Zur Homepage der Dissertation

Verteilung, Zusammensetzung und Aktivitäten mikrobieller Gemeinschaften in Wattsedimenten von der Oberfläche bis in mehrere Meter Tiefe

Distribution, composition and activities of microbial communities in tidal sediments from the surface down to several meters depth

Dissertation

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Für Gregor, Sylvi und Sabine

Für Opa Martin und Martin

Teilergebnisse aus dieser Arbeit sind als Artikel in wissenschaftlichen Zeitschriften bereits veröffentlicht, eingereicht oder werden eingereicht. Im Nachfolgenden ist mein Beitrag an der Erstellung dieser Manuskripte geschildert.

Beiträge in wissenschaftlichen Zeitschriften:

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Köpke, B., Feuerlein, T., Wilms, R., Engelen, B., Cypionka, H. und Sass, H. (2006). *Roseobacter pelophilus* sp. nov. and *Roseovarius pelophilus* sp. nov., facultatively anaerobic bacteria isolated from tidal flat sediments. (Eingereicht bei *Inter. J. Sys. Environ. Microbiol.*)

Vorarbeiten zur Physiologie der Isolate (Substratspektrum, Temperaturspektrum, Elektronenakzeptortest) durch T. F. (Diplomarbeit 2004) unter Anleitung von B. K.; Teile dieser Arbeit wiederholt (Elektronenakzeptortest) und erweitert (pH-Spektrum, Salinitätsspektrum, Wachstumsertrag) durch B. K.; Sequenzierung durch Andrea Schlingloff, T. F. und B.K.; phylogenetische Analyse durch B. K.; Erstellung des ersten Manuskriptes durch B. K.; Überarbeitung durch H. S., H. C.

Köpke, B., Böttcher, M. E., Köster, J., Freese, H., Wilms, R., Engelen, B., Cypionka, H., Rullkötter, J. und Sass, H. (2006). Presence and activity of microbial populations in the subsurface of a tidal flat. (In Vorbereitung zum Einreichen bei *Limnol. Oceanogr.*)

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TCC) durch B. K.; Sulfatreduktionsraten durch B.K.; Exoenzymaktivitäten durch R. W.; geochemische Analysen: Sulfat, Chlorid durch H. S., Eisen und Mangan durch M. B., TOC, DOC, TRS, $\delta^{13}C$, Korngröße durch J. K. und E. F., Ammonium durch Willm Martens-Habbena, Porosität und Bodendichte durch B. K.; Erstellung des ersten Manuskriptes durch B. K.; Überarbeitung durch H. S., M. B.

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Köpke, B., Wilms, R., Engelen, B., Cypionka, H., Rullkötter, J., and Sass, H. (2003). Cultivation of bacteria from a six meter long core from an intertidal sediment. Workshop on BioGeoChemistry of tidal flats, May 14-17, 2003, Hansewissenschaftskolleg Delmenhorst, Germany. Reports: Forschungszentrum Terramare 12: 75-76 (Extended Abstract).

Köpke, B., Wilms, R., Engelen, B., Cypionka, H., Rullkötter, J., and Sass, H. (2003). Cultivation of bacteria from a six meter long core of intertidal sediment. Poster presentation at the Annual Meeting of the Verein für Allgemeine und Angewandte Mikrobiologie, March 23-26, 2003, Berlin, Germany.

Köpke, B., Wilms, R., Engelen, B., Cypionka, H., Rullkötter, J., and Sass, H. (2004). Microbial communities in the „shallow subsurface“ of a North Sea tidal flat. Poster presentation at the International workshop on geomicrobiology – a research area in progress, January 28-31, 2004, University of Aarhus, Denmark.

Köpke, B., Wilms, R., Engelen, B., Cypionka, H., Rullkötter, J., and Sass, H. (2004). Microbial communities and activities in the "shallow biosphere" of a North Sea tidal flat. Oral presentation at the Annual Meeting of the Verein für Allgemeine und Angewandte Mikrobiologie, March 28-31, 2004, Braunschweig, Germany.

Zusammenfassung

In der vorliegenden Arbeit wurden die Zusammensetzungen und Aktivitäten mikrobieller Gemeinschaften in Sedimenten des Rückseitenwatts der Insel Spiekeroog untersucht. Dazu wurden verschiedene, bis zu 550 cm lange Sedimentkerne gewonnen und umfassend mittels mikrobiologischer, molekularbiologischer und geochemischer Methoden analysiert. Im Fokus stand die Erfassung vertikaler Veränderungen sowie die Beeinflussung der Bakteriengemeinschaften durch bestimmte geochemische Sedimenteigenschaften.

Im ersten Teil dieser Arbeit wurde die mikrobielle Zusammensetzung zweier Standorte unter der Verwendung verschiedener Kultivierungsmethoden (MPN-Technik und Gradientenröhrchen) analysiert. Die höchsten Kultivierungseffizienzen wurden mit einem Medium erzielt, das mit einem definierten Gemisch verschiedener Kohlenstoffquellen sowie unterschiedlichen Metalloxiden als Elektronenakzeptoren versetzt war. Damit konnten aus oberflächennahen Sedimentschichten bis zu 23 % als Lebendkeimzahl erfasst werden. Die Kultivierungsansätze wurden molekularbiologisch untersucht, um die einzelnen Methoden zu vergleichen und um herauszufinden, inwieweit diese sich eignen, abundante Vertreter zu isolieren. Dazu wurden Genfragmente der 16S rRNA aus Kultivierungsansätzen und Sedimentproben mittels PCR-DGGE analysiert und anschließend sequenziert. Der Vergleich der Sequenzen mit denen der Isolate zeigte jedoch, dass keines der isolierten Bakterien im Habitat abundant zu sein scheint.

Insgesamt umfasst die Kultursammlung 112 Stämme und zeichnet sich durch eine hohe Diversität aus. Die isolierten Stämme konnten sieben verschiedenen phylogenetischen Gruppen zugeordnet werden. Von den oberflächennahen Sedimentschichten wurden überwiegend Vertreter der *Proteobacteria* isoliert, während in den tieferen Lagen (≥200 cm) Vertreter der *Firmicutes* abundant waren. Die Mehrheit der Isolate waren fakultative oder strikte Anaerobier, die durch Fermentation oder anaerobe Atmung mit Nitrat, Eisenhydrit, Manganoxiden oder Sulfat als Elektronenakzeptor wuchsen. Einige Stämme wiesen eine geringere 16S rRNA Ähnlichkeit (≤93 %) mit bereits validierten, beschriebenen Arten auf und stellen wahrscheinlich neue Gattungen dar.

Im zweiten Teil dieser Arbeit werden fünf Stämme beschrieben, die von oberflächennahen Sedimentschichten isoliert wurden. Diese Isolate, die der *Roseobacter*-Gruppe zugeordnet werden, zeichneten sich besonders durch ihre physiologische Vielseitigkeit aus. Sie waren fähig, anoxischen Bedingungen zu widerstehen und anaerob zu wachsen. Aufgrund der phylogenetischen Analyse und der phänotypischen und

physiologischen Eigenschaften wurden zwei der fünf Stämme als neue Arten, *Roseobacter pelophilus* sp. nov. und *Roseovarius pelophilus* sp. nov., vorgeschlagen.

Im dritten Teil wurden in den Wattsedimenten vertikale Veränderungen mikrobielle Aktivitäten erfasst. Dabei wurde auch der Einfluss verschiedener geochemischer Sedimentparameter untersucht. Die höchsten mikrobiellen Aktivitäten wurden nahe der Sedimentoberfläche gemessen, wobei Sulfatreduktionsraten und Gesamtaktivitäten der Exoenzyme generell mit der Sedimenttiefe abnahmen. Allerdings änderten sich die spezifischen Exoenzymaktivitäten (pro Zelle) nur geringfügig mit der Tiefe. Hinsichtlich ihrer Aktivität unterschieden sich die Zellen in den tieferen Sedimentbereichen somit nicht maßgeblich von denen nahe der Sedimentoberfläche.

Die höchsten modellierten Netto-Sulfatreduktionsraten wurden in den Übergangszonen von Sulfat und Methan verzeichnet. Das deutet darauf hin, dass die Aktivität Sulfat reduzierender Bakterien nicht nur von der Verfügbarkeit organischen Materials, sondern auch von dem Vorhandensein alternativer Elektronendonatoren abhängig ist.

