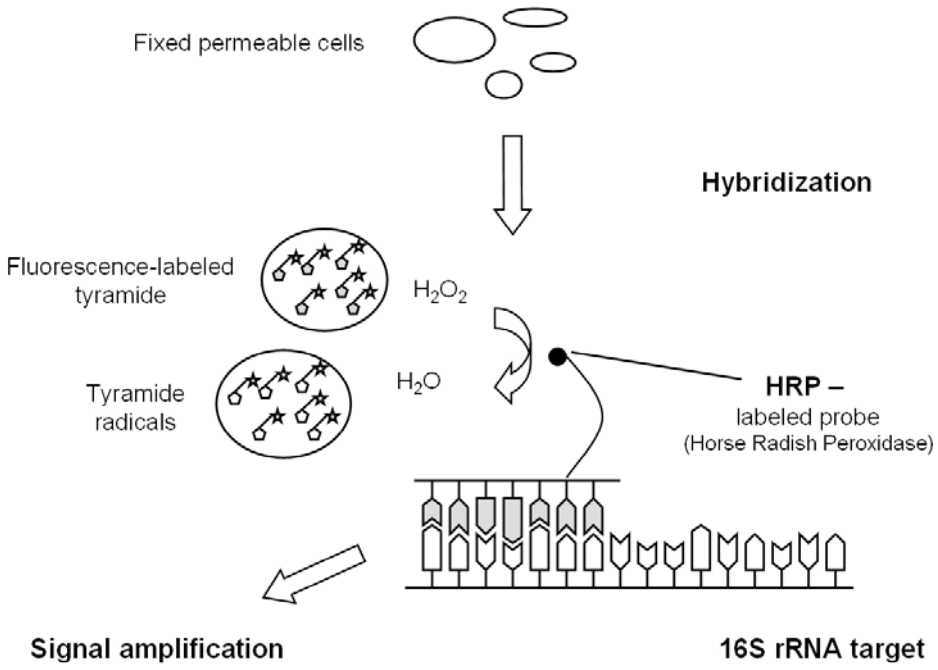

Figure 3: Principle of the CARD-FISH technique. The fluorescence signal is generated by the enzymatic reaction of fluorescence-labeled tyramides and H_2O_2 at the probe-bound HRP (Horse Radish Peroxidase).

measurements or radiotracer techniques (Jørgensen, 1978; Minz et al., 1999; Kuypers et al., 2003). For example, the high abundance of SRB in marine sediments together with elevated sulfate reduction rates indicated their actual involvement in the process *in situ* (Llobbet-Brossa et al., 2002; Ravenschlag et al., 2000; Leloup et al., 2006). Additionally, the calculation of cell-specific activity gives information about the physiological state of single populations or distinct parts of the microbial community and their contribution to nutrient fluxes in the environment.

Examples of combined highly sensitive methods recently developed to relate distinct populations or even single cells to a specific activity, are MAR-FISH (combination of microradioautography to fluorescence techniques, Lee et al., 1999; Ouverney and Fuhrman, 1999; Wagner et al., 2006), Raman-FISH (combination of Raman spectroscopy to fluorescence techniques, Huang et al., 2007) and mRNA-FISH to detect cells that express a particular gene (Pernthaler and Amann, 2004). These novel methods allow for the detection of single meta-

bolizing cells, directly link the uptake of labeled substrates to parts of mixed microbial communities or target on gene-expressing microorganisms.

1.5 Improvement and evaluation of cultivation by molecular techniques

Given the often-cited estimate that only a fraction of the microorganisms present in nature has yet been cultured (Keller and Zengler, 2004) and according to the repeatedly described discrepancy between molecular and cultivation-based studies (Felske et al., 1999; Lysnes et al., 2004), the development and application of the aforementioned techniques came into the focus of microbial ecologists. Nevertheless, the cultivation and isolation of indigenous members of the microbial community still remained the most appropiate tool to resolve physiolgical capacities and ecological relevances (Leadbetter, 2003).

Recently, the cultivation of physiologically novel and environmentally abundant microorganisms was significantly stimulated by insights from molecular ecological analyses (Giovannoni and Stingl, 2007). The application of sensitive molecular screening techniques promoted the cultivation and isolation of microorganisms found to grow only in low densities (Stevenson et al., 2004; Könneke et al., 2005). Simulating as "natural conditions" as possible using natural sea water or sediment extract media, low substrate concentrations and highly-diluted inocula remarkably promoted the cultivation of indigenous microorganisms (Vester and Ingvorsen, 1998; Rappé et al., 2002; Zengler et al., 2002). Additionally, discovering new metabolic pathways performed by microorganisms often resulted from integrated studies coupling the stimulation of microbial activity in enrichments or pure cultures to molecular screening tools (Schmid et al., 2005; Raghoebarsing et al., 2006). Although cultivation can be designed to target abundant members of the community, e.g. by dilution of the inoculum, it nevertheless remains essential to evaluate results revealed from cultivation-based approaches. Molecular techniques represent useful tools to both accompany the cultivation process and validate cultivation techniques (Süß et al., in preparation) as well as unravel the actual abundance of cultured organisms *in situ*.

1.6 Thesis outline

Within the scope of this thesis, a representative tidal sand flat in the German Wadden Sea ("Janssand", Fig. 4) was chosen to study the role of sandy sediments as "hot spots" of microbial activity. The "Janssand" extends over 11 km^2 and is covered with 1 - 2 m water during high tide. The marginal area of this tidal flat is sloping on average 1.6 $cm \cdot m^{-1}$ towards the low water line. Thus, the lower flat is longer inundated than the upper flat. Sampling for this study was performed at a lower flat site near the edge of the flat. Sediments at this site are characterized by a steep sulfate gradient pointing to elevated activity of sulfate-reducing prokaryotes. It was furthermore proposed that microbial communities within these sediments benefit from enhanced advective pore water transport and trapping of, e. g. dissolved nutrients (Billerbeck et al., 2006a). One might therefore hypothesize that the supply with nutrient-loaded pore water into these deep sediments and in consequence the stimulation of microbial activity has a remarkable, but yet unrecognized impact on the transfer and recycling of the elements in the tidal flat ecosystem.

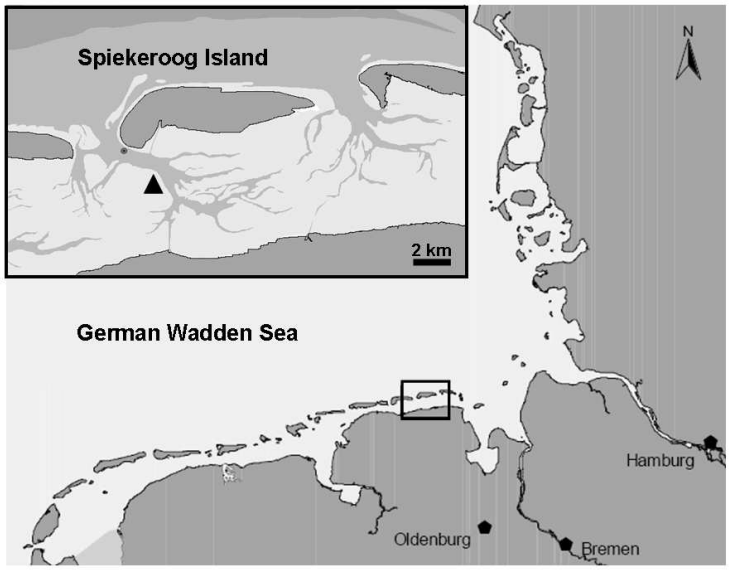

Figure 4: Location of the island Spiekeroog in the Northwestern part of the German Wadden Sea. The study site ("Janssand") is marked with the black triangle.

Sulfate-reducing bacteria appeared suited as "model organisms" as they act as terminal instance in the anaerobic degradation of organic matter in marine sediments and mainly utilize low molecular weight compounds as substrates. Thus, their presence and activity are coupled to the initial degradation of organic matter by hydrolizing and fermentating microorganisms. As they were previously shown to be highly abundant and active in surface sediments at site "Janssand", this thesis extends its focus to SRB in several meters deep sediments.

- As it was not only aimed to characterize the sulfate-reducing community with respect to structure and abundance, but to identify active microorganisms that are likely of ecological relevance, selective cultivation of SRB was evaluated by rRNA-based *in situ* quantification via CARD-FISH (see section 2.1). Growth of both organoheterotrophic as well as lithoautotrophic SRB should be stimulated in highly diluted cultures to target on predominant community members. The availability of pure cultures should furthermore reveal insights into physiological capacities and potential adaptations as well as facilitates the establishment of specific biomarkers.

- Five isolates from highly diluted sediment samples formed a distinct cluster within the *Desulfobulbaceae* and were only distantly related to any validly described species, *Desulfopila aestuarii*. They are assumed to be representatives of a highly abundant population and therefore possess physiological capacities that enable them to sustain a considerable population size. Based on phylogenetic analyses, physiological and chemotaxonomical characterization the novel genus *Desulfocucumis* gen. nov. is proposed with *Desulfocucumis infernus* sp. nov. as the type strain (see section 2.2).

- SRB in sediments at site "Janssand" were exposed to varying sulfate concentrations including sulfate-depleted conditions, but nevertheless, appeared to be highly abundant and constituted a significant fraction of the microbial community. Thus, one might argue on their actual metabolism expressed *in situ* and should – beside dissimilatory sulfate

reduction – consider alternative pathways of energy conservation, including fermentation and syntrophy. Sulfate reduction rates were determined in raw and substrate-amended sediment slurries to (i) quantify the endogenous activity of SRB and to (ii) identify potential substrates that are supposed to stimulate their activity (see section 2.3).

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Chapter 2

Publications

2.1 Directed isolation of *in situ* abundant representatives of the sulfate-reducing community in deep tidal sediments

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Summary

Sulfate-reducing bacteria (SRB) have recently been shown to form abundant and active populations in deep marine sediments, but only few of them have been brought into pure culture so far. In the present study, a selective cultivation approach was combined with a cultivation-independent quantification technique (CARD-FISH, catalysed reporter deposition fluorescence *in situ* hybridisation) to characterise the sulfate-reducing community in 5 meter deep sediments in the German Wadden Sea. Growth of organotrophic and lithotrophic SRB from sediment layers down to 4 meters depth was stimulated in liquid dilution cultures supplemented with lactate, acetate or hydrogen. Molecular screening of the highest dilutions showing sulfide formation by denaturing gradient gel electrophoresis (DGGE) and sequence analysis indicated that both dilution and substrate variations resulted in the selection for single, predominant phylotypes. The majority of the partial 16S rRNA gene sequences retrieved from these cultures shared highest identities with members of the *Desulfobacteraceae* or the *Desulfobulbaceae* within the *Deltaproteobacteria*. Ten sulfate-reducing strains were subsequently brought into pure culture. Based on 16S rRNA gene analysis all isolates belonged to the *Deltaproteobacteria* with six of them closely affiliating with members of the *Desulfobulbaceae*. These strains shared high identities with either candidatus *"Desulfobacterium corrodens"* or *Desulfobacterium catecholicum*. Four other isolates belonged to the *Desulfobacteraceae* and were related to *Desulfobacter psychrotolerans* or to *Desulfobacula phenolica*. CARD-FISH analysis with a set of seven oligonucleotide probes showed that at least two-thirds of all detected SRB in sediment layers beneath 50 cm were related to the *Desulfobacter-Desulfobacula* group and the *Desulfobulbaceae* confirming that the isolated SRB were representatives of *in situ* abundant populations. Both, rRNA-based quantification by CARD-FISH and cultivation indicated that SRB represented a significant fraction of the total microbial community (up to 7 %). Surprisingly, they were highly abundant in deep, sulfate-poor layers reaching even higher absolute and relative cell numbers than in sulfate-rich surface sediments. This relatively high community size in deep sandy sediments might be a result of a mutualistic association of SRB with other anaerobes and that they may be-

nefit from nutrient provision via enhanced pore water transport. Our results further suggest that anaerobic remineralisation processes in deep sediment layers contribute in as yet unrecognis ed significance to biogeochemical cycles in the tidal flat ecosystem.

Introduction

Tidal flat sediments are characterised by a high input of organic matter, since up to 50 % of the pelagic primary production reach the sediment surface (Wollast, 1992). Most of it is rapidly remineralised by microorganisms within the upper few centimetres. Compared to open ocean sites, tidal flat sediments show steep geochemical gradients, and may harbour typical "deep subsurface communities" even in a few meters depth (Wilms et al., 2006). As sulfate occurs at concentrations around 28 mM in seawater, it reaches sediment layers where oxygen, nitrate, ferric iron, and manganese oxides have already been consumed (Fenchel et al., 1998), and represents the major terminal electron acceptor in marine sediments (Jørgensen, 1982). Our study site ("Janssand", Fig. 1) appeared particularly suited to assess microbial communities in tidal flats, as its porous sediment structure and tidal pumping result in enhanced advective pore water transport that provides microorganisms with dissolved nutrients at least down to 50 cm deep sediment layers (Billerbeck et al., 2006). Current hydrologic models even suggest pore water drainage down to several meters depth (H. Røy and D. de Beer, personal communication). Additionally, methane was repeatedly observed to seep out of the sediment at lower flat sites pointing to high rates of methanogenesis within the sediment (unpublished).

Most studies on the community composition of sulfate-reducing bacteria (SRB), their activity and ecological role in marine sediments have focused on the uppermost centimetres (e. g. Ferdelman et al., 1997; Sahm et al., 1999; Ravenschlag et al., 2000; Llobbet-Brossa et al., 2002; Mußmann et al., 2005). More recently, SRB have been found to be abundant and highly diverse also in sediments down to several hundred meters of depth (Parkes et al., 2005; Leloup et al., 2006; Schippers and Neretin, 2006). Their high cell numbers indicate a significant impact on biogeochemical cycles within deep marine sediments. Nevertheless, only few SRB from the marine subsurface have been brought into

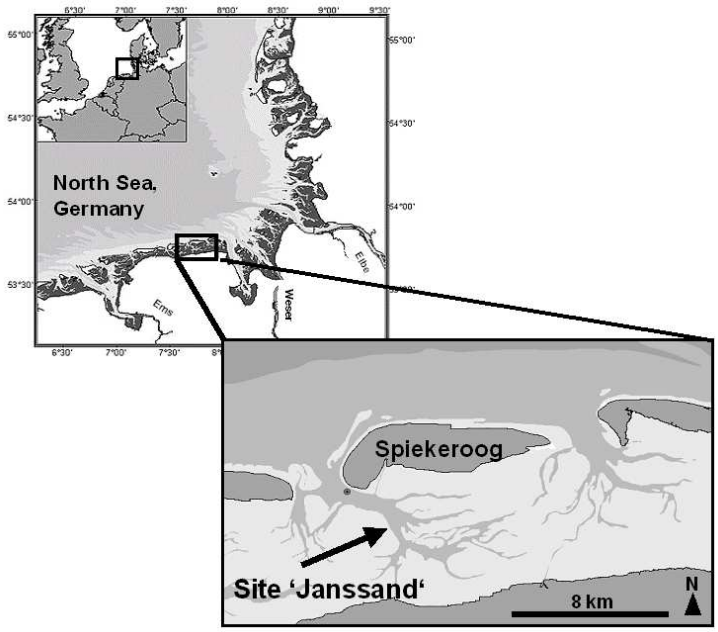

Figure 1: Location of site "Janssand", near the island of Spiekeroog, North Sea, Germany

pure culture, thus their physiology and ecological relevance in these habitats remained uncertain so far (Bale et al., 1997; Barnes et al., 1998; Sass et al., 2002; Süß et al., 2004; Köpke et al., 2005; Batzke et al., 2007).