Die Korngrößenverteilung der Sedimente beeinflusste nicht nur deren chemischen Eigenschaften sondern scheinbar auch die mikrobiellen Aktivitäten. Die Adsorption von organischem Material an Tonpartikel führte zur Anreicherung von organischem Kohlenstoff (TOC) in tonigen Sedimentschichten. Allerdings waren die mikrobiellen Aktivitäten in den tieferen sandigen Lagen höher als in den tonigen. Es wird daher angenommen, dass die geringere Porosität der tonigen Sedimente zu einer eingeschränkten Diffusion von Substraten und Exoenzymen und weniger Mobilität der Bakterien führt.

Die Tiefenprofile der Porenwasserkonzentrationen von Sulfat und Chlorid deuteten darauf hin, dass beide Standorte von unterirdischen Wasserzuströmen beeinflusst werden. Während in die tieferen Schichten des Standortes Gröninger Plate zum Teil Süßwasser eingetragen wurde, führte vermutlich ein zusätzlicher Einstrom von Meerwasser am Standort Neuharlingersieler Nacken zu einem zweiten Sulfatpeak in etwa 240 cm Tiefe. Diese Sulfatzufuhr spiegelte sich in der Akkumulation reduzierter Schwefelspezies, die hauptsächlich auf die Aktivität Sulfat reduzierender Bakterien zurückgeführt werden können, in tieferen tonigen Sedimentlagen wider.

Diese Studie beschreibt die Wattsedimente als ein hoch komplexes System, an dass sich die Bakteriengemeinschaften im Hinblick auf die Vielseitigkeit ihrer physiologischen Fähigkeiten gut angepasst haben. Auch wenn über die vertikale Ausdehnung der mikrobiell aktiven Wattsedimente keine Aussagen getroffen werden können, tragen die tieferen Sedimente dennoch deutlich zur Gesamtaktivität des Systems bei. Die unterschiedlichen

Resultate der molekularbiologischen und mikrobiologischen Untersuchungen machen zudem deutlich, dass eine Kombination aus kultivierungsabhängigen und –unabhängigen Methoden forciert werden sollte, um mikrobielle Gemeinschaften in Wattsedimenten zu untersuchen.

Summary

This thesis describes the composition and activities of microbial communities in sediments of the backbarrier tidal flat of the island of Spiekeroog. Several up to 550 cm long sediment cores were investigated by comprehensive microbiological, molecularbiological and geochemical methods.

In the first part of the study, microbial communities from two sites were analysed using different cultivation methods, the MPN technique and gradient tubes, respectively. The highest cultivation efficiencies (up to 23 %) were achieved near the sediment surface with medium supplemented with a defined mixture of carbon sources and metal oxides as electron acceptors. The different cultivation approaches were investigated molecularbiologically to compare the different methods and to proof their suitability to isolate abundant microbial community members. Therefore, gene fragments of the 16S rRNA obtained from the enrichment cultures and sediment samples were analysed by PCR-DGGE and subsequently sequencing. The comparison of the sequences to those of the isolates revealed that none of the isolated bacteria seem to be abundant in the habitat.

The culture collection of 112 strains showed a high diversity. The isolated strains are affiliated to seven different phylogenetic clusters. While predominantly members of the *Proteobacteria* were isolated from surface near sediments, members of the *Firmicutes* were more abundant in the deeper layers (≥200 cmbsf). The majority of the isolates were facultative or strict anaerobes that were able to grow by fermentation or anaerobic respiration with nitrate, ferrihydrite, manganese oxides or sulfate as electron acceptors. Several strains only showed marginal 16S rRNA similarities (≤93 %) to the next related validly described species and presumably represent new genera.

In the second part of this thesis, five strains were characterized, that were isolated from surface-near sediment layers. The isolates were related to the *Roseobacter* clade, nutritionally versatile and were able to sustain anoxic conditions and to grow anaerobically. Based on the phylogenetic analysis and the comparison of the phenotypic and physiological properties, two of the five strains were proposed as new species: *Roseobacter pelophilus* sp. nov. and *Roseovarius pelophilus* sp. nov.

The third part focused on vertical changes of microbial activities. Especially the impact of different geochemical sediment parameters were analyzed. Microbial activities were highest around the sediment surface. While sulfate reduction rates and total exoenzyme activities generally decreased with depth, specific exoenzyme activities

(referred to a single cell) changed only slightly. This finding indicates that the cells of the deeper layers were as active as those near the sediment surface.

The highest modelled net sulfate reduction rates were calculated for the sulfate-methane transition zone. Therefore, the activity of sulfate-reducing bacteria might not only depend on the availability of organic matter but also on the availability of alternative electron donors.

The sediment grain sizes seemed to influence not only the chemical sediment properties but also microbial activities. Due to the adsorption of organic matter on clay particles, muddy layers were enriched in total organic carbon (TOC). However, microbial activities were higher in deeper sand dominated than in mud dominated sediment layers. This might be the result of a lower diffusion of substrates and exoenzymes and a less bacterial mobility due to lower porosity in muddy sediments.

Porewater concentrations of sulfate and chloride lead to the assumption, that both study sites were influenced by subterranean water flow. While a freshwater inflow was measured for deeper layers of site Gröninger Plate, site Neuharlingersieler Nacken was affected by a lateral seawater intrusion. This presumably led to a second sulfate peak at around 240 cmbsf. The additional sulfate supply in these deeper layers was reflected in the accumulation of reduced sulfur species in the mud dominated layers, that as a result of the activity of sulfate-reducing bacteria.

The study describes tidal-flat sediments as a highly complex system in which bacterial communities are well adapted due to the versatility of their physiological skills. Although the extension of microbiologically active sediments is still unknown, it was shown that the subsurface layers clearly contribute to the total activity of the system. Due to the different results of the molecularbiological and microbiological investigations, the combination of culture-dependent and culture-independent approaches is proposed to be preferred to investigate microbial communities in these sediments.

Inhaltsverzeichnis

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Abkürzungen

Die Abkürzungen zu den englischen Erläuterungen stammen aus den Manuskripten.

ALC	mixture of alcohols
AQDS	2,6-anthraquinone disulfonate
CARD-FISH	catalysed reporter deposition-FISH
DAPI	4', 6-diamino-2-phenylindole
DGGE	denaturing gradient gel electrophoresis
DMF	dimethylformamide
DMS	dimethylsulfide
DMSO	dimethylsulfoxide
DMSP	dimethylsulfoniopropionate
DNA	desoxy ribonucleic acid
DOC	dissolved organic carbon
D_S	molecular diffusivity in seawater corrected for tortuosity
DSM, DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
D_{SW}	molecular diffusivity in seawater
EDTA	ethylene-diaminetetracetate
et al.	et alii
FISH	fluorescence in situ hybridization
G+C	(content of) guanidine and cytosine (in DNA)
GP	Gröninger Plate
GR	Gradientenröhrchen
HEPES	4-(2-hydroxyethyl)-1-piperazinethansulfonic acid
HPLC	high performance liquid chromatography
MB	Marine Broth
MCA	7-amino-4-methylcoumarine
MPN	most probable number
MUF	methylumbelliferone
n	number of samples
n. d.	not determined
NN	Normal Null
n. s.	not significant
NSL	Neuharlingersiel
NSN	Neuharlingersieler Nacken
OD	optical density

OM	organic matter
OTU	operational taxonimical units
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
S	Spearman rank correlation coefficient
SDS	sodium dodecyl sulfate
SGDW	submarine groundwater discharge
SRR	sulfate reduction rates
TCA	mixture of carboxylic acids
TE	tris(hydroxymethyl)aminomethane-EDTA
TCC	total cell counts
TMAO	trimethylammoniumoxide
TRIS	tris(hydroxymethyl)aminomethane
TRS	total reduced sulfur
TS	total sulfur
TOC	total organic carbon
YPG	yeast extract-peptone-glucose

Kapitel I

Einleitung

1. Das Wattenmeer der südlichen Nordsee

1.1 Lage und Entstehung

Wattengebiete sind von Gezeiten geprägte Systeme und weltweit an vielen Küsten anzutreffen, wie z.B. in Mauretanien, Bangladesch, aber auch in Australien und an den Küsten Südkoreas. Das Wattenmeer der südlichen Nordsee stellt eine der größten zusammenhängenden Wattflächen der Welt dar. Es umfasst ca. 9300 km^2 und erstreckt sich mit einer Gesamtlänge von 500 km von Den Helder in den Niederlanden bis Esbjerg in Dänemark und wird seeseitig von einer Inselkette begrenzt, die sich entlang der Nordseeküste erstreckt. Der Bereich zwischen den Inseln und dem Festland wird als Wattenmeer verstanden. Der Begriff Watt ist definiert als Küstenbereich, der zeitweise trockenfällt (Gripp, 1956) und nach Behre *et al.* (1979) auch das gesamte Gebiet zwischen Inseln und Festland berücksichtigt. Das Wattenmeer lässt sich nach Streif (1990) in vier große Ablagerungsräume untergliedern:

- das Sublitoral, die ständig von Salzwasser bedeckte Zone (Vorstrandbereich, Seegatts, tiefe Wattrinnen),
- das Eulitoral, das bei Hochwasser von Wasser bedeckt ist und bei Niedrigwasser trockenfällt (z.B. die Wattflächen zwischen den Inseln),
- das Supralitoral, das nur bei hoch auflaufenden Fluten von Salzwasser bedeckt wird (z.B. Trockenstrand oder unbedeichte Salzmarschen),
- die Dünen, die außerhalb des Einflussbereiches mariner Prozesse liegen.

Die Entstehung des Wattenmeeres der südlichen Nordsee wird auf die letzte Eiszeit zurückgeführt, in der große Teile des Beckens der heutigen Nordsee trockengefallen waren, da das Gebiet einerseits zum Atlantik hin entwässerte (Figge, 1980), andererseits aber auch Wasser als Eis im tiefgründig gefrorenen Boden verblieb. Mit der einsetzenden Erwärmung zu Beginn des Holozäns vor ca. 10.000 Jahren wurden diese Flächen überflutet (Eisma *et al.,* 1981). Vor 7500 Jahren (Meeresspiegel –20 m NN) wurde der pleistozäne Geestrücken erreicht, der zum Teil die ostfriesische Küstenlinie bildet (Streif, 1999). Der Meeresspiegel stieg weiter an, einhergehend mit einer Zunahme des Tidenhubes, und erreichte vor ca. 6500 Jahren –10 m NN mit einem Tidenhub von 2,2 m. Während dieser Zeit begann die Watten- und Marschenbildung (Flemming und Davis, 1994). Die Nordsee und die in sie mündenden Flüsse setzten unter der Mitwirkung von Gezeiten Schwemmmaterial an der Flachküste ab. Seit ca. 1000 Jahren wird das Wattenmeer der

südlichen Nordseeküste zusätzlich von anthropogenen Eingriffen, wie Landgewinnung und Deichbau, beeinflusst.

1.2 Charakteristika des Watts

Im Wattenmeer lassen verschiedene Sedimenttypen nach ihrer Korngrößenzusammensetzung unterscheiden (Gadow und Schaefer, 1973, Tardent, 2005):

- das Sandwatt mit weniger als 5 Gewichtsprozent Feinmaterial (Korngröße <63 µm),
- das Mischwatt mit 5 bis 50 Gewichtsprozent Feinmaterial,
- das Schlickwatt mit mehr als 50 Gewichtsprozent Feinmaterial.

Durch den bereits erwähnten Deichbau haben sich die Sedimentationsbedingungen derart verändert, dass im deichnahen Watt die Ablagerung feinerer Sedimente verhindert wird. Daher besteht das heutige Rückseitenwatt der Insel Spiekeroog aus reinen Sandwatten und Mischwatt. Schlickwatten sind nur vereinzelt in kleinen Flecken zu finden (Flemming und Davis, 1994).

Das Wattenmeer ist ein sehr dynamisches von den Gezeiten stark beeinflusstes System. Durch den Gezeitenstrom werden Wasserkörper horizontal und vertikal bewegt. Wattflächen fallen trocken und werden überflutet, wobei ein Tidenzyklus ca. 12,5 Stunden beträgt. Der mittlere Tidenhub im Untersuchungsgebiet, dem Spiekerooger Rückseitenwatt, liegt bei 2,7 m und somit im mesotidalen Bereich (Niesel,1999). Die ausgeprägte Hydrodynamik bewirkt große Schwankungen sowohl abiotischer als auch biotischer Parameter. Der Salzgehalt im Wattenmeer beträgt 23-30 PSU und ist damit geringer als der der Nordsee mit 35 PSU. Er unterliegt jahreszeitlichen Schwankungen. Bei hohen Niederschlägen kann es aufgrund der geringen Wassertiefe im Watt zu mehr oder weniger starken Verdünnungseffekten kommen. Der Salzgehalt des Wattbodens kann aber im Sommer aufgrund der Verdunstung auch über dem mittleren Gehalt des Meerwassers liegen (Reineck und Flemming, 1990). Die geringe Wassertiefe führt aber auch zu stärkeren Schwankungen der Wassertemperatur im Vergleich zur offenen See Die Temperaturen unterliegen nicht nur jahreszeitlichen und witterungsbedingten Veränderungen, sondern werden auch von den Gezeiten beeinflusst (Vugts und Zimmermann, 1975).

Wattsedimente zeichnen sich neben zeitlicher auch durch räumliche Variabilität aus. Durch die Bauten einiger Makrozoobenthosarten lassen sich oxische Mikronischen

noch in mehreren Zentimetern Sedimenttiefe finden (Kristensen, 2000a). Extremereignisse, wie Stürme, können größere Sedimentmengen in Bewegung setzen und damit auch einen Transport gelöster organischer und anorganischer Substanzen bewirken. Diese vielfältigen Sedimentumlagerungen bewirken letztendlich ein Mosaik von Mikronischen im Sediment (Kristensen, 2000a).

1.3 Probenahmestandorte

Die beiden Probenahmestandorte Neuharlingersieler Nacken und Gröninger Plate befinden sich im Rückseitenwatt der Insel Spiekeroog (Abb. 1) und liegen an der Hauptentwässerungsrinne.

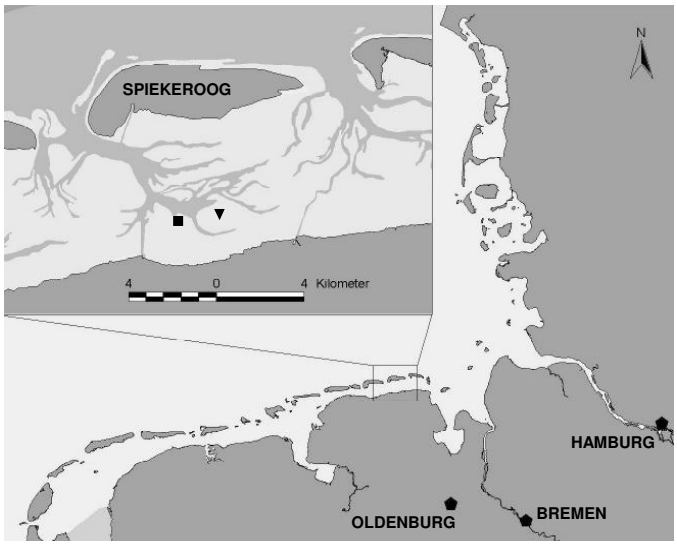

Abb. 1: Wattenmeer der südlichen Nordsee mit Focus auf das Rückseitenwatt der Insel Spiekeroog, in dem sich die beiden Probenahmestandorte Neuharlingersieler Nacken [■] und Gröninger Plate [▼] befinden.

Bis zu 200 cm Tiefe sind die Sedimente an beiden Standorten Sandwatt zuzuordnen. Unterhalb dieser unterscheiden sich die Standorte deutlich in ihrer lithologischen Stratigraphie. Während der Standort Gröninger Plate bis zu 550 cm dem Sand- und Mischwattmilieu zugeordnet werden kann, gehen am Standort Neuharlingersieler Nacken unterhalb von 200 cm Tiefe die Sand- und Mischwattfazies in eine Salzwiesen und Schlickwattfazies über (Chang *et al.,* 2003, Tilch, 2003). Die Koordinaten der Standpunkte sowie eine detaillierte stratigraphische Aufnahme der Sedimentkerne und die Darstellung chemischer Sediment- und Porenwasserparameter finden sich in Kapitel IV.