The potential of cultivation approaches to analyse microbial communities is controversially discussed (Nichols, 2007; Ritz, 2007). New molecular methods are available to get access to the microbial community structure without the need of cultivation and even without the amplification bias of genetic biomarkers (von Mehring et al., 2007). However, cultivation approaches aided by molecular screening tools remain still essential to resolve the physiological capabilities of novel phylotypes (Giovannoni and Stingl, 2007). In the present study, we chose selective cultivation conditions to facilitate growth of a distinct physiological and ecologically relevant group of microorganisms (SRB). The directed cultivation of SRB from diluted sediment samples was accompanied and evaluated by molecular techniques (DGGE, cloning and sequencing, CARD-FISH). Growth of organotrophic inclompete and complete oxidising, as well as lithotrophic SRB, was favoured by selective media amended with lactate, acetate or hydrogen as sole electron donor. Consistently, selective cul-

tivation and CARD-FISH analysis revealed that two groups predominated the sulfate-reducing community down to 5 meters depth indicating their ecological relevance *in situ*.

Results

Sediment and pore water characteristics of site "Janssand"

The sampling site was characterised by fine- to medium-sized sands dominating the lithological settings down to the analysed depth of 5 meters. However, our samples also comprised some thin mud-dominated layers with total organic carbon reaching up to 2.5 % of the dry weight (J. Köster, personal communication). The concentration of pore water sulfate, as the terminal electron acceptor for microbial sulfate reduction, decreased steeply with depth to concentrations of around 0.2 mM at 150 cm depth (Fig. 2A). Pore water from sediments deeper than 200 cm was characterised by less than 0.1 mM of dissolved sulfate. Short-chain organic acids like acetate, lactate and formate, previously reported as substrates for sulfate reducers in tidal flat surface sediments (Llobbet-Brossa et al., 2002), were detected even in 5 meters depth. Acetate concentrations ranged from 10 μM to 60 μM, whereas lactate and formate concentrations did not exceed 6 μM and 12 μM, respectively. Concentrations of propionate and butyrate were below the detection limit (1 μM).

Pore water analysis from the sampling campaign in summer 2006 revealed higher concentrations of sulfate at the sediment surface than in spring 2005 (Fig. 2A). The profile consistently showed a strong decrease of sulfate concentrations to about 2 mM in 200 cm depth. Noteworthy, pore water sulfate increased slighty in 250 cm and was almost depleted at 300 cm and below. Methane was detected within all sediment layers analysed. Concentration was highest in 200 cm depth and decreased to both the upper and the lower part of the sediment. The opposed depth profiles of sulfate and methane indicated the presence of an extended sulfate-methane transition zone (Fig. 2A).

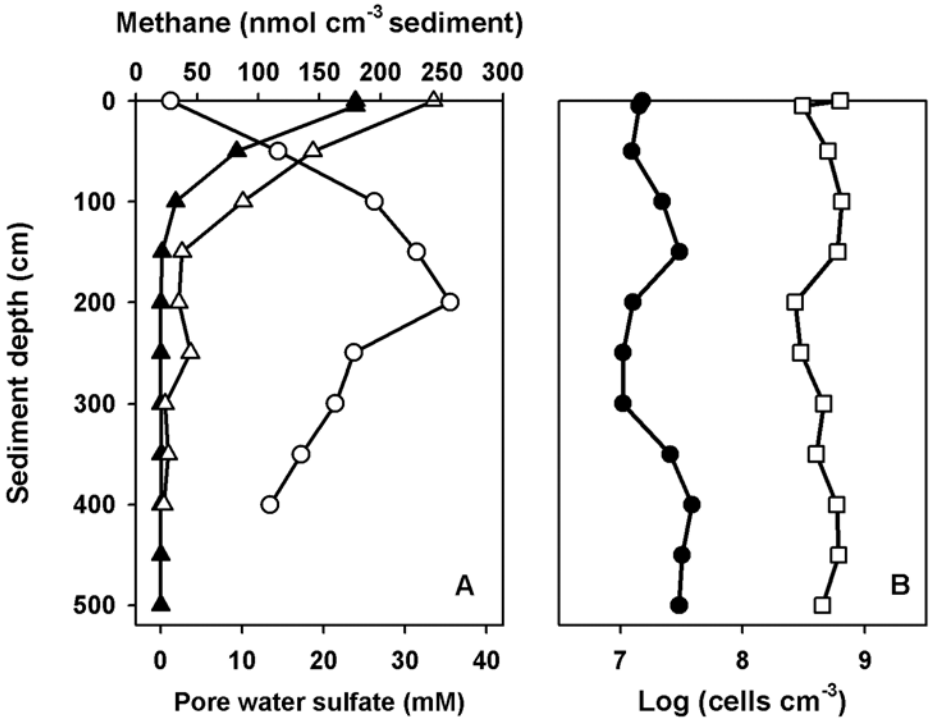

Figure 2: Depth profiles of (A) sulfate from pore water samples (April 2005: black triangles; July 2006: open triangles) and methane from sediment samples (July 2006, open circles). (B) Total cell counts (DAPI, open squares) and absolute abundance of SRB as detected with the chosen set of six CARD-FISH probes in April 2005 (filled circles, see also Tab. 2).

Desulfobulbaceae predominate highly diluted, sulfidogenic cultures

In order to cultivate the most abundant SRB, liquid dilution cultures (up to 10^{-8}) from different sediment layers were supplemented with lactate, acetate, or hydrogen as substrate. These substrates were selected to favour growth of different physiological types of SRB including organotrophs and lithotrophs. After 4 months of incubation, sulfide formation (>3 mM) was found in cultures from all depths and with all substrates used, while substrate-free controls showed no sulfide production. Highest sulfide-positive dilutions were observed with lactate showing growth in up to million-fold dilutions from 100, 250 and 400 cm deep sediments. Cultures from 50 and 100 cm with acetate or hydrogen as substrate showed sulfide formation in dilutions of at least 10^{-4}, whereas cultures from 250 and 400 cm were sulfide-positive only in lower dilutions (at least 10^{-2}).

The highest dilutions showing sulfide formation were chosen for molecular screening by *Bacteria*-specific 16S rRNA-based DGGE and sequence analysis. In general, DGGE yielded only one or two bands indicating that the dilution and substrate variations had resulted in an effective selection of predominant phylotypes (Fig. 3).

Partial sequencing and phylogenetic analysis revealed fifteen distinct 16S rRNA gene sequences (approx. 400 bp). Ten of these affiliated with marine deltaproteobacterial SRB that were previously shown to be abundant in Wadden Sea sediments (Llobbet-Brossa et al., 2002; Mußmann et al., 2005, Tab. 1). Among these were six sequences (labelled with A in Fig. 3) that were almost identical to each other with at least 99.7 % sequence identity and affiliated with members of the *Desulfobulbaceae*, in particular with candidatus *"Desulfobacterium corrodens"* (Dinh et al., 2004). These sequences were detected in both organotrophic and lithotrophic cultures originating from sediment layers between 100 cm and 400 cm depth.

Four more sequences were found to affiliate with the *Desulfobacteraceae*. Two of these (Fig. 3, labels C and D) were derived from the *Desulfosarcina-Desulfococcus* group, and shared highest identities with environmental sequences obtained from various marine sediments. Interestingly, one sequence (Fig. 3, label C) was related to that of an uncultured deltaproteobacterium identified as potential partner of an anaerobically methane-oxidising consortium in methane-seep sediments (95 %; Orphan et al., 2001). The two other sequences (Fig. 3, label B) were both highly similar to that of an uncultured member of the genus *Desulfobacula* detected in Antarctic sediments (Purdy et al., 2002) and were exclusively found in a culture containing hydrogen as electron donor. Five partial 16S rRNA gene sequences (Fig. 3, labels E to H) from cultures containing either lactate or acetate and originating from different sediment layers could not be assigned to any known sulfate reducer. All five sequences affiliated with the *Firmicutes* and shared high identities (90-98 %) with sequences retrieved from various sedimens, including a tidal flat located near site "Janssand" (Köpke et al., 2005). It remains unclear whether the corresponding organisms possess the capacity to reduce sulfate.

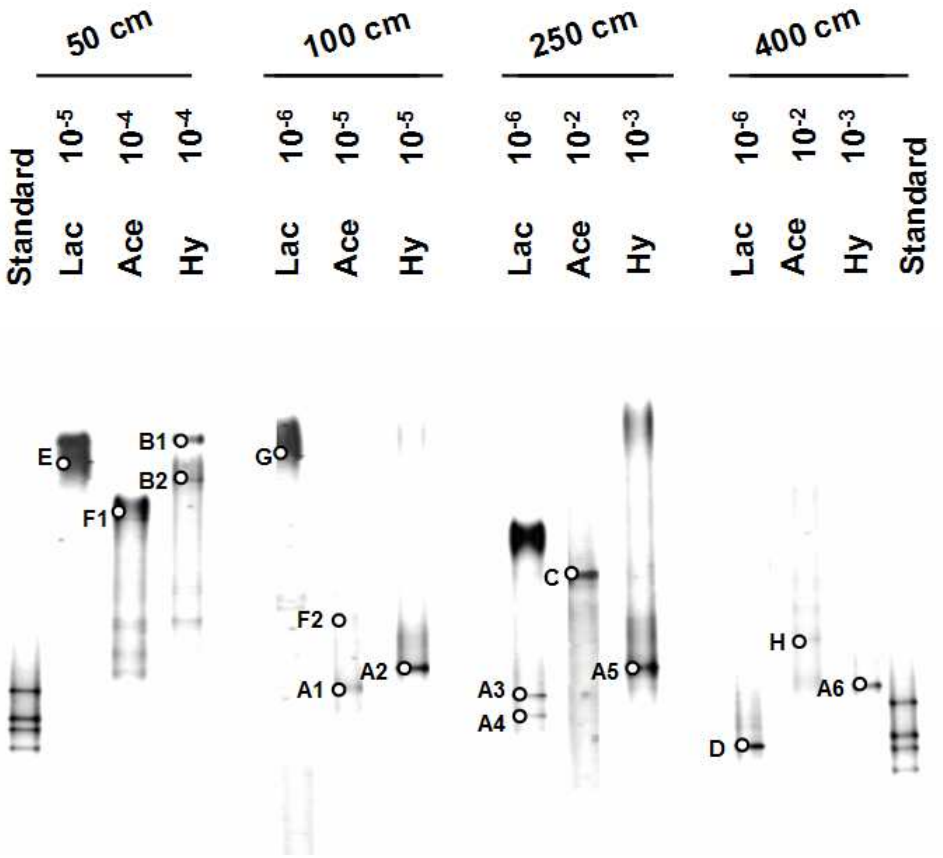

Figure 3: DGGE band patterns of 16S rRNA gene fragments revealed from "Janssand" sediment dilution cultures. DGGE lanes are labelled by the sediment depth that the inoculum originated from (50, 100, 250 and 400 cm), the extracted dilution step (e. g. 10^{-6}) and the electron donor offered (Lac: lactate, Ace: acetate, Hy: hydrogen). Circles indicate DGGE bands that were excised for reamplification and sequencing. Labels (A-H) refer to the data given in Tab. 1. Bands marked by the same letter point to sequences that appeared to be closely related to each other.

Table 1: Overview of phylotypes detected in highest dilutions showing sulfide formation by PCR-DGGE analysis

DGGE band		**Closest related phylotype** (accession no.)	**Sequence identity** (%)
A1	JS100AceS1	*Desulfobacterium corrodens* (AF228119)	95
A2	JS100Hy	*Desulfobacterium corrodens* (AF228119)	96
A3,4	JS250Lac	*Desulfobacterium corrodens* (AF228119)	95
A5	JS250Hy	*Desulfobacterium corrodens* (AF228119)	95
A6	JS400Hy	*Desulfobacterium corrodens* (AF228119)	95
B1,2	JS50Hy	Uncultured *Desulfobacula* sp. (AY177795)	98
C	JS250Ace	Uncultured deltaproteobacterium (AF354147)	95
D	JS400Lac	Uncultured bacterium clone KM88 (AY216443)	98
E	JS50Lac	*Dethiosulfatibacter aminovorans* (AB218661)	97
F1	JS50Ace	Uncultured *Clostridium* (AY370633)	96
F2	JS100AceS2	Uncultured *Clostridium* (AY370633)	90
G	JS100Lac	Uncultured Gram-positive bacterium (AY211678)	97
H	JS400Ace	Uncultured deltaproteobacterium (AJ889164)	98

Isolation of predominant sulfate-reducing bacteria

Ten strains of sulfate-reducing bacteria were isolated from the dilution cultures previously screened via DGGE. In general, the predominance of distinct members of the *Desulfobulbaceae* and the *Desulfobacteraceae* in these cultures was confirmed by their final isolation into pure cultures (Fig. 4). Five of the isolates (JS_SRB100Hy, 400Hy, 250Hy, 100Ace, and 250Lac) shared highest sequence identities (95-96.6 %) with the anaerobically iron-oxidising sulfate reducer candidatus *"Desulfobacterium corrodens"*. They all formed $2-4\,\mu$m long rod-shaped cells with round ends (Fig. 5a). Another isolate of the *Desulfobulbaceae* (JS_SRB400Ace) was related to *Desulfobacterium catecholicum* (98.6 %) and formed oval- to rod-shaped cells (Fig. 5b). Four isolates (JS_SRB250Ace, 100Lac, 50Lac, and 50Hy) belonged to the *Desulfobacteraceae* and shared highest identities with *Desulfobacter psychrotolerans* (98.3 %) or *Desulfobacula phenolica* (95-97 %), respectively. Like its closest relative *Desulfobacter psychrotolerans*, cells of the isolate JS_SRB250Ace formed dense aggregates of $1.5\,\mu$m wide, short rods (Fig. 5c). Cells of the isolates affiliating with the genus *Desulfobacula*, appeared as thick, slightly vibrio-shaped cells, $2-3\,\mu$m in length (Fig. 5d).

First growth experiments using various substrates showed that all isolates related to the *Desulfobulbaceae* shared a relatively high metabolic capacity with respect to their electron donor. They all utilised lactate, pyruvate, fumarate, and ethanol as well as formate and hydrogen as electron donor for sulfate reduction. All isolates related to the genus *Desulfobacula* were additionally able to utilise acetate as electron donor indicating their capacity to completely oxidise organic compounds to CO_2. Strain JS_SRB250Ace, affiliated with the genus *Desulfobacter*, appeared to be less metabolically versatile as growth was only observed with acetate and ethanol as sole electron donor. Additionally, all strains except JS_SRB250Ace showed fermentative growth with pyruvate and/or fumarate.