1.4 Bisherige mikrobiologische Untersuchungen in Wattsedimenten

Die mikrobiellen Gemeinschaften der Wattsedimente wurden sowohl mit kultivierungsabhängigen als auch –unabhängigen Methoden intensiv analysiert. Allerdings beschränkte man sich dabei auf die Untersuchung der oberen Sedimentschichten bis zu 50 cm Tiefe (Böttcher *et al.,* 2000, Llobet-Brossa *et al.* 2002, Rütters *et al.,* 2002). Dabei standen häufig ausgewählte mikrobielle Gruppen im Vordergrund, wie z.B. Sulfat reduzierende Bakterien (Wieringa *et al.,* 2000, Rütters *et al.,* 2002). Neben der Identifizierung der Bakterien lag ein weiteres Hauptaugenmerk auf der Untersuchung mikrobieller Aktivitäten. So wurden u.a. bakterielle Produktion (Poremba *et al.,* 1999), Exoenzymaktivitäten (Villbrandt *et al.,* 1999) und mikrobielle Umsatzraten, wie Sulfatreduktionsraten, gemessen. Letztere unterliegen einer komplexen Kontrolle von Temperatur und Verfügbarkeit organischen Materials (Böttcher *et al.,* 2000, Kristensen *et al.,* 1998). Sulfat reduzierende Bakterien wurden mit mikrobiologischen als auch molekularbiologischen Methoden quantifiziert. Offensichtlich stellen sie keine abundante Gruppe in den Sedimenten dar, auch in denen, die sich durch eine intensive Sulfatreduktion auszeichnen (Lobet-Brossa *et al.,* 2002).

2. Biogeochemie mariner Sedimente

Meere bedecken ca. 70 % der Erdoberfläche und stellen damit den weltweit größten Lebensraum dar. Die darin lebenden Mikroorganismen sind maßgeblich an biologischen Synthese- und Abbauleistungen beteiligt. Damit sind sie von globaler Bedeutung für Stoffkreisläufe der meisten chemischen Elemente (z.B. Canfield *et al.,* 2005, Schlegel, 1992). Im offenen Ozean sind Mikroorganismen für einen Großteil der Primärproduktion verantwortlich wie auch für die Mineralisierung des organischen Kohlenstoffs. Diese erfolgt dort meist unter oxischen Bedingungen. In Küstensedimenten, die meist geprägt sind von hohen Einträgen organischer Substanzen, dominieren anaerobe Prozesse (Henrichs und Reeburgh, 1987). Organisches Material, das nicht in der Wassersäule abgebaut wird, akkumuliert an der Sediment-Wassergrenze. Dort und in den obersten Sedimentschichten führen (mikro)biologische Mineralisationsprozesse zu einem erhöhten Sauerstoffbedarf. Der hauptsächlich über Diffusion ins Sediment eingetragene Sauerstoff reicht im Allgemeinen nicht aus, um diesen Bedarf zu decken. Dadurch sind die Sedimente bereits nach wenigen Millimetern Tiefe anoxisch. Allerdings kann sich durch Bioturbation (Kristensen, 2000a) und advektiven Transport (Huettel *et al.,* 1998) sowie durch Sedimentumlagerungen (Resuspension und Sedimentation) der Sauerstoffeintrag und die

Eindringtiefe ins Sediment erhöhen. Ist der molekulare Sauerstoff verbraucht, wird das organische Material über die anaerobe Nahrungskette (Gärung und anaerobe Atmungsprozesse) mineralisiert.

In Sedimenten findet man eine vertikale Abfolge der dabei genutzten Elektronenakzeptoren, die dem abnehmenden Redoxpotential und damit verbunden dem möglichen Energiegewinn folgt (Froelich *et al.,* 1979, Sørensen *et al.,*1979). Der größte Energiegewinn wird bei der aeroben Atmung mit Sauerstoff erzielt, gefolgt von Denitrifikation und Nitratammonifikation, der Reduktion von Mangan(IV) und Eisen(III), der Sulfatreduktion sowie der Methanogenese und Acetogenese mit dem Verbrauch von CO_2. Die Ausprägung der Gradienten der einzelnen Elektronenakzeptoren hängt vom Eintrag organischen Materials, den geochemischen Bedingungen sowie den mikrobiellen Aktivitäten am Standort ab.

Der größte Kreislauf, in den Mikroorganismen involviert sind, ist der des Kohlenstoffs. Die Mineralisation von organischem Kohlenstoff ist ein sehr komplexer Prozess, in den physiologisch unterschiedliche Organismen involviert sind. Die nachfolgenden Ausführungen beziehen sich nur auf bakterielle Prozesse. Im Gegensatz zum aeroben Abbau verläuft der anaerobe Abbau meist mehrstufig. In der Regel mineralisieren aerobe Bakterien die aufgenommenen Substrate vollständig. Bisher sind jedoch keine anaeroben Bakterien bekannt, die allein komplexes organisches Material vollständig verwerten. Stattdessen erweisen sich Anaerobier oft als Spezialisten einzelner Prozesse.

Beim anaeroben Abbau erfolgt als erster Schritt die enzymatische Spaltung polymerer organischer Substanz durch extrazelluläre hydrolytische Enzyme (Chrost, 1991). Das geschieht in der Regel durch primäre Gärer, die diese Enzyme ausscheiden und anschließend die entstandenen Monomere vergären (Abb. 2). Einige der Gärprodukte, wie z.B. Acetat, Wasserstoff, Kohlendioxid und andere C1-Verbindungen werden von methanogenen *Archaea* direkt zu Methan umgesetzt. Homoacetogene Bakterien können C1-Verbindungen zu Acetat umsetzten. Andere Gärprodukte, wie Alkohole und Fettsäuren, werden von sogenannten sekundären Gärern verwertet, die diese zu Wasserstoff, Kohlendioxid und Acetat disproportionieren. Primäre Gärprodukte werden auch von Bakterien oxidiert, die zur aneroben Atmung fähig sind. Diese Prozesse sind meist durch die Verfügbarkeit der dabei verwendeten Elektronenakzeptoren, wie Sulfat, Eisen(III), Mangan(IV) und Nitrat, begrenzt.

Viele Stoffwechselwege sind nur durch Syntrophie möglich. Darunter versteht man die Kooperation von zwei Organismen, wodurch Stoffwechselleistungen ermöglicht werden, zu denen ein Partner allein nicht fähig wäre (Fritsche, 1999). Der Abbau organischer Substanz ist dann energetisch nur möglich, wenn Produkte von nachfolgenden Bakterien verbraucht und somit deren Konzentrationen niedrig gehalten werden. Das gilt vor allem für Wasserstoff (Prinzip des „*interspecies hydrogen transfer*“, Bryant *et al*., 1967, Schink, 1997).

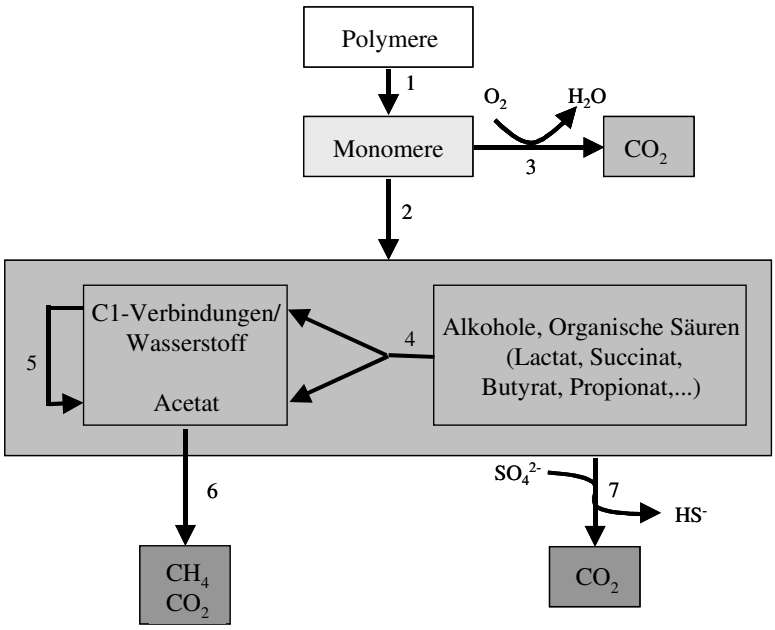

Abb. 2: Kohlenstoff- und Elektronenfluss durch die verschiedenen metabolischen Gruppen, die am Abbau komplexer, organischer Verbindungen beteiligt sind. 1) und 2) primäre Gärer, 3) aerobe, heterotrophe Bakterien, 4) sekundäre Gärer, 5) homoacetogene Bakterien, 6) Wasserstoff oxidierende sowie Acetat verwertende Methanogene, 7) Sulfatreduzierer (modifiziert nach Cypionka, 1999).

In marinen Sedimenten sind die aerobe Atmung sowie die Sulfatreduktion die entscheidenden Prozesse bei der Mineralisierung organischen Materials (Jørgensen, 1982). Aufgrund der relativ hohen Sulfatkonzentrationen von ca. 28 mM im Meerwasser (Fenchel *et al.*, 1998), was etwa der hundertfachen Konzentration an Sauerstoff entspricht, ist Sulfat auch im Sediment in höheren Konzentrationen vorhanden. Vor allem für tiefere Sedimenthorizonte, in denen ein negatives Redoxpotential herrscht, wird die Sulfatreduktion als charakteristisch angesehen (Jørgensen und Bak, 1991). Bis zu 50% des organischen Materials in marinen Sedimenten werden anaerob durch Sulfatreduktion mineralisiert (Jørgensen, 1982). Allerdings wurden an marinen Standorten auch schon

ähnlich hohe Umsatzraten von Eisen(III) und Mangan(IV) wie von Sulfat beobachtet (Canfield *et al.,* 1993, Thamdrup, 1994).

2.1 Der Schwefelkreislauf in marinen Sedimenten

Der Schwefelkreislauf ist aufgrund der Vielzahl der Oxidationsstufen des Schwefels sehr komplex. Transformationen sind sowohl auf mikrobiologische als auch auf chemische Prozesse zurückzuführen. Trotz verschiedener intermediärer Schwefelverbindungen, wie z.B. Sulfit und Thiosulfat, liegt Schwefel hauptsächlich in drei verschiedenen Oxidationsstufen vor: -2 (Sulfid), 0 (elementarer Schwefel) und +6 (Sulfat).

Schwefel liegt in der mikrobiellen Biomasse als Bestandteil von Aminosäuren, Coenzymen, Vitaminen und Elektronenüberträgern vor. Der Einbau von Schwefel in die Biomasse erfolgt über assimilatorische Sulfatreduktion. Die dissimilatorische Sulfatreduktion (Abb. 3) ist ein ausschließlich mikrobieller Prozess und stellt die größte Sulfidquelle in marinen Sedimenten dar. Allerdings wird der größte Teil des Sulfids (bis zu 90%) wieder zu Sulfat oxidiert (Jørgensen, 1987). Nur ein geringer Teil wird in Form von Eisensulfiden gefällt oder wird an die Atmosphäre abgegeben (Jørgensen, 1987, Kristensen *et al.,* 2000b). Neben Sulfat kann auch Schwefel reduziert werden, was vor allem ein weit verbreiteter Prozess innerhalb der *Archaea* ist (Jan *et al.,* 1999, Kletzin *et al.,* 2004).

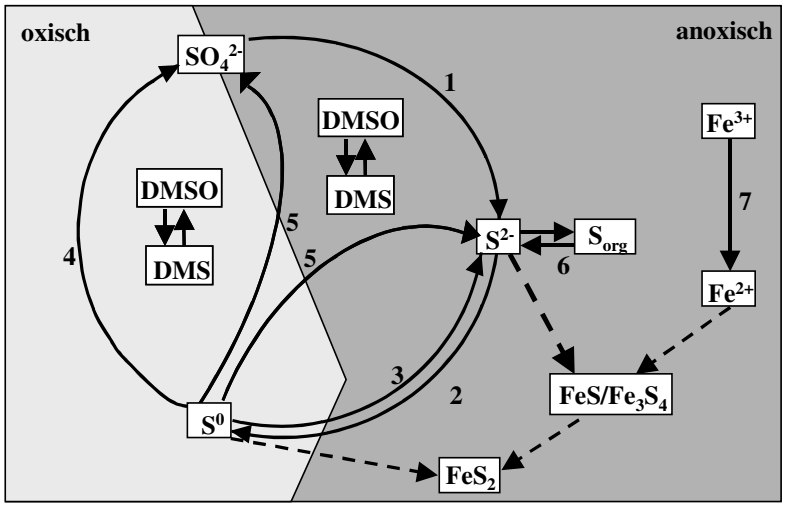

Abb. 3: Der Schwefelkreislauf in marinen Sedimenten mit den hauptsächlichsten mikrobiellen und anorganischen Transformationen (modifiziert nach Cypionka, 1999 und Oschmann, 2000). 1 = Sulfatreduktion, 2 = Sulfidoxidation, 3 = Schwefelreduktion, 4 = Schwefeloxidation (chemisch und mikrobiell), 5 = Schwefeldisproportionierung, 6 = Desulfurylation, 7 = Eisenreduktion. Durchgezogene Pfeile: mikrobielle Prozesse, gestrichelte Pfeile: chemische Prozesse.

Viele verschiedene Bakteriengruppen vermögen Sulfid oder reduzierte Schwefelverbindungen zu oxidieren. Chemolithoautotrophe Bakterien verbinden die Sulfidoxidation mit der Reduktion von Sauerstoff oder Nitrat (Canfield *et al.,* 2005). Die Energie wird dazu benutzt, um Kohlendioxid (CO_2) zu assimilieren. Aber auch chemolithoheterotrophe Mikroorganismen sind dazu in der Lage (Canfield *et al*., 2005). Ist Licht verfügbar, können reduzierte Schwefelverbindungen auch von phototrophen Schwefelbakterien oxidiert werden. Die anoxygene Photosynthese ist mit einer CO_2-Fixierung verbunden (Canfield *et al.,* 2005).

Durch Sulfatreduktion und Sulfidoxidation können verschiedene intermediäre Schwefelverbindungen, wie Sulfit und Thiosulfat entstehen, die neben den beschriebenen Prozessen wie Schwefel auch disproportioniert werden können (Bak und Cypionka, 1987, Thamdrup *et al.*, 1993).

Neben den vielen anorganischen Schwefelverbindungen werden auch organische Verbindungen von Mikroorganismen verwertet. Die häufigste organische Schwefelverbindung ist Dimethylsulfid (DMS). Es entsteht als Abbauprodukt von Dimethylsulfoniumpropionat (Abb. 3), einem Osmolyt verschiedener Phytoplanktongruppen (Turner *et al.*, 1988, Yoch, 2002). Dimethylsulfid kann in Oberflächenwasser oder in der Atmosphäre z.B. zu Dimethylsulfoxid (DMSO) photooxidiert werden (Brimblecombe und Shooter, 1986) oder es wird von verschiedenen Bakteriengruppen konsumiert. Dazu zählen sowohl Aerobier als auch Anaerobier (Jonkers *et al.* 1998, Tanimoto und Bak, 1994, Zeyer *et al.,* 1987). Dimethylsulfoxid spielt u.a. für Mikroorganismen als Elektronenakzeptor eine Rolle (Griebler und Slezak, 2000). Zusammen mit dem schon erwähnten Dimethylsulfid können Schwefelgase wie Carbonylsulfid (COS) und Kohlendisulfid (CS_2), die ebenfalls durch biologische Abbauprozesse, chemische Photolyse oder chemische Oxidation entstehen, bis zu 20 % der gesamten Schwefelemission in Wattsedimenten ausmachen (Kristensen *et al.,* 2000b).

2.2 Ökophysiologie der in den Schwefelkreislaufs involvierten Bakterien der *Roseobacter*-Gruppe

Viele phylogenetische Cluster basieren auf Sequenzvergleichen der 16S rDNA und enthalten keine kultivierten Vertreter (Rappé und Giovannoni, 2003). Im Gegensatz dazu wird die Gruppe der *Roseobacter*, die den α-*Proteobacteria* zuzuordnen ist, durch eine Vielzahl von Isolaten repräsentiert, so dass durch deren Charakterisierung Rückschlüsse auf ihre Funktion im Habitat möglich sind. Alle Isolate der *Roseobacter*-Gruppe,

abgesehen von denen der Gattung *Ketogulonicigenium*, stammen aus marinen oder salzhaltigen Habitaten, wie z.B. Meerwasser, marinen Sedimenten sowie hypersalinen Mikrobenmatten (Allgaier *et al.,* 2003, Eilers *et al.,* 2000, Fuhrman and Overney, 1998, Jonkers und Abed, 2003). Sie wurden ebenfalls in Assoziation mit marinen Algen, Schwämmen und Cephalopoden nachgewiesen (Allgaier *et al.,* 2003, Barbieri *et al.,* 2001, Webster *et al.,* 2004). Die *Roseobacter*-Gruppe ist in marinen Systemen weit verbreitet (Selje *et al.,* 2004) und zählt, basierend auf Untersuchungen der 16S rRNA Gene, zu einer der größten Gruppen des marinen Bakterioplanktons (González und Moran, 1997, Rappé *et al.,* 1997). Allerdings können sie auch im Sediment signifikant zur mikrobiellen Gemeinschaft beitragen (González *et al.,* 1999). Mit Ausnahme von *Roseobacter denitrificans* und *Roseovarius crassostreae*, die Nitrat als terminalen Elektronenakzeptor nutzen können, sind die bisher beschriebenen Stämme der *Roseobacter*-Gruppe strikte Aerobier (Boettcher *et al.,* 2005, Shiba, 1991). Die zur *Roseobacter*-Gruppe zugehörigen Bakterien sind an vielen ökologischen Prozessen beteiligt und haben eine hohe metabolische Diversität. Einige physiologische Fähigkeiten sind jedoch charakteristisch für diese Gruppe.