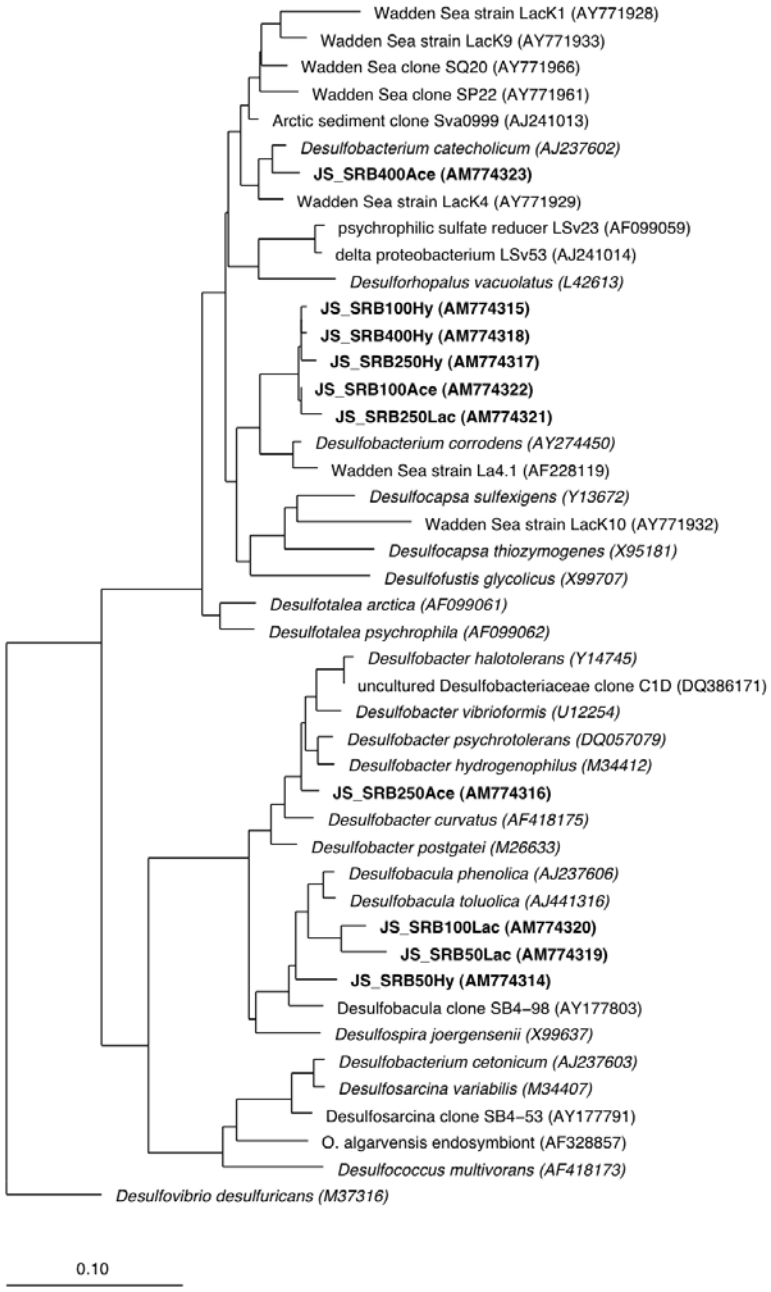

Figure 4: Phylogenetic affiliation of isolated SRB with selected *Deltaproteobacteria* based on 16S rRNA gene sequences analyses. The tree was calculated with nearly full-length sequences (>1400 nucleotides) by maximum-likelihood analysis. Sequences obtained from this study are written in bold. Scale bar corresponds to 10 % estimated sequence divergence.

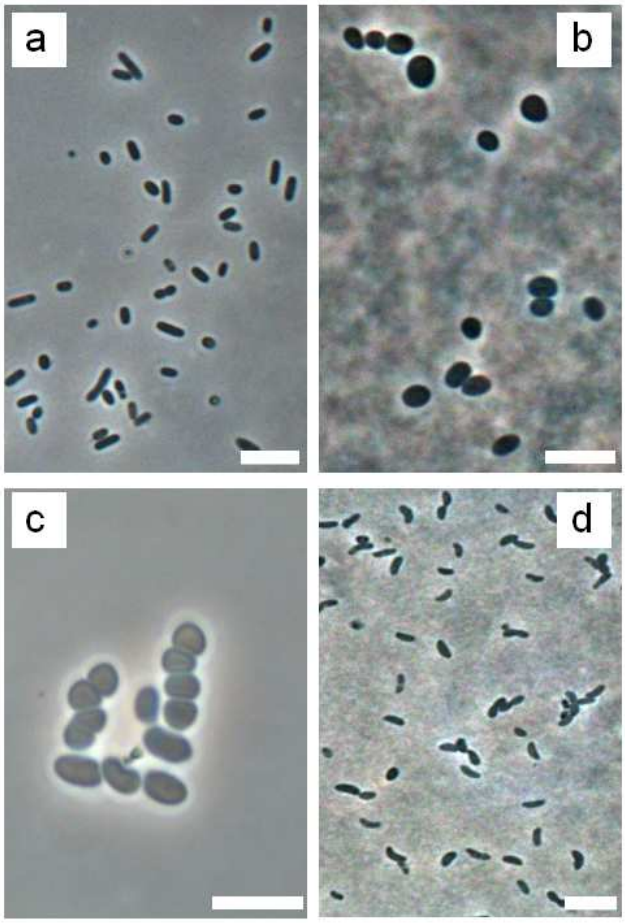

Figure 5: Phase-contrast micrographs of SRB isolated from site "Janssand". Strains JS_SRB400Hy (a) and JS_SRB400Ace (b) belong to the *Desulfobulbaceae* and are related to candidatus *"Desulfobacterium corrodens"* and *Desulfobacterium catecholicum*, respectively. Strains JS_SRB250Ace (c) and JS_SRB50Lac (d) are members of the *Desulfobacteraceae* and belong to the genera *Desulfobacter* and *Desulfobacula*. Scale bars represent 5 μm.

CARD-FISH confirms the predominance of the isolated SRB

Based on the sequences retrieved from the molecular screening of our dilution cultures, CARD-FISH was applied to fixed sediment samples using a set of seven different 16S rRNA oligonucleotide probes. Beside a *Bacteria*-specific probe (probe EUB338), this set included group-specific probes targeting on the phylogenetic groups that were detected in our dilution cultures (probes

DSB985, Sval428, DSS658) as well as such that were not detected, but have previously been found to be abundant in marine sediments (probes DSR651, DSV698, DFMI227a).

Total cell numbers (DAPI counts) showed only minor variations with respect to the sediment depth (2.7 to $6.5 \cdot 10^8$ cells $\cdot$ cm^{-3} sediment; Fig. 2, Tab. 2). CARD-FISH analysis with the *Bacteria*-specific probe EUB338 showed that the microbial community over the entire vertical profile was dominated by *Bacteria* accounting for up to 96 % of the total cell counts.

SRB were detected in all sediment layers and, based on the sum of the six SRB-specific probes, accounted for at least 2.4 % ($1.5 \cdot 10^7$ cells $\cdot$ cm^{-3}) in the surface layer and up to 6.8 % ($3 \cdot 10^7$ cells $\cdot$ cm^{-3}) of the total microbial community at five meters depth (Fig. 2, Tab. 2). Highest numbers of targeted SRB were found at 400 cm depth ($3.9 \cdot 10^7$ cells $\cdot$ cm^{-3}). The most abundant SRB were members of the *Desulfobacter-Desulfobacula* group and of the *Desulfobulbaceae* targeted by the probes DSB985 and Sval428, respectively. The latter also targets at *"Desulfobacterium corrodens"* and its relatives. While these two groups showed only low abundance at the sediment surface, they represented at least two-thirds of all detected SRB in deeper layers. This finding confirmed that our cultivation approach resulted in the isolation of representatives of the most abundant members of the sulfate-reducing community.

The abundance of the *Desulfosarcina-Desulfococcus* group (probe DSS658) was relatively low as their members accounted for less than 0.7 % of the total microbial community. *Desulfovibrio* species (probe DSV698) were only found in the surface sample where they represented 1.1 % of all DAPI-stained cells. Numbers of bacteria affiliating with the genus *Desulforhopalus* and its relatives (probe DSR651) remained almost constant (about 1 % of the total community) along the entire sediment column. *Desulfotomaculum*-related bacteria (probe DFMI227a) were only detected at the sediment surface and at 200 and 500 cm depth, where they accounted for less than 0.3 % of all DAPI-stained cells.

Table 2: Total cell counts and abundance of different phylogenetic groups of SRB in a five meter sediment core from site "Janssand" as studied by CARD-FISH (given in cells per cm^3 sediment)

Depth	**DAPI**	**Probe counts**[a]							**Sum of all SRB probes**	
(cm)	($\cdot 10^8$)	EUB338 ($\cdot 10^8$)	DFMI227a ($\cdot 10^6$)	DSB985	DSR651	DSS658	DSV698	Sval428	($\cdot 10^7$)	% DAPI
0	6.3 ± 1.5	6.0 ± 1.2	0.76 ± 0.01	2.46 ± 0.03	1.07 ± 0.01	nd	6.93 ± 0.7	3.84 ± 0.04	1.51 ± 0.08	2.4 ± 0.13
5	3.1 ± 0.8	3.0 ± 0.6	nd	2.79 ± 0.14	3.13 ± 0.16	0.65 ± 0.01	nd	7.63 ± 0.99	1.42 ± 0.13	4.6 ± 0.42
50	5.0 ± 1.4	4.7 ± 0.9	nd	1.90 ± 0.04	2.20 ± 0.04	0.6 ± 0.01	nd	7.65 ± 0.54	1.24 ± 0.06	2.5 ± 0.13
100	6.5 ± 3.0	6.3 ± 2.5	nd	5.33 ± 0.11	7.76 ± 0.31	nd	nd	8.97 ± 0.36	2.21 ± 0.08	3.4 ± 0.12
150	6.0 ± 2.3	5.6 ± 1.6	nd	19.8 ± 2.00	2.58 ± 0.03	1.50 ± 0.01	nd	6.84 ± 0.21	3.07 ± 0.22	5.1 ± 0.37
200	2.7 ± 1.3	2.2 ± 0.4	0.76 ± 0.01	6.18 ± 0.25	nd	0.70 ± 0.01	nd	5.02 ± 0.20	1.27 ± 0.05	4.7 ± 0.17
250	3.0 ± 1.3	2.7 ± 0.4	nd	4.26 ± 0.04	nd	nd	nd	6.21 ± 0.06	1.05 ± 0.01	3.5 ± 0.03
300	4.6 ± 1.7	4.0 ± 0.6	nd	nd	2.94 ± 0.12	nd	nd	7.59 ± 0.76	1.05 ± 0.09	2.3 ± 0.19
350	4.0 ± 1.4	3.3 ± 0.4	nd	11.2 ± 1.80	2.88 ± 0.12	2.76 ± 0.11	nd	8.80 ± 1.14	2.56 ± 0.32	6.4 ± 0.79
400	5.9 ± 2.6	3.6 ± 0.5	nd	30.5 ± 9.15	nd	2.07 ± 0.04	nd	6.25 ± 0.38	3.88 ± 0.96	6.6 ± 1.62
450	6.1 ± 1.5	5.6 ± 1.4	nd	14.0 ± 0.42	2.20 ± 0.02	3.66 ± 0.04	nd	12.0 ± 0.24	3.19 ± 0.07	5.2 ± 0.12
500	4.5 ± 1.6	3.8 ± 1.1	1.17 ± 0.01	7.74 ± 0.08	1.13 ± 0.02	2.75 ± 0.03	nd	17.6 ± 0.35	3.04 ± 0.05	6.8 ± 0.11

[a] Numbers were corrected by substracting NON338 counts. Means and standard deviations were calculated from the counts of two subcores. (nd: not detected)

Discussion

In the present study we have demonstrated that two phylogenetic groups of SRB dominate the sulfate-reducing community in deep sediment layers of a sandy tidal flat. Both, their cultivation as well as rRNA-based *in situ* quantification indicated that our isolate apparently represent active and potentially ecological relevant members of the microbial community.

SRB community structure in deep tidal flat sediments

Previous studies using FISH techniques have revealed that SRB constitute an important fraction of the microbial community within the upper 40 cm of tidal flat sediments (Llobbet-Brossa et al., 2002; Ishii et al., 2004; Mußmann et al., 2005; Musat et al., 2006). The present study reports for the first time on the abundance and vertical distribution of different phylogenetic groups of SRB in 5 meter deep sediments of a tidal sand flat. In contrast to other studies on deep tidal flat sediments (Köpke et al., 2005; Wilms et al., 2007), we chose selective cultivation conditions and a rRNA-based quantification approach to specifically characterise the active fraction of the sulfate-reducing community.

Our study revealed that the relative abundance of SRB varied only slightly with depth accounting for up to 7 % of the total microbial community. This is consistent with previous estimations of 5 % SRB detected in several meters deep tidal mud flat sediments via quantification of specific marker genes (*dsrAB*, dissimilatory (bi)-sulfite reductase; Wilms et al., 2007). Characterisation of the microbial community within these deep mud flat sediments revealed a pronounced community shift from *Proteobacteria* dominating the upper, sulfate-rich layers to spore-forming members of the *Firmicutes* in deeper, sulfate-depleted sediments (Köpke et al., 2005; Wilms et al., 2006). In contrast, the SRB community at site "Janssand" was numerically dominated by *Deltaproteobacteria*, in particular members of the *Desulfobulbaceae* and the *Desulfobacter-Desulfobacula* group, throughout the whole sediment column. According to this finding, only representatives of these two phylogenetic groups were isolated from highly diluted sediment samples. Six isolates retrieved from this study belonged to the family of the *Desulfobulbaceae* and shared highest identities with SRB that were previously detected in various other marine

sediments (Powell et al., 2003; Purdy et al., 12003; Mußmann et al., 2005).

In both muddy and sandy surface sediments, members of the polyphyletic *Desulfosarcina-Desulfococcus* group were described to predominate the SRB community in the upper 40 cm of the sediment and at different seasons (Ishii et al., 2004; Mußmann et al., 2005; Musat et al., 2006). In difference, members of this group were detected in rather low abundance in deeper sediments within the scope of our study. Although we were able to stimulate growth of representatives of this phylogenetic clade in dilution cultures, sub-cultivation and subsequent isolation selected for another phylotype that was initially not detected by molecular screening.

Being aware that the chosen cultivation conditions and the limited number of obtained cultures only fractionally reflects the diversity of the indigenous SRB community, we apparently cultured members of the predominating phylogenetic groups assumed to be of ecological relevance.

High abundance of sulfate-reducing bacteria in sulfate-poor sediments

A surprising outcome of this study was the high abundance of SRB in deep, sulfate-poor layers of the sediment. Similar observations were made at several meters deep Black Sea sediments, where SRB were present in significant numbers throughout the methane zone (Leloup et al., 2006). In contrast to DNA-based molecular surveys that target on 16S rRNA genes or specific marker genes (*dsrAB*), we applied a 16S rRNA-based quantification technique that presumably detects only cells in a physiologically active state. The high abundance of supposedly active SRB in comparison to the low availability of sulfate leads to the questions how they may stand the competition for substrates and how they gain sufficient energy to maintain a considerable community size even in sulfate-poor sediments.

First, based on the sulfate profile in April 2005 we suggest that the activity of SRB in sediments down to 150 cm depth was not limited by sulfate, as concentrations were in the range of the half-saturation constants recently described for marine sulfate-reducing communities (0.1 - 0.3 mM; Pallud and van Cappellen, 2006). The chemical pore water profile from July 2006 even

indicated a supply of sulfate in deeper sediment layers. Enrichment and isolation of lithotrophic as well as organotrophic SRB with hydrogen, acetate and lactate suggest that a broad spectrum of compounds may serve as potential electron donor for sulfate reduction *in situ*. Additionally, the pronounced sulfate-methane transition zone provides an indication that reducing equivalents for microbial sulfate reduction could also originate from biogenic methane. Members of the *Desulfosarcina-Desulfococcus* group and more recent, of the *Desulfobulbaceae* were reported to mediate the anaerobic oxidation of methane via sulfate reduction in association with certain archaea (Orphan et al., 2001; Lösekann et al., 2007). But it requires more detailed investigations to clarify if a fraction of the SRB detected in our study are involved in this reaction.

In sediments deeper than 150 cm with less than 0.1 mM of pore water sulfate, SRB are forced to sustain these conditions by energy conservation via alternative metabolic pathways. Since compounds that potentially substitute sulfate as electron acceptor were assumed to be not available in these deep sediments, fermentative growth and mutualism appear as most feasible alternatives. For instance, all isolates, except the *Desulfobacter*-related strain JS_SRB250Ace, shared the capacity to ferment fumarate and/or pyruvate in the absence of sulfate. Since both compounds are usually not released during anaerobic degradation processes, we suggest that SRB within these sediments rather exist in syntrophic association. In this case, methanogenic or homoacetogenic partners would be essential to scavenge the hydrogen produced by SRB. Interspecies hydrogen transfer could create thermodynamically feasible conditions for sulfate reducers to oxidise various organic substrates in the absence of sulfate (Bryant et al., 1977; Plugge et al., 2002; Stolyar et al., 2007). Interaction of our isolates with methanogenic or homoacetogenic cultures has yet not been tested, but this syntrophy was proposed to be widely distributed in anoxic environments (Schink, 1997). However, both scenarios presume that the sulfate-reducing community within these deep sediment layers is supplied with utilisable, not yet identified substrates providing at least sufficient energy for survival.