Besonderes Merkmal der ersten beiden Isolate der *Roseobacter*-Gruppe (*Roseobacter litoralis, Roseobacter denitrificans*) war das Vorkommen von Bacteriochlorophyll *a* sowie photosynthetische Aktivität (Shiba, 1991). Mittlerweile sind weitere Vertreter bekannt, die Bacteriochlorophyll *a* synthetisieren, wie *Staleya guttiformis* (Labrenz *et al.,* 2000), *Roseovarius tolerans* (Labrenz *et al.,* 1999), *Roseivivax halotolerans* und *Roseivivax halodurans* (Suzuki *et al.,* 1999). Molekularbiologische Untersuchungen der *pufLM*-Gene, die für die Untereinheit des Photosynthesereaktionszentrums kodieren, weisen darauf hin, dass Vertreter der *Roseobacter*-Gruppe in die **aerobe anoxygenen Photosynthesen** involviert sind (Oz *et al.,* 2005).

Bakterien der *Roseobacter*-Gruppe sind über viele Reaktionen in den **Schwefelkreislauf** eingebunden. Einige vermögen das Osmolyt Dimethylsulfoniopropionat (DMSP), das von Algen produziert wird, abzubauen (González *et al.,* 2003, Malmstrom *et al.,* 2004). Dabei wird u.a. Dimethylsulfid (DMS) gebildet, das einige *Roseobacter*-Verwandte ebenfalls verwerten können (González *et al.,* 1999, Schaefer *et al.,* 2002). Außerdem vermögen einige anorganische Schwefelverbindungen wie z.B. Schwefel, Sulfit oder Thiosulfat zu oxidieren (Ivanova *et al.,* 2004, Sorokin, 1995).

Einige Vertreter der *Roseobacter*-Gruppe sind in der Lage, **aromatische Verbindungen abzubauen** (Buchan *et al.,* 2000). Diese können, wie z.B. Huminstoffe, in größeren Mengen zum Kohlenstoffpool in Küstengebieten beitragen (Moran und Hodson, 1994). Der Abbau von Aromaten erfolgt u.a. über den β-Ketoadipat-Weg. In *Roseobacter*-Isolaten wurde das *pcaH*-Gen nachgewiesen, das ein Ring spaltendes Enzym dieses Abbauweges kodiert (Buchan *et al.,* 2001, Buchan *et al.,* 2004).

Einige Bakterien der *Roseobacter*-Gruppe produzieren **Sekundärmetabolite**, die zum Teil probiotische oder antibiotische Wirkung haben. *Roseobacter gallaeciensis* verhält sich antagonistisch zu Stämmen der γ-Proteobakterien (Ruiz-Ponte *et al.,* 1998) hat aber probiotische Auswirkungen auf Larven der Kammmuschel (Ruiz-Ponte *et al.,* 1999). Brinkhoff *et al.* (2004) beschrieben einen *Roseobacter* verwandten Stamm, der ein Antibiotikum produziert, das Bakterien- und Algenwachstum inhibiert. *Oceanibulbus indolifex* produziert u.a. Indol, Indolderivate und eine antimikrobielle Verbindung (Wagner-Döbler *et al.,* 2004).

3. Methodische Ansätze zur Untersuchung mikrobieller Gemeinschaften

Die Schwerpunkte der mikrobiellen Ökologie liegen in der Untersuchung der Biodiversität der Mikroorganismen, was deren Quantifizierung, Isolierung und Identifizierung in einem Habitat beinhaltet. Denn allein die phylogenetische Einordnung von Bakterien gibt keinen Aufschluss über deren physiologische Leistungen und Funktionen *in situ.* Dazu bedarf es zum Einen der Charakterisierung von Isolaten und zum Anderen der Messung mikrobieller Aktivitäten und deren Interpretation mit Hilfe chemischer Parameter (z.B. Konzentration potentieller Elektronenakzeptoren und –donatoren sowie möglicher Stoffwechselzwischen- und endprodukte). Die Kombination von molekularbiologischen und mikrobiologischen Untersuchungen ermöglicht die Schaffung eines komplexeren Bildes der mikrobiellen Gemeinschaft sowie ein besseres Verständnis ihrer Funktion *in situ*.

3.1 Kultivierungsmethoden – Vor- und Nachteile

Im Gegensatz zu Makroorganismen können Bakterien nicht allein über ihren Phänotyp identifiziert werden, da sie nur von geringer Größe sind und nur geringe morphologische Unterschiede aufweisen. Mit Hilfe mikroskopischer Techniken kann man sie nach ihrer Gestalt, der Begeißelung sowie dem Gramverhalten unterscheiden (Schlegel, 1992) als auch quantifizieren. Mikroorganismen werden häufig mit Substanzen gefärbt, die selektiv

an Zellbestandteile binden. Dazu werden häufig fluoreszierende Farbstoffe wie Acridinorange, DAPI, Sybr Green und Sytox verwendet, die mit DNA und/oder RNA spezifisch reagieren (Kepner und Pratt, 1994, Klauth *et al.,* 2004, Porter und Feig, 1980, Weinbauer *et al.,* 1998).

Die Identifizierung von Bakterien mittels Kultivierungstechniken ist nur zum Teil möglich. Denn Farb- und Formenvielfalt in und auf Agarmedien wachsender Kolonien sind begrenzt. Zudem können sich diese in Abhängigkeit von den Kultivierungsbedingungen, wie z.B. der Zusammensetzung des Mediums, Inkubation im Licht oder im Dunkeln, usw. unterscheiden. Außerdem können Organismen verschiedener phylogenetischer Gruppen einen ähnlichen oder gar gleichen Phänotyp aufweisen. Trotz dieser Einschränkung wurden und werden phänotypische und physiologische Merkmale von Bakterien zu deren Identifizierung und Klassifikation herangezogen.

Die physiologische Charakterisierung von Mikroorganismen ist erst nach erfolgreicher Kultivierung und Isolierung möglich, was ein sehr zeitaufwendiges Unterfangen darstellt. Durch den Einsatz ausgewählter Substrate (z.B. Kohlenstoffquellen, Elektronenakzeptoren) in Selektivmedien sowie durch die Wahl von Inkubationsbedingungen (z.B. oxisch/anoxisch) lässt sich die Isolierung bestimmter physiologischer Gruppen forcieren. Kultivierungsansätze werden auch zur Quantifizierung von Mikroorganismen verwendet, indem die Lebendkeimzahl[1] bestimmt wird. Allerdings haben diese Quantifizierungsmethoden einen entscheidenden Nachteil. Jannasch und Jones (1959) wiesen erstmalig auf die Diskrepanz zwischen der Gesamtzellzahl und der „Plattenzellzahl“ der Bakterien hin, was später auch als *great plate count anomaly* bezeichnet wurde (Staley und Konopka 1985). Darunter ist zu verstehen, dass die Zahl der Bakterien *in situ*, meist deutlich größer ist als die Zahl der aus diesem Habitat kultivierten Bakterien. Schätzungsweise lassen sich nur ca. 1 % der gesamten bakteriellen Gemeinschaft eines Standortes kultivieren (Amann *et al.,* 1995). Zu diesem Resultat führten auch DNA-Reassoziationsanalysen von Torsvik *et al.* (1994), die in Bodenproben

[1] Mit dieser Methode werden nur vermehrungsfähige Zellen erfasst. Dazu werden verschieden Verdünnungen einer Umweltprobe auf einem Agarnährboden verteilt und beberütet. Aus der Zahl der Kolonie bildenden Einheiten kann unter Berücksichtigung der Verdünnungsstufe die Lebendkeimzahl berechnet werden (Bast, 1999). Ähnlich erfolgt die Bestimmung Bestimmung der wahrscheinlichsten Keimzahl, m(ost) p(robable) n(umber). Aus der höchtsen positiven Verdünnungsstufe wird statistisch die wahrscheinlichste Zahl lebender Keime bestimmt. Man geht davon aus, dass die niedrigen Verdünnungsstufen von schnell wachsenden, die höheren Verdünnungsstufen von abundanten Mikroorganismen dominiert werden (Großkopf *et al.,* 1998). Allerdings wiesen Jackson *et al.* (1998) in verschiedenen Verdünnungsstufen von Anreicherungskulturen mittels DGGE Mikroorganismen nach, die in der Umweltprobe nicht detektiert werden konnten. Es ist daher kritisch zu betrachten, ob abundante Vertreter isoliert wurden oder ob aufgrund der geringen Abundanz bestimmter Mikroorganismen eine Detektion mittels DGGE in der Standortprobe nicht möglich war (Muyzer *et al.,* 1996).

eine 200-fach höhere genetische Diversität der mikrobiellen Gemeinschaft fanden als die Diversität der isolierten Bakterien. Verdeutlicht wird diese Diskrepanz auch durch die Annahme von Rappé und Giovannoni (2003), dass nur 26 der 52 bisher identifizierten Phyla der Domäne der Bakterien kultivierte Vertreter aufweisen. Für eine geringe Kultivierungseffizienz wurden bisher vielfältige Gründe diskutiert:

Meist wird die Zahl der kultivierten Bakterien zur Gesamtzellzahl ins Verhältnis gesetzt. Da aber z.B. der Fluoreszenzfarbstoff DAPI Zellen unabhängig von ihrem metabolischen Zustand färbt, kann die **Anzahl lebender Zellen überschätzt** werden. Vor allem wenn man berücksichtigt, dass in marinen Habitaten der Anteil toter Zellen an der Gesamtzellzahl bis zu 70 % betragen kann (Luna *et al.,* 2002).

Ein schwieriges Unterfangen stellt die Nachbildung der physikochemischen *in situ* Bedingungen im Labor dar. Aufgrund der meist Substrat limitierten Bedingungen im Sediment wird ein „Substratschock" der Mikroorganismen im Kultivierungsansatz für den geringen Kultivierungserfolg verantwortlich gemacht (Straskrabova, 1983, Shiba *et al.,* 1995). Dieses Phänomen ist auch als **„Substrat beschleunigter Tod"** bekannt (Postgate und Hunter, 1963, Postgate und Hunter, 1964).

Weiterhin wird vermutet, dass einige Bakterien nicht kultiviert werden können, da sie mit **temperenten Viren** infiziert sind. Der lytische Zyklus der Bakteriophagen, die aquatischen Ökosystemen zahlreich vorhanden sind (Hara *et al.,* 1991). u.a. durch das Sonnenlicht (UV-C-Strahlung) oder durch chemische Substanzen (z.B. Wasserstoffperoxid, Antibiotikum Mitomycin C) induziert werden (Weinbauer und Suttle, 1996, 1999).

Der **programmierte Zelltod** wird in Zusammenhang mit verschiedenen mikrobiellen Prozessen beschrieben. Dazu zählen u.a. die Lyse der Mutterzelle während der Sporulation bei *Bacillus subtilis* sowie die Lyse vegetativer Zellen bei der Fruchtkörperbildung bei *Myxococcus xanthus* (Lewis, 2000).

Zellschäden können durch verschiedene Stressoren verursacht werden, wie durch sogenannte *reactive oxygen species* (ROS), UV-Strahlung, hohe oder niedrige Temperaturen, hohe Salzkonzentrationen. Dem kann durch die Synthese spezifischer Enzyme entgegengewirkt werden (Wood und Sorensen, 2001, Ehling-Schulz und Scherer, 1999, Vorob´eva, 2003). Um einen osmotischen Schock zu verhindern, akkumulieren Mikroorganismen *compatible solutes* (Osmolyte). Damit wird dem Ausstrom des Wassers aus der Zelle entgegengewirkt (Kempf und Bremer, 1998).

Die eben geschilderten Stressfaktoren können dazu führen, dass aktiv wachsende Bakterienzellen in einen **Ruhezustand** übergehen, in dem sie nur eine geringe metabolische Aktivität haben. In diesem Zustand, der reversibel ist (Stevenson *et al.,* 1977), können sie über einen längere Zeitraum verbleiben ohne zu wachsen und sich zu vermehren (Kaprelyants *et al.,* 1993). In Verbindung mit dem Ruhezustand von Zellen wird der ***viable but not culturable*** (***VBNC***)-Zustand (Bloomfield *et al.,* 1998) kontrovers diskutiert. Der Unterschied dieser zwei Zustände besteht darin, dass ruhende Zellen inaktiv aber totzdem „kultivierbar" sind, während Zellen im *VBNC*-Zustand metabolisch aktiv (bestimmt durch z.B. *direct viability count,* Reduktion von Tetrazoliumsalz usw.), aber nicht „kultivierbar" sind (Kell *et al.,* 1998). Allerdings ist es umstritten, ob ein Bakterium, was noch nicht kultiviert wurde, unkultivierbar ist oder ob man es bisher einfach noch nicht kultivieren konnte.

Auch **interspezifische Wechselwirkungen** können sich negativ auf die Kultivierung auswirken. Manche Mikroorganismen leben in einer syntrophen Assoziation (siehe Kapitel 2) und können auch nur unter diesen Bedingungen kultiviert werden.

Aufgrund dieser Einschränkungen ist man ständig darum bemüht, Kultivierungsmethoden zu modifizieren und effizienter zu gestalten. So werden u.a. „natürliche" Medien durch Aufarbeitung von Habitatproben hergestellt (Vester und Ingvorsen, 1998), natürlicher Habitate nachgebildet (Kaerberlein *et al.,* 2002), Signalmolekülen zugegeben (Bruns *et al.,* 2002), Zellen in Geltropfen eingeschlossen(Zengler *et al*., 2002) oder der einzelne Bakterienzellen mittels Mikromanipulator isoliert (Fröhlich und König, 2000). Bei der Untersuchung der Isolate sollte immer auch die Frage nach deren Abundanz und Beitrag zur Gesamtaktivität des Systems berücksichtigt werden. In vielen Untersuchungen konnte nachgewiesen werden, dass die kultivierten Arten am natürlichen Standort nicht immer die häufigsten sind (Eilers *et al*., 2000, Felske *et al.,* 1999), so dass deren Bedeutung für den Substratumsatz *in situ* fraglich ist.

3.2 Molekularbiologische Methoden – Vor und Nachteile

Das Problem der eingeschränkten Detektion und ungenauen Klassifizierung von Bakterien wurde mit der Entwicklung molekularbiologische Methoden gelöst, mit denen man mikrobielle Gemeinschaften *in situ* allein über deren 16S rRNA Gene analysieren kann. Bereits 1965 wiesen Zuckerandl und Pauling darauf hin, dass Biomoleküle zur Untersuchung der Evolutionsgeschichte herangezogen werden können. Aber erst in den

70er Jahren des letzten Jahrhunderts wurde die ribosomale RNA als Werkzeug zur Aufklärung der Phylogenie innerhalb der Prokaryonten eingeführt. Woese und Fox (1977) erkannten die Vorteile der rRNA, wie universelles Auftreten, hohe Konservierung, angemessene Variabilität und minimalen lateralen Gentransfer. Über einen Sequenzvergleich der 16S rRNA Gene wurde eine relativ stabile Klassifizierung der bekannten Bakterienarten sowie die Einordnung neuer Arten ermöglicht.

Im Laufe der Zeit wurden verschiedene molekularbiologische Methoden entwickelt, die meist auf der Ebene der 16S rRNA angewendet wurden. Häufig wurden diese Methoden bereits im medizinischen Bereich eingesetzt und für die Anwendung in der mikrobiellen Ökologie modifiziert. Der Vorteil dieser Methoden liegt vor allem in der Zeitersparnis und der Unabhängigkeit von Kultivierungserfolgen. Die Aufklärung der Diversität einer mikrobiellen Gemeinschaft kann über Sequenzvergleiche der 16S rDNA erfolgen. Dazu werden u.a. Klonbibliotheken erstellt, die mittlerweile nicht mehr auf die Ebene der 16S rRNA Gene beschränkt sind. Klonbibliotheken können auch andere funktionelle Gene enthalten, die zur Abschätzung des metabolischen Potentials unkultivierter Bakterien herangezogen werden (Béjá *et al.,* 2000).