Transport of solutes into deep sediment layers

Sediments at site "Janssand" showed an almost homogenous distribution of microorganisms over several meters depth. This phenomenon might result from the transport of organic as well as inorganic nutrients into deep sediment layers. It was previously proposed that the microbial communities benefit from the higher permeability of sands and the resulting enhanced advective pore water transport in porous sediment layers (Huettel and Rusch, 2000; de Beer et al., 2005; Billerbeck et al., 2006). Billerbeck and colleagues showed that the drainage of pore water at site "Janssand" through the sediment from the upper flat directed towards the low water line affects at least the sediment layers down to 50 cm depth. Recent hydrologic modelling approaches suggest that this transport might even affect several meters deep layers (H. Røy, D. de Beer, personal communication). However, there is only little information available on the chemical composition of these deep pore waters. Considerable seasonal variations in sulfate concentration within sediment layers at 2.5 meters depth indicate a transport of solutes from the upper flat. Additionally, a variety of organic acids, potential substrates to e. g. sulfate-reducing bacteria, were detected even at several meters depth. Thus, it is feasible that these compounds were microbially formed through fermentation processes within these sediment layers or more likely originated from upper layers and were subsequently transported into deeper sediments. Nevertheless, we assume that local nutrient concentrations represent the result of both the transport of nutrients from the upper part of the sand flat into deep sediment layers at lower flat sites and variations in the activity of the microbial community. In conclusion, the relatively high abundance of microbial cells and the presence of active and abundant sulfate reducers might be regarded as indication for a beneficial effect of pore water drainage and of the provision with as yet undefined nutrients to the microbial community of deep sediments.

Experimental procedures

Study site and sampling

Five meter long sediment cores were taken in April 2005 and July 2006 at the Northeastern margin of the "Janssand" tidal flat (53°44.177'N, 007°41.970'E) that is located in the back barrier area of the island of Spiekeroog, North Sea, Germany (Fig. 1). The biogeochemistry and hydrography of the site have been described elsewhere (Billerbeck et al., 2006). Up to six meter long aluminium liners were driven into the sediment by vibro-coring (Wilms et al., 2006). Near-surface samples were taken separately with short plastic liners (20 cm) or sterile cut-off syringes. Long sediment cores were immediately taken to the laboratory and processed within 6 h after sampling (2005) or samples were taken immediately after recovering (2006). To avoid pore water mixing they were transported horizontally. The cores were cut into 50 cm sections and samples for various applications were taken from the innermost part of the freshly exposed surfaces.

Pore water analyses

Pore water was gained by centrifugation of sediment in closed vessels under inert gas (N_2) and filtered through 0.2 μm membrane filters. Pore water sulfate was determined by ion chromatography with conductivity detection (Sykam, Fürstenfeldbruck, Germany) as described previously (Sass et al., 2001).

Short-chain organic acids were analysed on an ICS-2000 ion chromatography system (Dionex, Sunnyvale, CA) equipped with a self-regenerating suppressor unit (Dionex ASRS-Ultra II 4-mm) and a conductivity detector. Undiluted pore water samples (injection volume 2 μL) were transferred into vials and placed in an autosampler unit, cooled to 4 °C, prior to analysis. Anions were separated on an Ionpac AS15 column (4 x 250 mm, Dionex, Sunnyvale, CA) at temperature of 30 °C and a flow rate of 0.8 mL $\cdot$ min^{-1}. An eluent gradient was applied with a flow of 0.6 mM KOH for 28 min, followed by an increase of 29.5 mM KOH for 2 min and a final 10 min with 65 mM KOH. Concentrations of short-chain organic acids were calculated with the Chromeleon software package (Dionex, Sunnyvale, CA) using standard calibration curves.

For measuring methane concentrations, 2 ml of sediment were transferred into 18 ml of sodium hydroxide solution (2.5 %) in gas-tight tubes immediately after recovering the core. Fixed samples were stored at 4 °C in the dark until further proceeding. Headspace samples were analysed on a Varian CX 3400 gas chromatograph (Varian, Darmstadt, Germany) with nitrogen as the carrier gas isothermic at 40 °C. The GC system was equipped with a Plot Fused Silica column (No. 7517; 25 m by 0.53 mm, Al_2O_3/KCl coated; Chromopack, Middleburg, The Netherlands) and a flame ionisation detector. Temperature of the injector and the detector were 150 °C and 200 °C, respectively.

Enrichment and isolation of sulfate-reducing bacteria

Liquid dilution cultures were initiated with sediment samples from layers down to 4 meters depth. 2 cm^3 sediment were suspended into 18 mL of a defined, bicarbonate-buffered, sulfide-reduced seawater medium (Widdel and Bak, 1992) under an atmosphere of N_2/CO_2 (90:10, v/v). These sediment slurries were diluted ten-fold in glass tubes with air-tight rubber septa up to a final dilution of $1:10^8$. Lactate (20 mM), acetate (20 mM) or H_2/CO_2 (90:10, v/v) served as substrates. Cultures were incubated at 15 °C for 4 months. Growth and activity of sulfate reducers were monitored by phase-contrast microscopy and by measuring sulfide (Cord-Ruwisch, 1985). The highest dilution steps showing sulfide production were chosen for molecular screening and isolation following the deep agar dilution method of Widdel and Bak (1992). Purity of the cultures was proven microscopically and by transferring the cultures to a complex medium containing yeast extract (1 %, w/v), glucose (10 mM) and peptone (1 %, w/v).

Substrate utilisation of the isolates was tested by substituting the electron donor used for isolation with potential electron donors including short-chain organic acids or ethanol each at a final concentration of 10 mM. The capacity for fermentative growth was tested in medium without an additional electron acceptor supplemented with lactate, malate, pyruvate or fumarate (10 mM each).

Molecular analyses of dilution cultures and pure cultures

For extraction of genomic DNA from the highest liquid dilutions showing sulfide production 1 mL of each culture was centrifuged and pellets were resuspended in 50 μL sterile distilled water. After adding lysozyme (40 $\mu g \cdot mL^{-1}$) and sodium dodecyl sulfate (SDS, 1 % w/v) five freeze and thaw cycles (- 80 °C to 70 °C, 3 min each) were conducted to release nucleic acids. DNA was directly amplified from the lysate using bacterial primers GC-341f and 907r (Muyzer et al., 1995). PCR, DGGE and sequence analysis were carried out as described by Süß et al. (2004).

Genomic DNA from cell pellets of harvested pure cultures was extracted by a modified phenol-chloroform-isoamylalcohol extraction protocol including enzymatic digestion as well as treatment with SDS and CTAB (Cetyltrimethylammoniumbromide, 10 %, w/v) (Wilson, 2001). 16S rRNA gene-targeted bacterial primers 8f and1492r (Overmann and Tuschak, 1997) were used to amplify almost complete gene fragments. PCR products were separated by agarose gel electrophoresis, and amplicons of the expected size were either directly sequenced (Mußmann et al., 2005) or cloned into chemically competent *E. coli* JM109 cells using the pGEM® -T Easy vector system (Promega Corporation, Madison, Wisconsin, USA) according to the manufacturer's instructions. Clones were screened for unique inserts by PCR with the M13 primer pair and purified PCR products were sequenced at GATC Biotech (Konstanz, Germany).

To affiliate partial 16S rRNA gene sequences retrieved from excised DGGE bands to the closest phylotypes they were compared to those in GenBank using the BLAST function at the National Center for Biotechnology Information server (www.ncbi.nlm.nih.gov). Nearly complete 16S rRNA gene sequences of our isolates were analysed using the ARB program package (Ludwig et al., 2004). 16S rRNA gene sequences most closely related to our isolates were retrieved from the EMBL database using the ARB Fast Aligner feature (http://www.arb-home.de). The phylogenetic tree was calculated by maximum-likelihood criteria with 16S rRNA gene sequences consisting of at least 1400 nucleotides.

Nucleotide sequence accession numbers

All 16S rRNA gene sequences obtained in this study are available from EMBL under accession numbers AM410927 to AM410939 for DGGE bands and AM774314 to AM774323 for the isolates.

Cell counts and fluorescence *in situ* hybridisation (CARD-FISH)

Sediment samples were fixed in 4 % paraformaldehyde over night and further treated as described previously (Mußmann et al., 2005). CARD-FISH procedure was carried out as described by Ishii et al. (2004). 16S rRNA-targeted oligonucleotide probes labelled with horseradish peroxidase (HRP) at the 5'-end were purchased from Biomers (Ulm, Germany). Probes and hybridisation conditions are listed in Tab. 3. For each probe and sample, counter-staining was performed with DAPI and at least 600 DAPI stained cells were counted. Hybridisation with probe NON338 was performed as a negative control (Wallner et al., 1993). The stringency conditions for the HRP-labelled probes Sval428, DFMI227a and DSB985, that were previously used only for FISH, were evaluated in formamide (FA) gradients using reference strains (Tab. 3).

Table 3: 16S rRNA-targeted oligonucleotide probes used in this study[a].

Probe	**Target**	**Reference strain**	Sequence (5'-3') of probe	**FA**[c] **(%)**	**NaCl**[d] **(mM)**	**Reference**
Eub338	*Bacteria*	*Desulforhopalus vacuolatus* (DSM9700)	GCTGCCTCCCGTAGGAGT	55	20	Amann et al. (1995)
NON338	None		ACTCCTACGGGAGGCAGC	55	20	Wallner et al. (1993)
DFMI227a	*Desulfotomaculum* spp.	*Desulfotomaculum thermoacetoxidans* (DSM5831)	ATGGGACGCGGACCCATC	50	28	Loy et al. (2002)
DSB985[b]	*Desulfobacter*, *Desulfobacula*	*Desulfobacter hydrogenophilus* (DSM3380)	CACAGGATGTCAAACCCAG	20	225	Manz et al. (1998)
DSR651	*Desulforhopalus*, some *Desulfocapsa*	*Desulforhopalus vacuolatus* (DSM9700)	CCCCCTCCAGTACTCAAG	55	20	Manz et al. (1998)
DSS658	*Desulfosarcina*, *Desulfococcus*	*Desulfococcus multivorans* (DSM2059)	TCC ACT TCC CTC TCC CAT	60	14	Manz et al. (1998)
DSV698	Most *Desulfovibrio*	*Desulfovibrio desulfuricans* (DSM642)	GTTCCTCCAGATATCTACGG	35	80	Manz et al. (1998)
DSV698c	Competitor to DSV698		GTTCCTCCAGATATCTACGC	35	80	Mußmann et al. (2005)
Sval428[b]	*Desulfotalea*, some *Desulfocapsa*	JS_SRB100Hy (this study)	CCATCTGACAGGATTTTAC	20	225	Sahm et al. (1999)

[a] If not stated otherwise, hybridisation and washing was performed at 35 °C and 37 °C, respectively.
[b] Hybridisation at 46 °C, washing at 48 °C
[c] Formamide (FA) concentrations in hybridisation buffer
[d] NaCl concentration in washing buffer

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2.2 Description of *Desulfocucumis infernus* gen. nov., sp. nov., an abundant sulfate-reducing bacterium in deep tidal flat sediment

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Summary

A novel, mesophilic sulfate-reducing bacterium (strain JS_SRB250Lac^{T}) was isolated from 2.5 meters deep, tidal flat sediment from the German Wadden Sea. The isolate used a variety of short-chain organic acids, ethanol and hydrogen as electron donors for sulfate reduction. Sulfate and sulfite were utilized as electron acceptors. Thiosulfate and nitrate were not reduced. Fermentative growth was observed with fumarate and pyruvate, but not with lactate and malate. Strain JS_SRB250Lac^{T} grew lithoautotrophically with hydrogen as electron donor and carbon dioxide/bicarbonate as sole carbon sources. Growth occurred between 10 °C and 35 °C with a growth optimum at 28 °C. Highest growth rates were found at marine NaCl concentrations between 20 ‰ and 30 ‰ (w/v). The major cellular fatty acids were *n*-16:0, *n*-17:0, 16:1*c*9, 16:1*c*11 and cyc17:0. Based on 16S rRNA gene sequence analysis, strain JS_SRB250Lac^{T} belonged to the *Deltaproteobacteria* and shared 96.3 % sequence identity with its closest cultured relative, strain IS4 (*"Desulfobacterium corrodens"*), a not validly described sulfate-reducing bacterium. The closest validly described species was *Desulfopila aestuarii* MSL86T (94.2 % sequence identity). Based on the phenotypic and phylogenetic differences to its closest relatives the establishment of a novel genus and species, *Desulfocucumis infernus* gen. nov., sp. nov., is proposed with strain JS_SRB250Lac^{T}(= DSM 19738) as the type strain.

Introduction

The dissimilatory reduction of sulfate is a strictly anaerobic process that enables distinct bacteria and archaea to gain energy via the reduction of sulfate to sulfide. Typical electron donors for sulfate reducers are metabolic products released by fermentative microorganisms like organic compounds with low molecular weight (e.g. organic acids and alcohols) or hydrogen (Rabus et al., 2006). As sulfate occurs in high concentration in seawater, dissimilatory sulfate reduction represents the major terminal degradation process of organic matter in marine sediments (Jørgensen, 1982). As most studies on the activity, abundance and ecology of sulfate-reducing bacteria (SRB) in coastal areas have been restricted to the uppermost centimetres of the sediment (e. g. Llobbet-Brossa et al., 2002; Ishii et al., 2004; Mußmann et al., 2005; Billerbeck et al., 2006b; Musat et al., 2006), there is only little information about the SRB community in deeper layers. Additionally, only few SRB from the marine subsurface have been brought into pure culture, thus their physiology and ecological relevance in these habitats remained uncertain so far (Bale et al., 1997; Barnes et al., 1998; Sass et al., 2002; Süß et al., 2004; Köpke et al., 2005; Batzke et al., 2007).

In the present study, we describe the phenotypical and phylogenetic characteristics of a marine, mesophilic SRB that was isolated from a 2.5 meter deep, tidal sand flat sediment located in the German Wadden Sea. In general, sandy sediments were assumed to be less active than mud-dominated sediments as they were characterized by low organic matter content and harbour less microbial cells (Bergamaschi et al., 1997; Llobet-Brossa et al., 1998; Rusch et al., 2003). More recent studies, however, indicated that microbial remineralization may occur almost as rapid as in organic-rich sediments. It was suggested that enhanced advective pore water transport in permeable sediment layers provides microorganisms with dissolved nutrients and therefore stimulates microbial activity (de Beer et al., 2005; Billerbeck et al., 2006a).

An approach combining cultivation-based and molecular methods on a sand flat sediment revealed that SRB are abundant and active even within several meters depth, and therefore contribute to the recycling of nutrients in tidal flat systems (Gittel et al., *submitted*). Six out of ten SRB isolated from different

sediment depths affiliated with the family of the *Desulfobulbaceae*. Five of them shared almost identical 16S rRNA gene sequences exhibiting less than 95 % sequence identity with any other validly described microorganism. Here, one of these strains (JS_SRB250Lac^T) is phylogenetically and phenotypically characterized. Based on its taxonomical differences to other described species, we propose strain JS_SRB250Lac^T as the type strain for a novel genus and species within the *Deltaproteobacteria*.

Methods

Source of organism

Strain JS_SRB250Lac^T was isolated from a tidal flat sediment originating from a depth of 2.5 meter (Gittel et al., *submitted*). The sampling site is a typical sand flat in the German Wadden Sea ("Janssand", 53°44.177' N, 007°41.970' E).

Enrichment, isolation and cultivation

Strain JS_SRB250Lac^T was enriched, isolated and routinely cultivated in an anoxic, carbonate-buffered, mineral medium consisting of (L^{-1} distilled water): 26.0 g NaCl, 11.2 g $MgCl_2 \cdot 6\,H_2O$, 1.4 g $CaCl_2 \cdot 2\,H_2O$, 4.0 g Na_2SO_4, 0.7 g KCl, 0.1 g KBr, 0.16 g NH_4Cl, 0.2 g KH_2PO_4, 1.0 mL trace element solution SL10 (Widdel and Bak, 1992), 1.0 mL resazurin solution ($50\,g \cdot L^{-1}$), 1.0 mL selenite-tungstate solution (Widdel and Bak, 1992), 1.0 mL vitamine solution (Balch et al., 1979), 30 mL $NaHCO_3$ (1 M) and 1.0 mL sodium sulfide (1 M) as reducing agent. The pH was adjusted to 7.2 - 7.4. Unless otherwise noted, incubations were carried out at 20 ° C in the dark using an inoculum volume of 5 % (v/v). Strain JS_SRB250Lac^T was enriched and isolated using lactate (20 mM) as the electron donor and sulfate (28 mM) as the electron acceptor. The isolation was performed by repeated application of the deep-agar dilution method (Widdel and Bak, 1992) with the described seawater medium solidified with 15 g washed agar L^{-1}. The purity of the culture was repeatedly checked by microscopy. In addition, the culture was transferred to a complex medium containing yeast extract (1 %, w/v), glucose (10 mM) and peptone (1 %, w/v).

Physiology and metabolism

Growth experiments were performed in duplicate or triplicate in Hungate culture tubes or 250 mL glass flasks closed with air-tight rubber septa. In general, growth was monitored by phase-contrast microscopy combined with the photometric measurement of sulfide (Cord-Ruwisch, 1985), if sulfate was amended as the electron acceptor, and by determination of cellular protein content (Bradford, 1976).

Substrate utilization was tested by substituting lactate with potential electron donors that were added from sterile stock solutions (10 mM each, Table 1). Lithotrophic growth was tested either in the presence of acetate (2 mM) or with a headspace of H_2/CO_2 (90:10, v/v) with CO_2/HCO_3^- as the sole carbon source to test the capacity for autotrophic growth. Growth tests on different electron acceptors were prepared in sulfate-free medium which was supplied with lactate (20 mM) as electron donor and either nitrate (5 mM), sulfite (2 mM) or thiosulfate (10 mM) as electron acceptor. For fermentation tests, medium without an additional electron acceptor was supplemented with lactate, malate, pyruvate or fumarate at a final concentration of 10 mM. Growth was determined microscopically and defined as positive after the third sucessful transfer.

Determination of the temperature cardinal points of growth was performed for six temperatures ranging between 4 and 35 °C. Growth rates were calculated from the linear regression of produced sulfide and/or formed cellular protein as a function of time. The NaCl requirement for growth was monitored in media with eight different NaCl concentrations between 1 and 50 g NaCl L^{-1}.

G + C content of genomic DNA

The G + C content of the genomic DNA was determined by HPLC at the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany).

Gram-staining

Gram-staining was performed with heat-fixed cells as described by Murray et al. (1994).

Fatty acid analysis

Cells for determination of fatty acid composition were cultivated in 250 mL glass flasks closed with air-tight rubber septa containing 150 mL seawater medium with lactate (20 mM) and sulfate (28 mM). Cells from the late exponential phase were harvested by centrifugation. Fatty acid methyl ester (FAME) were obtained by saponification, methylation and extraction as previously described by Sasser (1997). FAME were quantified by GC-FID (Hewlett Packard HP 5890 Series II gas chromatograph, Hewlett Packard, Waldbronn, Germany) equipped with a DB-5HT capillary column (30 m · 0.25 mm, 0.1 μm film thickness, J & W, Folsom, CA, USA) and identified by GC-MS using a Finnigan MAT SSQ 710B mass spectrometer (Finnigan-Thermoquest, San Jose, CA, USA). The carrier gas was helium (constant pressure of 12 psi). The oven temperature was run from 60 °C (isothermal for 2 min) to 360 °C at a rate of 3 °C min^{-1} and hold for 5 min. Mass spectra were collected in full scan mode (m/z 50-650, ionization energy 70 eV). Positions of double bonds were tentatively assigned by comparison with retention times of standards (Bacterial Acid Methyl Esters CP Mix; Supelco, Bellefonte, PA. USA).

Nucleic acid extraction, PCR amplification and sequencing

Nucleic acid extraction, amplification, cloning and sequencing of nearly full-length 16S rRNA gene fragments were performed as described previously (Gittel et al., *submitted*). Additionally, specific primers DSR1F and DSR4R (Wagner et al., 1998) were used to target on the *dsrAB* gene sequence and to amplify almost complete gene fragments for cloning and sequencing.

Phylogenetic analysis

The nearly complete 16S rRNA gene sequence of strain JS_SRB250Lac^{T} was analysed using the ARB program package (Ludwig et al., 2004). 16S rRNA gene sequences most closely related to our strain were retrieved from the EMBL database using the ARB Fast Aligner feature (http://www.arb-home.de). The phylogenetic tree was calculated by neighbour-joining methods with 16S rRNA gene sequences consisting of at least 1400 nucleotides.

The *dsrAB* amino acid sequence of strain JS_SRB250Lac^T was compared to those in GenBank using the *blastx* function at the National Center for Biotechnology Information server (www.ncbi.nlm.nih.gov).

Results

The cells of strain JS_SRB250Lac^T were Gram-negative, straight rods with rounded ends, 0.3 - 0.5 μm wide and 1.0 - 2.0 μm long (Fig. 1). Longer cells of up to 5 μm were observed under low temperature conditions. Cells of strain JS_SRB250Lac^T formed light-brown colonies in agar tubes and aggregated during growth in liquid medium. The cells were non-motile. Formation of endospores was not observed.

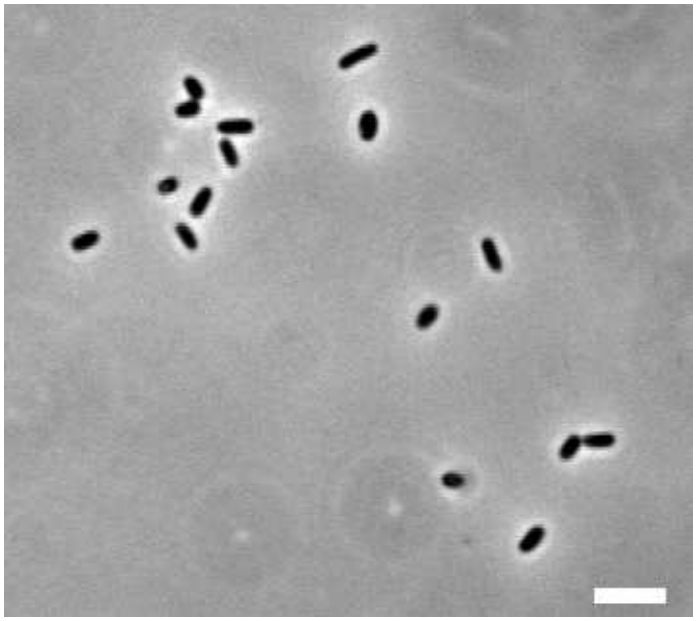

Figure 1: Phase contrast micrograph of strain JS_SRB250Lac^T. The scale bar corresponds to 2 μm.

Strain JS_SRB250Lac^T grew in the presence of NaCl at concentrations from 5 ‰ to 50 ‰ (w/v). Optimum growth occured between 20 ‰ and 30 ‰ (w/v) NaCl. The temperature range for growth was 10 to 35 °C with an optimum at 28 °C. Strain JS_SRB250Lac^T utilized lactate, formate, fumarate, pyruvate, ethanol and hydrogen as electron donors for sulfate reduc-

tion (Tab. 1). Acetate, propionate and malate were not utilized as electron donors. Thus, strain JS_SRB250Lac^{T} seems to perform an incomplete type of oxidation of electron donors. Shortest doubling time of 3.6 days was observed at sulfate-reducing conditions with lactate (20 mM) at 28 °C. In addition to sulfate, strain JS_SRB250Lac^{T} utilized sulfite as electron acceptor with lactate as the electron donor. In the absence of an electron acceptor, fermentative growth was observed with fumarate and pyruvate.

Phylogenetic analyses of the 16S rRNA gene and *dsrAB* amino acid sequences of strain JS_SRB250Lac^{T} consistently grouped the strain within the deltaproteobacterial family of the *Desulfobulbaceae* (Fig. 2). Based on 16S rRNA gene sequence analysis the phylogenetically closest relative of strain JS_SRB250Lac^{T} was strain IS4 (*"Desulfobacterium corrodens"*; Dinh et al., 2004) sharing 96.3 % 16S rRNA sequence identity.

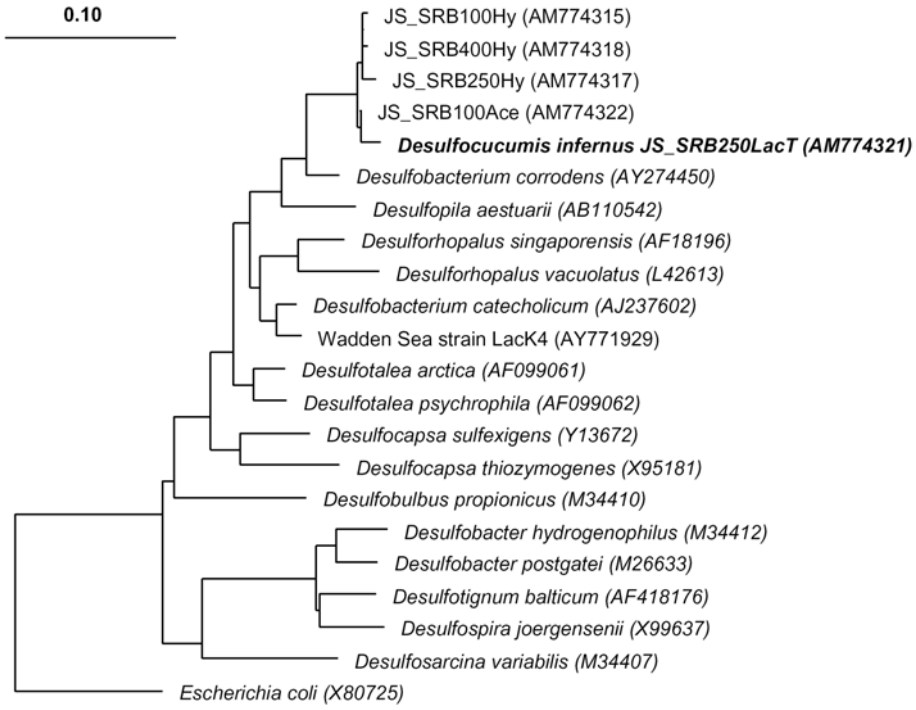

Figure 2: Neighbour-joining phylogenetic tree showing the relationship of strain JS_SRB250Lac^{T} and selected reference taxa of the *Deltaproteobacteria*. The scale bar represents 10 % sequence divergence.

Based on phylogenetic analysis candidatus *"D. corrodens"* should not remain within the genus *Desulfobacterium* but should be rather reclassified as a novel genus within the *Desulfobulbaceae* (J. Kuever, *personal communication*). The most closely related and validly described species to strain JS_SRB250Lac^{T} was *Desulfopila aestuarii* MSL86T (94.2 %), which was isolated from an estuarine sediment (Suzuki et al., 2007). Strain JS_SRB250Lac^{T} formed a distinct cluster with four sulfate-reducing strains isolated from different sediment depths at site "Janssand" (Gittel et al., *submitted*). The sequence identity between these strains was at least 98.4 %.

No *dsrAB* amino acid sequences are currently available for *"D. corrodens"* and *D. aestuarii*. However, *dsrAB* amino acid analysis of strain JS_SRB250Lac^{T} supported its grouping within the *Desulfobulbaceae* sharing 87 % and 76 % identity with *Desulforhopalus singaporensis* and *Desulfofustis glycolicus*.

Comparison of major physiological and chemotaxonomic characteristics of strain JS_SRB250Lac^{T} to its two closest relatives revealed several phenotypical differences (Tab. 1). Beside its striking property to utilize iron as electron donor for sulfate reduction, *"D. corrodens"* utilized only few other electron donors. In contrast, strain JS_SRB250Lac^{T} is nutritionally versatile utilizing a variety of short-chain organic acids, ethanol as well as hydrogen as electron donors. Additionally, sulfate was found to be the only electron acceptor for candidatus *"D. corrodens"*, whereas strain JS_SRB250Lac^{T} both reduced sulfate and sulfite. With respect to its different physiology and phylogenetic distance, strain JS_SRB250Lac^{T} should not be affiliated with the potential novel genus that is needed to accommodate candidatus *"D. corrodens"*.

With respect to their physiology, strain JS_SRB250Lac^{T} and *D. aestuarii*, share the capacity to grow on a wide spectrum of organic electron donors. However, strain JS_SRB250Lac^{T} remarkable differs from *D. aestuarii* in its capacity to grow lithoautotrophically by using hydrogen as sole electron donor and CO_2/HCO_3^- as the sole carbon source. Moreover, thiosulfate as electron acceptor instead of sulfate was not utilized by strain JS_SRB250Lac^{T}, but by *D. aestuarii*.

Table 1: Comparison of selected characteristics of *"Desulfobacterium corrodens"*, *Desulfopila aestuarii* and JS_SRB250Lac^T

Characteristic	***"Desulfobacterium corrodens"***[a]	***Desulfopila aestuarii***[b]	**JS_SRB250Lac^T**
Isolation source	Marine sediment	Estuarine sediment	Tidal flat sediment
Morphology	Rod	Rod	Rod
G + C content (mol %)	51.9	54.5	
Optimum salinity ($g\,NaCl\,L^{-1}$)	10-15	10	20-30
Optimum temperature (°C)	28-30	35	28
Utilization of electron donors (10 mM each)			
Lactate	+	+	+
Acetate	-	-	-
Formate	+	+	+
Propionate	-	-	-
Fumarate	-	+	+
Pyruvate	+	+	+
Malate	-	-	-
Ethanol	-	+	+
H_2	+	-	+
H_2 + Acetate (2 mM)	+	-	+
Utilization of electron acceptors			
Nitrate (5 mM)	nd	-	-
Sulfite (2 mM)	-	+	+
Thiosulfate (10 mM)	-	+	-
Fermentative growth	pyruvate, (lactate)	pyruvate, fumarate	pyruvate, fumarate

[a] Dinh (2003)
[b] Suzuki et al. (2007)
+ growth detected, - no growth detected, nd not determined

G + C content of genomic DNA of strain JS_SRB250Lac^T was xy mol %[1]. The main fatty acids of strain JS_SRB250Lac^T were the saturated straight-chained *n*-16:0 (23.3 %) and *n*-17:0 (8.3 %), the unsaturated straight-chained 16:1*c*9 (18.3 %) and 16:1*c*11 (11.4 %) and the cyclopropane fatty acid cyc17:0 (15.3 %). The latter was not detected in *D. aestuarii* and may represent a chemotaxonomic marker to distinguish both SRB from each other (Tab. 2). The cyclopropane fatty acid cyc17:0 is a common fatty acid in strict or facultative anaerobic bacteria and, depending on growth conditions and growth phase, is a major component in the cellular fatty acid composition in *Desulfobacter* species (Kuever et al., 2001; Könneke and Widdel, 2003). As there are no data on fatty acids available for *"D. corrodens"*, it remains open whether cyc17:0 is also present in large amounts in this species.

Table 2: Cellular fatty acid composition (%) of strain JS_SRB250Lac^T and its closest described relative *Desulfopila aestuarii* (Suzuki et al., 2007)

Fatty acid	**JS_SRB250Lac^T**	***Desulfopila aestuarii***
n-14:0	1.1	1.4
n-15:0	1.6	–
n-16:0	23.3	33.6
16:1*c*7	0.7	–
16:1*c*9	18.3	6.0
16:1*c*11	11.4	17.1
n-17:0	8.3	3.4
17:1	–	13.7
cyc17:0	15.3	–
n-18:0	11.5	2.5
18:1	8.6	5.7

[1] in progress

Discussion

Ecological relevance of the genus *Desulfocucumis*

Strain JS_SRB250Lac^T and four closely related isolates formed a distinct cluster within the *Desulfobulbaceae* with less than 95 % sequence identity to the closest validly described relative, *Desulfopila aestuarii*. These strains originated from highly diluted cultures initiated with sediment samples from different depths of a five meter long sediment core. Their frequent cultivation and affiliation with the *Desulfobulbaceae*, identified to be a predominant group of SRB within these sediments, indicated that the isolates are representatives of an *in situ* highly abundant population. Strain JS_SRB250Lac^T was characterized as nutritionally versatile with respect to its electron and carbon sources and to possess the capacity of fermentative growth. Thus, one might assume that representatives of this novel genus are adapted to both the variable supply of substrates through pore water transport within these sediments as well as to thrive under sulfate-depleted conditions.

Description of *Desulfocucumis* gen. nov.

On the basis of phylogenetic analyses as well as its physiological properties and chemotaxonomic characteristics, strain JS_SRB250Lac^T represents the type species of a novel genus and species in the class *Deltaproteobacteria*, for which we propose the name *Desulfocucumis infernus* gen. nov., sp. nov.

Desulfocucumis (De.sul.fo.cu.'cu.mis. L. pref. *de* from; L. n. *sulfur* sulfur; L. m. n. *cucumis* cucumber; sulfate reducer shaped like a cucumber)

Mesophilic, strictly anaerobic, marine. Cells are straight rods, Gram-negativ and non-spore-forming. Lactate is oxidized incompletely to acetate. Chemolithoautotrophic growth on H_2 plus CO_2/HCO_3^-. The type strain is *Desulfocucumis infernus*.

Description of *Desulfocucumis infernus* sp. nov.

Desulfocucumis infernus (in.'fer.nus. from lower site/underground/subsurface).

Cells are straight rods with rounded ends, 0.3 - 0.5 μm in width and 1.0 - 2.0 μm in length. Non-motile. The NaCl range for growth is 5 ‰ to 50 ‰

(w/v) with an optimum between 20 ‰ and 30 ‰ (w/v). The temperature range for growth is 10 - 35 °C with an optimum at 28 °C. Utilizes lactate, formate, fumarate, pyruvate, ethanol and hydrogen as electron donors for sulfate reduction. Does not utilize acetate, propionate and malate. Sulfate and sulfite serve as electron acceptors. Pyruvate and fumarate are fermented in the absence of electron acceptors. G + C content of genomic DNA is xy mol %[2]. The major cellular fatty acids are *n*-16:0, *n*-17:0, 16:1 *c*9, 16:1*c*11 and cyc17:0. The type strain is JS_SRB250Lac^{T} (= DSM 19738).

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We wish to thank Katharina Deutz for her assistance in preparation and analysis of fatty acid methyl esters. Michael Pilzen is acknowledged for assistance in measuring sulfide and cell protein. This work is part of the research group "Biogeochemistry of Tidal flats" and was financially supported by the Deutsche Forschungsgemeinschaft (DFG).

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2.3 Activity and stimulation of sulfate-reducing prokaryotes in deep tidal flat sediments

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Summary

Activity of sulfate-reducing prokaryotes (SRP) and the effect of substrate amendments were studied in sediment slurries from several meters deep tidal sand flat sediments (Wadden Sea, Germany) using radiolabeled sulfate (^{35}S). Acetate and lactate as well as hydrogen and methane were chosen as electron donors as they are common subtsrates to SRP and/or were described to be present within sediments at the sampling site. Chemical analyses revealed opposed vertical profiles for sulfate and methane. Although sulfate steeply decreased with depth, sulfate reduction was not limited by the availability of sulfate as concentrations reached at least 2 mM in all sediment layers. Methane was highly concentrated between 1 and 2.5 meters depth (up to $260\,\mathrm{nmol \cdot cm^{-3}}$). Highest activity of SRP was observed in surface sediments ($20\,\mathrm{nmol \cdot cm^{-3} \cdot d^{-1}}$), and additional maxima occurred within the methane-rich zone at 1.5 and 2 meters depth (7.4 and $4.6\,\mathrm{nmol \cdot cm^{-3} \cdot d^{-1}}$, respectively). These activity peaks were accompanied by elevated total cell numbers being even higher than at the sediment surface ($> 10^9$ cells per cm^3 sediment). Additionally, pore water profiles indicated an inflow of sulfate within this region. These findings supported the hypothesis that deep pore water flows stimulate microbial communities within deep tidal flat sediments. Activity of the resident SRP community was significantly enhanced by adding hydrogen as well as methane to the sediment slurries. In contrast, the addition of acetate and lactate showed no effect or was even inhibitory to sulfate reduction. Next to the presence of an active, hydrogen-consuming SRP community, our results indicate that SRP mediating the anaerobic oxidation of methane are abundant at site "Janssand" and that this process is of so far unquantified significance within this moderate environment.

Introduction

Dissimilatory sulfate reduction may account for half or more of the total organic carbon remineralization in marine environments (Jørgensen, 1982; Canfield et al., 1993). It is potentially the major anaerobic degradation process in sediments that lack alternative electron acceptors like nitrate, ferrous iron and manganese oxides. *In situ* activity of sulfate-reducing prokaryotes (SRP) has been investigated in a variety of marine surface sediments (Jørgensen, 1977; Ravenschlag et al., 2000; Llobbet-Brossa et al., 2002; de Beer et al., 2005) and, to minor extent, in sediments that exceed to several hundreds of meters the deepest (Parkes et al., 2000, 2005; Engelen et al., submitted). It was generally observed that the activity of SRP decreased with depth and was significantly affected by the limited availability of readily degradable organic substrates (Parkes et al., 2000). However, deep maxima of sulfate reduction together with specific archaeal and bacterial populations were found at discrete sulfate-methane transition zones (Orphan et al., 2001; Thomsen et al., 2001; Parkes et al., 2005; Leloup et al., 2006).

Sandy sediments, that were previously described as "microbial deserts", have recently been characterized as biofilters stimulating microbial activity by trapping and concentrating suspended particles, algae and bacteria (Huettel and Gust, 1992; D'Andrea et al., 2002; de Beer et al., 2005). Studies on sandy sediments in the German Wadden Sea revealed that SRP were most active directly beneath the oxygen penetration depth (Billerbeck et al., 2006b; Werner et al., 2006) and might be stimulated by the provision with nutrient-loaded pore water in deeper layers. Indeed, SRP have been shown to be highly abundant in several meters deep, sulfate-poor sediments reaching even higher cell numbers than in sulfate-rich surface sediments (Gittel et al., submitted). Moreover, a high metabolic potential of the SRP community was indicated by the abundance of both organoheterotrophic as well as lithoautotrophic SRP that shared the capacity to oxidize a variety of substrates and even to grow in the absence of sulfate.

Thus, the objective of the present study was to quantify the activity of SRP within deep sediments of a tidal sand flat and to identify the substrates that are likely to be relevant *in situ*. Therefore, we studied the effect of poten-

tial electron donors on sulfate reduction rates by adding common substrates for different physiological types of SRP (acetate, lactate and hydrogen). As methane is highly concentrated within these sediments and might theoretically provide reducing equivalents for sulfate reduction, both tracer incubations and a long-time experiment were performed to clarify whether sulfate reducers are stimulated and/or methane oxidation is coupled to the reduction of sulfate (AOM) under atmospheric pressure.

Methods

Sampling and sediment handling

A four meter long sediment core was taken in July 2006 at the Northeastern margin of the "Janssand" tidal flat (53°44.177' N, 007°41.970' E) that is located in the back barrier area of Spiekeroog Island (German Wadden Sea, Fig. 1). Aluminium liners (six meters in length) were driven into the sediment by vibrocoring and the recovered core was immediately cut into 50 cm sections (Wilms et al., 2006). Samples for various applications were taken from the innermost part of the freshly exposed surfaces with sterile tipless syringes. Near-surface samples were taken separately with short plastic liners (20 cm).

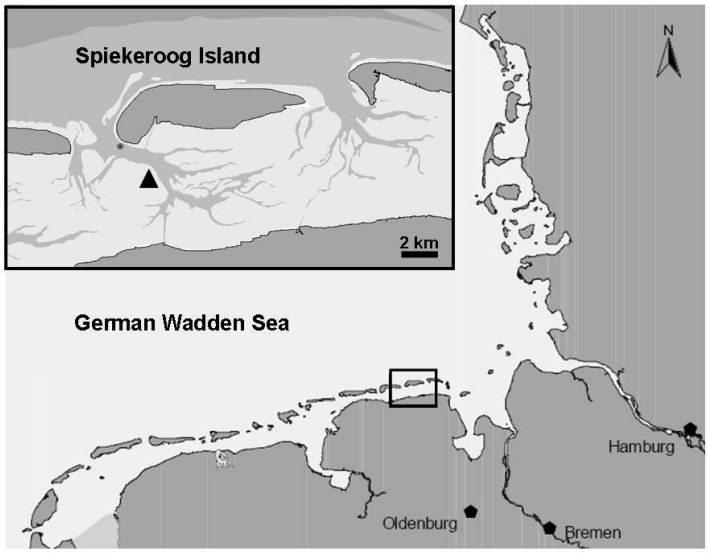

Figure 1: Location of the island Spiekeroog in the Northwestern part of the German Wadden Sea. The study site ("Janssand") is marked with the black triangle.

For the determination of sulfate reduction rates and long-time incubations, approximately 100 cm^3 of sediment from different depths were transferred to sterile glass flasks. The headspace was flushed with nitrogen gas, flasks were closed with air-tight rubber septa and stored at 4 °C in the dark. Rate measurements were started at the most 48 hours after sampling.

Separate subsamples were taken for the determination of sediment porosity and for analysis of pore water sulfate and methane concentrations.

For the determination of total cell counts, sediment samples were fixed in 4 % paraformaldehyde over night, washed and stored as described previously (Mußmann et al., 2005).

Determination of sulfate reduction rates in raw and substrate-amended sediment slurries

Sediment slurries were prepared by mixing one volume of sediment with two volumes of anoxic, sulfate-free seawater medium (Widdel and Bak, 1992) under a constant flow of nitrogen gas. Sulfate concentration in the slurries was adjusted to the *in situ* concentration by adding the appropiate volume of a 2 M sodium sulfate stock solution. 10 mL of sediment slurry were transferred to 20 mL culture tubes, headspaces were flushed with N_2/CO_2 (90:10, v/v) and sealed with butyl rubber septa and screw caps. Substrates and tracer were added by injection with a hamilton syringe through the septa to avoid oxygen influx.

Endogenous sulfate reduction rates were determined in substrate-free sediment slurries. Acetate and lactate were each added at a final concentration of 2 mM. Gaseous hydrogen and methane were added by exchanging N_2/CO_2 in the headspace with either H_2/CO_2 (90:10, v/v) or pure methane of atmospheric pressure (0.1 MPa). With an incubation temperature of 15 °C, this corresponded to a concentration of approximately 0.7 mM dissolved hydrogen and 1.3 mM dissolved methane, respectively (Yamamoto et al., 1976; Wiesenburg and Guinasso, 1979). After a pre-incubation of about 4 hours at 15 °C, carrier-free ^{35}S-sodium sulfate was injected to obtain a radioactivity of about 170 kBq in each culture tube. All incubations were carried out at 15 °C in the dark for an incubation time of 4 hours starting with the tracer injection.

Incubations were stopped by adding 10 mL 20 % zinc acetate solution followed by freezing at -20 °C. The ^{35}S-labeled reduced sulfur fraction was extracted using the cold chromium distillation method (Kallmeyer et al., 2004). Sulfate reduction rates were calculated according to Jørgensen (1978).

Preparation of anoxic incubations with methane

4 mL of the original sediment were transferred to 72 mL of an anoxic, artificial seawater medium (Widdel and Bak, 1992) in 250 mL Meplat flasks under an atmosphere of N_2/CO_2 (90:10, v/v). Flasks were closed with air-tight, tapered rubber stoppers. By means of a nitrogen-flushed syringe, 50 mL pure methane were applied to the headspace corresponding to a methane concentration of approximately 12 mM methane in the headspace. Flasks were gently shaken and pre-incubated for several hours to facilitate diffusion of methane. After depressurization, flasks were incubated horizontally to extend the culture surface. Incubations were carried out at 28 °C. Set-ups without sediment or without methane in the headspace served as controls.

Samples for analysis of sulfide from the liquid phase and methane from the headspace were taken regularly via N_2-preflushed syringes and analyzed immediately.

Determination of total cell counts

Aliquots of fixed sediment samples were dispersed by sonication at minimum power for 30 s with a sonication probe. The sonicated samples were filtered onto white polycarbonate filters (pore size 0.2 μm, GTP, Millipore, Eschborn, Germany) and washed twice with 5 to 10 mL phosphate-buffered saline (1 x PBS; 130 mM NaCl, 5 mM Na_2HPO_4, 5 mM NaH_2PO_4). For cell attachment, dried filters were dipped with both sides into 0.2 % (w/v) low gelling point agarose (MetaPhor, FMC Bioproducts, Maine, U.S.A.). After drying, 7 μL of a Sybr-Green I staining and mounting solution (Lunau et al., 2005) were directly pipetted onto the cover slip and placed onto the microscopic slide. After 2 hours of incubation at 4 °C in the dark, counting was performed by epifluorescence microscopy. A minimum of 600 cells per filter were counted.

Analytical techniques

Sulfate from pore water samples was determined by ion chromatography with conductivity detection (Sykam, Fürstenfeldbruck, Germany) as described previously (Sass et al., 2001).

For measuring *in situ* methane concentrations, 2 mL of sediment were transferred into 18 mL of sodium hydroxide solution (2.5 %) in gas-tight tubes immediately after subsampling. Fixed samples were stored at 4 °C in the dark until further proceeding. Headspace samples were analyzed on a Varian CX 3400 gas chromatograph (Varian, Darmstadt, Germany) equipped with a Plot Fused Silica column (No. 7517; 25 m by 0.53 mm, Al_2O_3/KCl coated; Chromopack, Middleburg, The Netherlands) and a flame ionization detector. Headspace samples from enrichment experiments with methane were thousand-fold diluted by injecting 50 μL into 50 mL serum flasks that were one-fourth filled with glass beads and closed with air-tight butyl rubber septa. Gas mixing was achieved by inverting the flasks manually for about one minute. Gaschromatographic analysis was similar to that of the sediment samples.

Sulfide was determined spectrophotometrically by the methylene-blue method (Cline, 1969). Sediment porosity was determined as described by Bak and Pfennig (1991).

Statistics

The effect of substrate amendments on the endogenous sulfate reduction rate were tested using Student's t-test on the means of the measured sulfate reduction rates. The terms significant ($P < 0.05$) and very significant ($P < 0.01$) were used.

Results

Specific sediment characteristics

Based on pore water sulfate profiles from different sampling campaigns at identical position at site "Janssand" (Fig. 2A), it is suggested that deep sediments might be supplied with sulfate through advective pore water transport (Fig. 2A). A peak in the sulfate concentration was detected at 2.5 meters depth in summer 2006 with a significantly higher sulfate concentration (appr. 3.6 mM) than in spring and autumn 2005 (0.02 mM and 0.8 mM, respectively).

According to the data of the summer sampling in 2006, concentrations of sulfate in pore water and methane in sediments showed opposite depth profiles with steeply decreasing sulfate concentrations and a methane-rich part of the sediment between 1 and 2.5 meters depth (Fig. 2A + B). Down to this depth sulfate concentrations were higher than 2 mM, whereas in deeper layers less than 1 mM sulfate were detected. Lowest methane concentrations of about 30 nmol $\cdot$ cm^{-3} sediment were measured in samples from the sediment surface. Between 1 and 2.5 meters depth, methane concentrations were almost nine times higher (up to 260 nmol $\cdot$ cm^{-3} sediment).

Stimulation and enrichment experiments were carried out with surface sediment and sediment from different layers of this methane-rich, not sulfate-depleted zone to study the activity of the sulfate-reducing community and the effect of substrate amendments.

Enhanced activity of sulfate reducers in methane-rich sediments accompanied by high total cell numbers

Endogenous sulfate reduction rates were determined in sediment slurries without any additions except the ^{35}S-labeled tracer (Fig. 2D). The highest activity was found in samples from the sediment surface (20 nmol $\cdot$ cm^{-3} $\cdot$ d^{-1}) being within the range previously reported for sand flats in the German Wadden Sea (de Beer et al., 2005; Billerbeck et al., 2006b). Two additional activity peaks were found in the methane-rich zone at 1.5 and 2 meter depth with sulfate reduction rates of 7.4 nmol $\cdot$ cm^{-3} $\cdot$ d^{-1} and 4.7 nmol $\cdot$ cm^{-3} $\cdot$ d^{-1}, respectively. This increase in activity was significant ($P < 0.05$) in comparison to the activ-

ity in sediment slurries prepared with sediments from 1 and 2.5 meter depth ($< 1\,\mathrm{nmol \cdot cm^{-3} \cdot d^{-1}}$).

Increasing activity was accompanied by slightly increasing total cell counts that were even higher than at the sediment surface reaching $1.1 \cdot 10^9$ cells $\cdot\, \mathrm{cm^{-3}}$ sediment at 1.5 meters depth (Fig. 2C). High and even relatively stable total cell numbers have been previously reported for sandy surface sediments (Musat et al., 2006), but also for several meters deep layers at site "Janssand" (Gittel et al., submitted). Here, both enhanced activity of a distinct group of microorganisms as well as high microbial cell numbers indicated a highly active microbial community within these sediments.

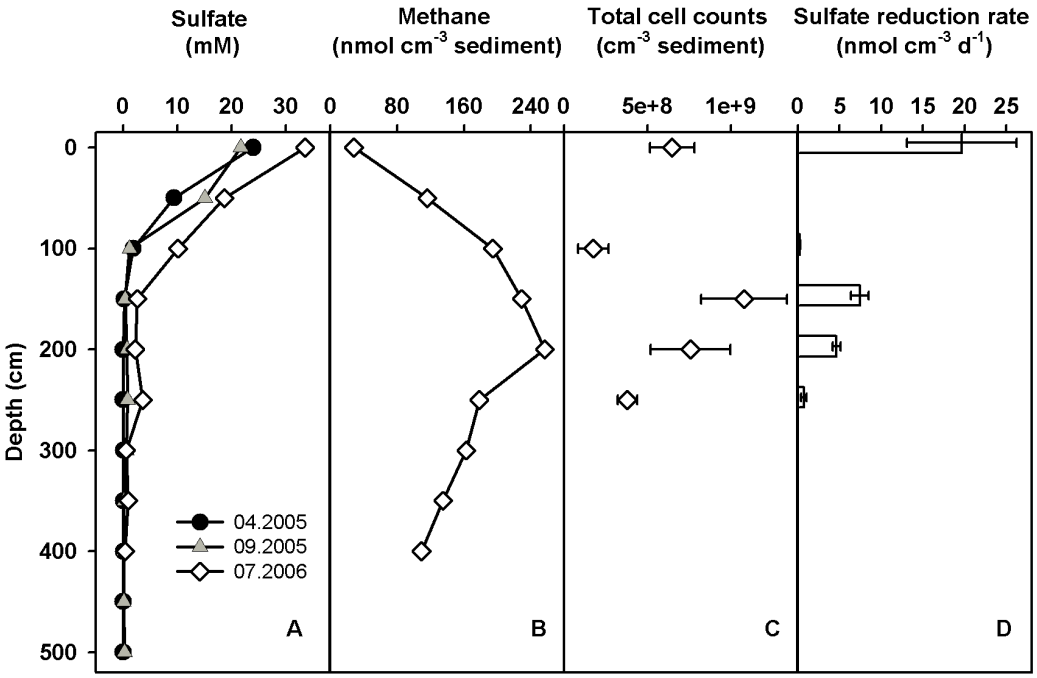

Figure 2: (A) Depth profiles of pore water sulfate at three different time points. (B-D) Depth profiles from sampling in July 2006; (B) Methane concentrations, (C) Total cell counts as determined with SybrGreen as staining dye and (D) Endogenous sulfate reduction rates for the surface sediment and a methane-rich zone from 1 to 2.5 m depth.

Addition of hydrogen and methane to sediment slurries results in elevated sulfate reduction rates

Stimulation of sulfate reduction in sediment slurries was investigated by adding different electron donors known to be common substrates for SRP (i. e. acetate, lactate, hydrogen) as well as methane. In general, the activity of sulfate reducers was only significantly enhanced by the additon of methane and hydrogen to the headspace when compared to incubations without any additions (Fig. 3). In contrast, the amendment of acetate and lactate resulted in reduced activity, although this observation was not found to be significant for all incubations (Tab. 1).

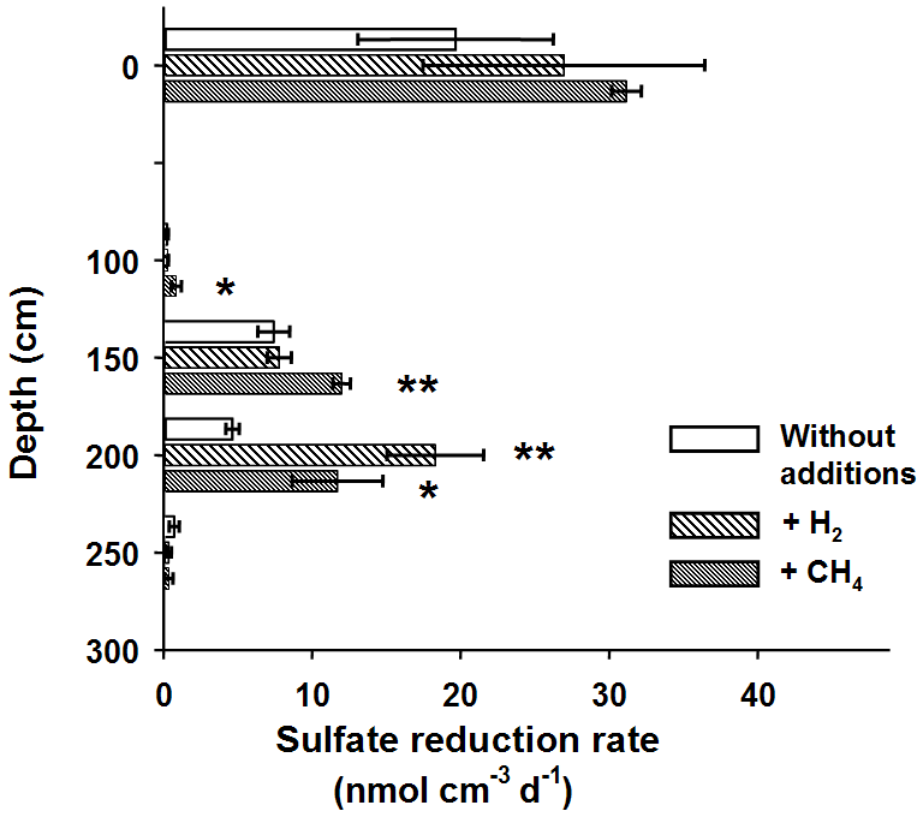

Figure 3: Endogenous sulfate reduction rates and stimulation through adding hydrogen (H_2) and methane (CH_4) to the headspace. Standard deviations result from 3 replicates and are depicted as error bars. Significance of stimulation by addition of substrates is indicated right hand side of the bars; *, significant ($P < 0.05$); ** very significant ($P < 0.01$).

Table 1: Pore water sulfate concentrations, porosity and sulfate reduction rates in sediment slurries from different depths without additions (endogenous) and amended with substrates (H_2, CH_4, acetate and lactate).

Depth (cm)	**Sulfate (mM)**	**Porosity (mL cm^{-3})**	**Sulfate reduction rate nmol · cm^{-3} · d^{-1}**				
			Endogenous	+ H_2	+ CH_4	+ Acetate	+ Lactate
0	33.48	0.35	19.7 ± 6.6	27.0 ± 9.5	31.2 ± 1.0	6.82	12.8 ± 6.1
50	18.67	0.35			nd		
100	10.13	0.33	0.25 ± 0.07	0.31 ± 0.01	0.86 ± 0.34	0.24	0.44 ±0.2
150	2.65	0.37	7.42 ± 1.07	7.79 ± 0.81	12.0 ± 0.6	2.38 ± 1.29	2.38 ± 0.67
200	2.23	0.34	4.64 ± 0.45	18.3 ± 3.3	11.7 ± 3.1	2.08 ± 0.64	3.58 ± 1.85
250	3.66	0.36	0.72 ± 0.33	0.36 ± 0.16	0.36 ± 0.28	0.22 ± 0.01	0.27 ± 0.05
300	0.54	0.51			nd		
350	0.95	0.46			nd		
400	0.38	0.55			nd		

nd: not determined

Stimulation through either methane or hydrogen in slurries from surface-near sediment was not found to be significant ($P > 0.1$). However, incubations that were prepared with samples from the methane-rich part of the sediment were significantly ($P < 0.05$) or even very significantly ($P < 0.01$) stimulated by the addition of hydrogen or methane. With regard to methane-stimulated sulfate reduction, the most remarkable effect was obtained with sediment from 1.5 meter depth. Here, the sulfate reduction rate was nearly two times higher than in the unamended replicates.

Methane stimulates sulfate reduction in enrichment cultures

To investigate the simultaneous production of sulfide and consumption of methane, sediment slurries with methane as electron donor were monitored for an incubation time of more than 200 days under atmospheric pressure (0.1 MPa). Sulfide production occurred in all incubations but with regard to a control containing sediment, but no methane, only incubations with more than 3 mM sulfide produced were considered as potentially methane-stimulated. Only the slurry prepared with sediment from 1.5 meters depth showed both significant sulfide production (5 mM) and methane consumption (Fig. 4). Methane concentration in the headspace decreased from 9.4 mM at the beginning of the experiment to 5.8 mM after 220 days. However, stoichiometric calculations based on the amounts of sulfide and methane could not be validly performed. The gas phase was relatively large (175 - 185 mL) compared to the aqueous phase (72 - 82 mL), so that small changes in the amount of the substances could hardly be resolved by the applied analytical techniques. Hence, it remains open whether the production of sulfide was equimolar to the consumption of methane as described for the microbially-mediated anaerobic oxidation of methane via sulfate reduction.

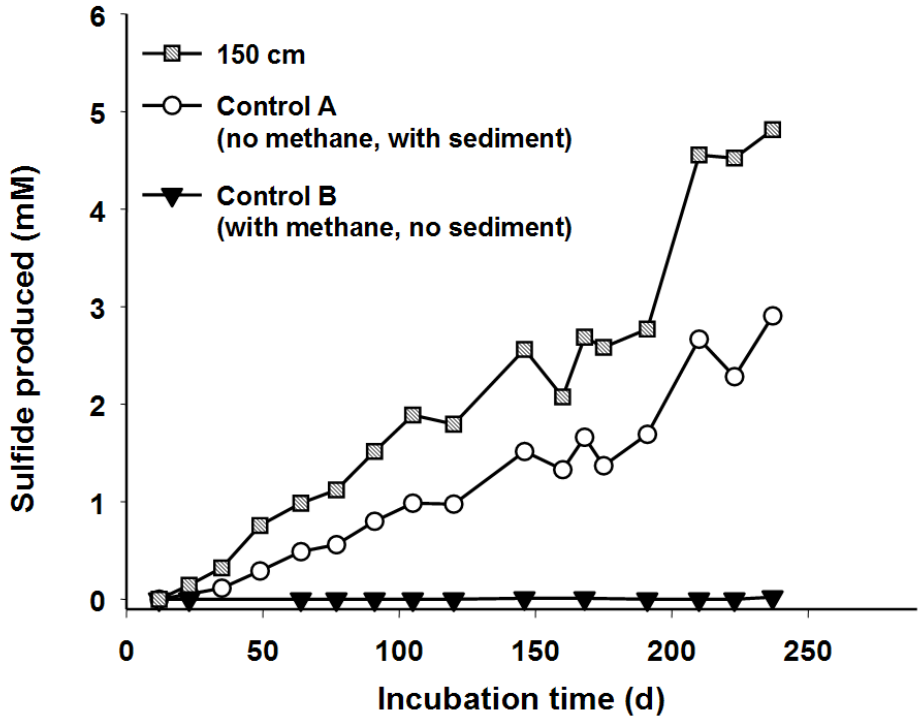

Figure 4: Formation of sulfide from sulfate with methane added as potential electron donor to a sediment slurry (sediment from 150 cm depth) incubated under strictly anoxic conditions. Control A contained no methane. Control B consisted of pure medium with methane in the headspace.

Discussion

Activity peaks and high cell numbers indicate the supply of nutrients into deep sediment layers

Besides high activity of SRP at the sediment surface, additional activity peaks were found in 1.5 to 2 meters deep sediment layers at site "Janssand". Sulfate reduction rates for slurries with surface sediment were in the range of previously reported data for sand flat sites obtained with the whole core incubation method (de Beer et al., 2005; Billerbeck et al., 2006b). The presence of subsurface maxima of activity might point to the previously proposed advective transport of pore water loaded with nutrients to deep regions of the sand flat (Billerbeck et al., 2006a). This hypothesis is supported by considerable sea-

sonal changes in sulfate concentrations between 1.5 and 3 meters depth and the presence of a sulfate peak in a layer that is not affected by diffusive transport from the overlying sediment (Fig. 2A). The supply with both sulfate and organic compounds might stimulate the sulfate-reducing community in distinct layers leading to enhanced activity in contrast to layers not influenced by pore water exchange. Peaks of SRP activity were accompanied by elevated total cell counts indicating that this stimulation not only influences a distinct physiological group, but the microbial community in general.

Suitability of sediment slurries to study stimulation effects

The application of sediment slurries to quantify *in situ* activities was controversially discussed, but appeared most suitable to assess the effects of both liquid and gaseous substrate additions. It was previously described that sulfate reduction rates might be significantly underestimated compared to tracer injections into undisturbed subcores (Meier et al., 2000). The reasons for these discrepanies in activity between disturbed and undisturbed sediment samples seem to be manifold and hardly predictable. First, the mixing of sediment samples destroys both lithological structures as well as microbial associations. Moreover, the addition of e. g. artificial seawater leads to the dilution of substrates and numerically diminishes the microbial community. Therefore, certain processes might be enhanced or inhibited making sediment slurries a rather artificial environment and not reflecting *in situ* activities. However, in contrast to intact core incubations sediment slurries are more suitable to study the response of initial reaction rates to changes in environmental variables like substrate availability. Besides, flow-through reactors with undisturbed slices of sediment are an elegant tool to study the effect of substrate amendements to the activity of SRP (Roychoudhury et al., 1998). They have just recently been used to investigate the potential of sulfate reduction in intertidal surface sediments (Pallud and van Cappellen, 2006). However, as also gaseous substrates were tested in our study, the sediment slurry approach appeared to be more practical and required less technical effort.

Hydrogen and methane are potential substrates of SRP in deep tidal flat sediments

SRP were previously shown to be highly abundant, potentially active and metabolically versatile in deep sediments at site "Jannsand" (Gittel et al., submitted). In general, sulfate-reducing communities in sediment slurries responded differently to the addition of substrates. Significant increases in activity were observed for hydrogen- and, most remarkably, methane-amended slurries indicating the presence of both a lithotrophic community and of sulfate reducers involved in the anaerobic oxidation of methane (AOM).

Hydrogen is an important fermentation product and intermediate in syntrophic relationships and rapidly consumed *in situ*. It is furthermore a competitive substrate for SRP and hydrogenotrophic methanogens, the latter being favoured in sulfate-depleted sediments. However, sediment layers studied here contained at least 2 mM of pore water sulfate and, based on the stimulation effect of hydrogen, seem to harbour a potentially active hydrogen-consuming SRP community. This finding is additionally supported by the isolation of lithotrophic SRP from different sediment depths of this sand flat (Gittel et al., submitted).

Both the pronounced sulfate-methane transition zone as well as the enhancement of sulfate reduction through methane indicate that AOM is a relevant process within these moderate sediments. Most interestingly, stimulation of sulfate reduction was shown either in tracer incubations or in a long-term experiment that aimed to enrich microorganisms involved in AOM under atmospheric pressure. Again sediments from 1.5 and 2 meter deep, probably pore water-influenced layers appeared to be most promising with respect to microbial activity. Members of the *Desulfosarcina-Desulfococcus* group and, more recently, of the *Desulfobulbaceae* have been described to be involved in methane cycling in methane-rich sediments (Orphan et al., 2001; Lösekann et al., 2007). However, it remains open whether their members participate in AOM at our study site. Although both phylogenetic groups have been shown to be highly abundant (Ishii et al., 2004; Mußmann et al., 2005; Gittel et al., submitted), the linkage between their abundance and specific *in situ* activities and functions is missing so far.

Although acetate and lactate are typical substrates for SRP, their addition did not lead to a stimulation during incubation. As both acetate- and lactate-utilizing SRP are common within these sediments, the resident community was probably carbon-limited and needed more time to adapt to the provision of labile substrates.

In conclusion, our study demonstrated that deep tidal flat sediments harbour an active sulfate-reducing community that is most likely stimulated by the provision with nutrient-loaded pore water from the upper flat. Besides hydrogen-driven sulfate reduction, we could show that the addition of methane significantly enhanced the activity of sulfate reducers. It needs to be further investigated which microorganism(s) is (are) actually involved in this reaction *in situ*.

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Chapter 3

Discussion & Outlook

Sandy sediments are discussed as potential "hot spots" of microbial activity and therefore to harbour microbial communities that contribute significantly to the degradation of organic matter and to the nutrient cycling in tidal flat ecosystems. Aim of this thesis was to characterize the active part of the sulfate-reducing community as representative members of the anaerobic microbial community in tidal sand flat sediments down to five meters depth.

3.1 Identification of abundant and active sulfate-reducing bacteria

Beside the enormous impact of cultivation-independent molecular tools to study microbial communities, cultivation and isolation of microorganisms is essential to study physiological capabilities and the ecological relevance of phylotypes (Leadbetter, 2003; Giovannoni and Stingl, 2007). Within the scope of this thesis, the selective cultivation of a distinct physiological and ecologically relevant group of marine microorganisms, the sulfate-reducing bacteria, was (i) accompanied and evaluated by molecular tools, complemented by (ii) rRNA-based, cultivation-independent quantification and (iii) radiotracer techniques to quantify endogenous as well as potential activities. This approach consistently demonstrated that SRB form abundant and active populations within tidal sand flat sediments down to five meters depth. In contrast to previous DNA-based molecular surveys on several meters deep marine sediments (Wilms

et al., 2007; Leloup et al., 2006; Parkes et al., 2007), this approach specifically targeted on active cells, i. e. cells that are not only culturable, but detectable due to a sufficient rRNA content. Additionally, their endogenous activity was evident and could be stimulated by the amendment of gaseous substrates (see section 2.3).

Members of the *Desulfobulbaceae* and the *Desulfobacter-Desulfobacula* group were identified to predominate the sulfate-reducing community at site "Janssand". Both, their frequent cultivation from highly-diluted sediment samples as well as rRNA-based *in situ* quantification indicated that the isolated SRB apparently represent active and ecologically relevant members of the microbial community. As distinct from previous studies on deep mud flat sediments that revealed a pronounced community shift from *Proteobacteria* dominating the upper, sulfate-rich layers to spore-forming members of the *Firmicutes* in deeper, sulfate-depleted sediments (Köpke et al., 2005; Wilms et al., 2006), deltaproteobacterial SRB were highly abundant throughout the whole sediment column at site "Janssand" even in sulfate-depleted sediment layers. The community shift in mud flat sediments was assumed to be correlated to the low availability of easily degradable substrates in deep sediment layers. Thus, the presence of an abundant and active SRB community within deep sediments at site "Janssand" might be an indication of substantially different environmental settings (e. g. advective pore water transport; see section 3.3) or the adaptation of unique populations to remain in a physiologically active state under sulfate-depleted conditions (see section 3.2.2).

Five out of ten isolates showed almost identical 16S rRNA gene sequences and formed a distinct cluster within the *Desulfobulbaceae*. They were only distantly related to cultured and validly described SRB and therefore proposed to represent a novel genus – *Desulfocucumis* gen. nov. (see section 2.2). These isolates were retrieved from up to a million-fold diluted sediment samples and included both organoheterotrophic as well as lithoautotrophic physiotypes. As in general members of the *Desulfobulbaceae* were found to be highly abundant *in situ*, these isolates are assumed to be representatives of an ecological relevant population. However, the use of more specific, recently described rRNA-targeted probes (Dblb1032, Mußmann et al., 2005) or designing new probes based on the 16S rRNA gene sequences of the isolates

could unravel their actual abundance and contribution to the sulfate-reducing community. Growth tests identified representatives of the novel genus as nutritionally versatile with respect to their electron donor and carbon source. As they were additionally able to grow fermentatively, this generalistic "life style" probably facilitates them to sustain a variable supply with substrates as well as temporary sulfate-depleted conditions.

In sandy surface sediments (depths < 40 cm), members of the *Desulfosarcina-Desulfococcus* group were described to predominate the SRB community over depths and seasons (Ishii et al., 2004; Mußmann et al., 2005; Musat et al., 2006). In difference, members of this group were detected in rather low abundance in deeper sediments, but could nevertheless be stimulated to grow in dilution cultures with either acetate or lactate as electron donor. However, sub-cultivation and subsequent isolation finally selected for another phylotype related to *Desulfobacter* that was initially not detected by molecular screening. It was previously suggested that members of the *Desulfosarcina-Desulfococcus* group might be associated with members of the *Bacteroidetes* (Mußmann, 2003). The latter are a highly abundant, probably heterotrophic group of microorganisms that are thought to grow fermentatively and therefore provide common substrates for SRB (Eilers et al., 2000; Llobet-Brossa et al., 1998; Rossello-Morá et al., 1999). One might speculate that this association is obligate to this sulfate reducer and might hinder the isolation into pure culture. Following this hypothesis, future cultivation attempts should be designed to favour this interaction, e. g. by the establishment of co-cultures with defined *Bacteroidetes* strains or the addition of complex substrates for fermentation.

3.2 Ecophysiology of sulfate-reducing bacteria in sand flat sediments

Both pore water sulfate data from a transectional sampling as well as repeated sampling at the edge of the sand flat indicated that SRB within these sediments are forced to tolerate not only fluctuations in sulfate concentration, but even sulfate-depleted conditions (< 0.1 mM). Nevertheless, SRB reached even higher relative and absolute cell numbers within deep, sulfate-depleted sediment layers than in the sulfate-rich surface sediments.

As recently shown by Pallud and van Cappellen (2006) for a marine site in the Scheldt estuary, pore water sulfate concentrations $>0.1\,\mathrm{mM}$ are not assumed to limit microbial sulfate reduction. Therefore, sulfate reduction coupled to the oxidation of organic compounds or hydrogen is proposed to be the dominating pathway of energy conservation of SRB within these sediments. Isolation of lithoautotrophic as well as organoheterotrophic SRB and their broad spectrum of utilizable electron and carbon sources suggest a high nutritional versatility what makes them potentially adapted to a changing supply with substrates *in situ*.

However, there is only few information available on the composition of dissolved organic carbon within these sediments. Based on carbon isotopic signatures, organic matter in the upper 50 cm was characterized as mainly of marine origin ($>80\,\%$ of the total organic matter, J. Köster). Although DOC concentrations generally increased with depth at a nearby upper flat location (Beck et al., 2007) indicating the accumulation of hardly degradable organic compounds in deep sediment layers, a variety of short-chain organic acids (including acetate and lactate), that may serve as potential substrates to e. g. sulfate-reducing bacteria, were detected even at several meters depth at the lower flat. It is feasible to argue that these compounds were microbially formed through fermentation processes within these sediment layers or originated from upper layers and were subsequently transported into deeper sediments.

3.2.1 Anaerobic oxidation of methane

Beside a pronounced sulfate-methane transition zone, it was evident from stimulation experiments that reducing equivalents for sulfate reduction could also originate from biogenic methane (see section 2.3). There are only few studies so far that demonstrated the relevance of anaerobic methane oxidation (AOM) under moderate conditions, i. e. an *in situ* atmospheric pressure and therefore low methane partial pressure (Thomsen et al., 2001; Leloup et al., 2006; Parkes et al., 2007). Within the scope of these studies, peaks of microbial activity (sulfate reduction, AOM) at discrete sulfate-methane transition zones were correlated to the presence of specific archaeal and bacterial populations. Beside the common observation that SRB related to the *Desulfosarcina-Desulfococcus*

group are involved in this process (Orphan et al., 2001), just recently members of the *Desulfobulbaceae* were reported to mediate AOM in submarine mud volcano sediments (Lösekann et al., 2007). Thus, it appears likely that there exists a not yet definded fraction of SRB involved in methane oxidation. It remains to be clarified whether the identified and highly abundant SRB at site "Janssand" are involved in this process.

3.2.2 Energy conservation under sulfate limitation

Generally, methanogens are favored to stand the competition for substrates with SRB under sulfate depletion. In fact, both the presence of high methane concentrations as well as the cultivation of physiologically diverse methanogens from site "Janssand" point to an active resident community. Nevertheless, SRB were highly abundant and potentially active in deep, sulfate-depleted sediments. It is therefore proposed that fermentative growth and mutualism are the most feasible alternatives of energy conservation in the absence of sulfate as electron acceptor. Other electron acceptors (like nitrate, ferrous iron or manganese oxides) are not assumed to be highly concentrated within these deep sediment layers. Thus, it appears unlikely that they substitute sulfate as electron acceptor resulting in a numerically stable SRB community. In fact, almost all isolated SRB shared the capacity to grow fermentatively on fumarate and/or pyruvate. However, both compounds are important intermediates of anaerobic degradation processes and usually not released during the remineralization of organic matter. Fermentation of other compounds was not tested yet, but the availability of pure cultures of *in situ* abundant SRB now allows for further studies on their metabolic capacities.

Anyway, it appears more likely that SRB within these sediments exist in syntrophic association with methanogens or homoacetogens that scavenge hydrogen produced by the sulfate reducer. This interspecies hydrogen transfer results in thermodynamically feasible conditions for sulfate reducers to oxidize various organic substrates in the absence of sulfate (Schink, 1997; Plugge et al., 2002; Stolyar et al., 2007). Interaction of our isolates with methanogenic or homoacetogenic cultures has yet not been tested, but this syntrophy was proposed to be widely distributed in anoxic environments (Schink, 1997). More-

over, diverse methanogens have been cultured from the same sediment samples facilitating future syntrophy studies with indigenous species.

3.3 Tidal sand flats appear to be "hot spots" of microbial activity

Sandy sediments at site "Janssand" showed an almost homogenous distribution of microorganisms over several meters depth with SRB accounting for up to 7 % of the total microbial community. Beside a high activity of SRB in organic-rich surface sediments, additional activity peaks were found in sediments at 1.5 to 2 meters depth. This finding was unexpected, as microbial activities are generally assumed to decrease with sediment depth and are strongly coupled to the accompanying decrease in easily available organic matter. At site "Janssand", both high total cell counts as well as enhanced activity of SRB – being repesentative members of the microbial community – might result from the transport of organic as well as inorganic nutrients into deep sediment layers. It was previously proposed that microbial communities might benefit from the higher permeability of sands and the resulting enhanced advective pore water transport in porous sediment layers (Huettel and Rusch, 2000; de Beer et al., 2005; Billerbeck et al., 2006). Billerbeck and colleagues showed that the drainage of pore water at site "Janssand" through the sediment from the upper flat directed towards the low water line affects at least the sediment layers down to 50 cm depth. Recent hydrologic modeling approaches suggest that this transport might affect even several meter deep layers (H. Røy, D. De Beer, personal communication). It was additionally suggested that such deep flows of pore water are loaded with metabolic products and may contribute to the high pore water solute concentrations emerging at lower flat sampling sites. Considerable seasonal variations in sulfate concentration within sediment layers at 2.5 meters depth at the edge of the flat might indicate a transport of solutes from the upper flat to deep layers at lower flat sites. Nevertheless, local nutrient concentrations just represent the result of both the transport of nutrients from the upper part of the sand flat into deep sediment layers at lower flat sites as well as variations in the activity of the microbial community. In

conclusion, the relatively high abundance of microbial cells and the presence of active and abundant sulfate reducers might indicate a beneficial effect of pore water drainage and of the provision with as yet undefined nutrients to the microbial community of deep sediments. Thus, tidal sand flats have the potential to be microbial "hot spots" and are assumed to possess a significant impact on remineralization processes and nutrient cycling.

3.4 Future perspectives

As SRB are "only" the terminal instance in anaerobic degradation processes, they strongly depend on the activity of hydrolizing and fermentating microorganisms. Most promising, the highly diverse *Gammaproteobacteria* and *Bacteroidetes* were found to be abundant and probably associated to sulfate reducers within "Janssand" sediments. Both selective cultivation (including coculturing, selective (heavy isotope-labeled) substrates, and molecular monitoring) as well as highly specific techniques like MAR-FISH or mRNA-FISH should be performed to attribute specific functions and activities to distinct populations within the microbial community.

The availability of isolates that are assumed to represent abundant members of the indigenous sulfate-reducing community now facilitates to study their metabolic capacities and physiological adaptations that obviously enable them to sustain highly abundant populations in this dynamic environment. Additionally, the phylogenetic analysis of these isolates provides specific biomarkers (16S rRNA and *dsrAB* gene sequences) to track their actual *in situ* abundance and ecological relevance.

More detailed analyses of pore water fluxes and the characterization of dissolved organic carbon should provide information on the *in situ* available substrates as well as clarify whether and to which extend microbial communities are actually stimulated through pore water transport. Higher seasonal and spatial resolution of pore water profiles and community analyses might result in a more detailed picture of the linkage between environmental parameters, community structure and microbial activities.

As it could be shown that microorganisms are highly abundant, active and metabolically diverse also in several meter deep sediments, they are supposed

to have a yet not quantified impact on remineralization processes. Without fail, processes within these deep sediments should therefore be considered in the future budgeting of nutrient fluxes and cycling in tidal flat ecosystems.

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Hiermit versichere ich, dass ich die vorliegende Arbeit selbstständig verfasst und nur die angegebenen Hilfsmittel verwendet habe.