Mikrobielle Gemeinschaften können auch durch die sequenzspezifische Auftrennung von DNA-Fragmenten mit der Denaturierenden Gradienten Gelelektrophorese (DGGE) untersucht werden (Muyzer *et al.,* 1993). Einen andere Möglichkeit stellt die Restriktion der 16S rDNA dar, wobei die DNA-Fragmente nach ihrer Länge aufgetrennt werden (Restriktions-Fragment-Längen-Polymorphismus, RFLP, Marsh, 1999). Diese *Fingerprint*-Methoden ermöglichen auch eine Identifizierung der Bakterien durch anschließende Sequenzierung der in den Banden enthaltenen DNA-Fragmente. Die dabei erhaltenen Sequenzen können in Genbanken hinterlegt und mit bereits vorhandenen Sequenzen verglichen werden, um so die mikrobielle Gemeinschaft am Standort zu charakterisieren.

Aufgrund der großen Zahl von 16S rRNA Gensequenzen konnten spezifische Oligonukleotidsonden entwickelt werden, die u.a. bei der Fluoreszenz-*in-situ*-Hybridisierung (FISH) zum Einsatz kommen (Amann *et. al.,* 1995). Die Sonden, die mit einem Fluoreszenzfarbstoff markiert sind, binden dabei an die 16S rRNA innerhalb der Zellen. Die Organismen können somit bei Fluoreszenzanregung mikroskopisch sichtbar gemacht werden. Damit ist ein direkter und spezifischer Nachweis von Organismen im Habitat möglich (DeLong *et al.,* 1989, Amann *et al.,* 1990). Allerdings wird die Sensitivität dieser Methode durch die Zahl der Ribosomen beschränkt (Amann *et al.,*

1995). Bei der Weiterentwicklung der Methode (Catalyzed-Reporter-Deposition (CARD)-FISH) wird die Signalintensität unabhängig vom Ribosomenanteil und damit der metabolischen Aktivität erhöht (Speel *et al.,* 1999, Pernthaler *et al.,* 2002).

Die anfängliche Euphorie, mit der die neuen Techniken aufgenommen wurden, ist dem bewussteren Umgang mit den molekularbiologischen Methoden gewichen, da man erkennen musste, dass jede dieser Methoden systematische Fehler besitzt. Zunächst stellt schon die quantitative Gewinnung von DNA aus Umweltproben ein Problem dar, da bereits die Extraktion durch die Adsorption von DNA an Sedimentpartikel erschwert werden kann und die Effizienz der Aufschlussmethoden von Bakterienzellen von deren Zellwandaufbau beeinflusst wird (Head *et al.,* 1998, Frostegård *et al.,* 1999). Meist geht der molekularbiologischen Analyse eines Zielgens dessen Amplifikation mittels Polymerase-Kettenreaktion (PCR) voran. Allerdings werden dabei bestimmte Sequenzen bevorzugt amplifiziert oder es können Artefakte entstehen (Suzuki und Giovannoni, 1996, von Wintzingerode *et al.,* 1997). Mittels PCR-DGGE werden überwiegend abundante Vertreter der mikrobiellen Gemeinschaft erfasst (mit einem Anteil von mehr als 1 % an der Gesamtgemeinschaft; Muyzer *et al.,* 1996).

3.3 Bestimmung mikrobieller Aktivitäten

Die Kultivierung von Bakterien und Erfassung ihrer physiologischen Eigenschaften im Labor ermöglichen nur eine Abschätzung potentieller Aktivitäten. Diese sagen aber nichts über die *in situ* Aktivität im Habitat aus. Mittlerweile stehen vielfältige Methoden zur Verfügung, die dies ermöglichen.

Zum Einen lassen sich Umsatzraten eines radioaktiv markierten Substrates untersuchen. Dieses wird Proben zugesetzt und anschließend wird die Radioaktivität des Stoffwechselproduktes gemessen. Auf diese Weise lassen sich z.B. die Aktvität Sulfat reduzierender und heterotropher Bakterien abschätzen. Sulfatreduktionsraten können aus der Aktivität des gebildeten ^{35}S-Sulfids ermittelt werden, nachdem ^{35}S-Sulfat mit bekannter Aktivität in die Umweltproben injiziert wurde (Fossing und Jørgensen, 1989, Kallmeyer *et al.,* 2004). Um auf das „heterotrophe Potential“ einer Bakteriengemeinschaft zu schließen, werden ^{14}C markierte organische Substrate Umweltproben zugesetzt und die Aktivität des gebildeten ^{14}C-Kohlendioxid wird bestimmt (Hoppe, 1978, Novitsky und Kepkay, 1981). Weiterhin kann die bakterielle Produktion ermittelt werden, indem die Inkorporation von ^{3}H-Thymidin in die DNA oder die Inkorporation von ^{3}H-Leucin in Proteine untersucht wird (Fuhrman und Azam, 1982, Kirchman *et al.,* 1985). Radioisotope

werden auch bei der Mikroautoradiographie eingesetzt. Dabei wird der Anteil an Bakterienzellen, der ein bestimmtes radioaktiv markiertes Substrat aufgenommen hat, quantifiziert (Hoppe, 1976). In Kombination mit FISH[2] (siehe vorhergehendes Kapitel) kann die Aktivität bzw. die Substrataufnahme phylogenetischen Gruppen zugeordnet werden. Daurch ist eine Einschätzung möglich, ob und inwieweit diese zur Gesamtaktivität *in situ* beitragen.

Eine weiter Möglichkeit zur Untersuchung mikrobieller Stoffumsätze stellt die Bestimmung des Verhältnisses stabiler Isotope eines Elementes dar. Die meisten biochemischen Reaktionen sind mit einer Isotopenfraktionierung verbunden. Schwerere Isotope werden gegenüber leichteren diskriminiert, wodurch sich das Isotopenverhältnis in den Endprodukten im Vergleich zum Substrat ändert (Madigan *et al.,* 2000). Durch Isotopenverhältnisse erhält man Hinweise auf die Entstehung einer Verbindung, ob sie geochemischen Ursprunges ist oder auf mikrobielle Aktivitäten zurückgeführt werden kann (Kaplan und Rittenberg, 1964, Rees, 1973).

Eine sensitive Methode für die Untersuchung mikrobieller Aktivitäten stellt die Analyse von Exoenzymaktivitäten dar. Es ist bekannt, dass Bakterien die Fähigkeit besitzen, verschiedenste polymere organische Kohlenstoffverbindungen mittels spezifischer Exoenzyme abzubauen (Chrost, 1991). Bakterien können nur organische Verbindungen mit einem Molekulargewicht < 600 Da inkorporieren (Weiss *et al.,* 1991) Deshalb stellt die extrazelluläre enzymatische Hydrolyse einen entscheidenden Prozess beim Abbau organischen Materials dar (Meyer-Reil, 1991). Da die Produktion von extrazellulären Hydrolasen meist ein Substrat induzierter Prozess ist (Priest, 1984), kann die potentielle hydrolytische Aktivität eines spezifischen Enzyms Aufschluss über Verfügbarkeit des jeweiligen organischen Substrates geben. Um Enzymaktivitäten zu messen, werden künstliche Substrate verwendet, bei deren enzymatischer Hydrolyse fluoreszierende Substrate freigesetzt werden. Häufig werden Methylumbelliferon (MUF) oder 7-Aminomethylcoumarin (MCA) eingesetzt (Hoppe, 1993).

Aufgrund der beschriebenen Vor- und Nachteile der jeweiligen Methoden sollten für die Analyse von Bakteriengemeinschaften Kultivierungsansätze mit molekularbiologischen Methoden und Aktivitätsmessungen kombiniert werden. Diese

[2] Dafür gibt es verschiedenen Bezeichnungen wie MAR-FISH (microautoradiogarphy-fluorescence *in situ* hybridization; Lee *et al.,* 1999), STAR-FISH (substrate tracking autoradiogarphy-fluorescence *in situ* hybridization; Ouverney und Fuhrman, 1999) oder MICRO-FISH (microautoradiogarphy-fluorescence *in situ* hybridization; Cottrell und Kirchman, 2000), wobei sich diese Methoden nur geringfügig unterscheiden.

Herangehensweise wird immer häufiger dazu angewendet (Bruns *et al.,* 2002, Jaspers *et al.*, 2004, Inagaki *et al.,* 2003, Mußmann *et al.,* 2005).

4. Zielsetzung der Arbeit

Diese Arbeit gliedert sich ein in das Projekt der Forschergruppe „BioGeoChemie des Watts“, dessen Schwerpunkt in der Untersuchung der biologischen, chemischen und physikalischen Wechselwirkungen und deren Bedeutung für die Entwicklung und Strukturierung von Watt-Systemen liegt. Mikrobiologisch-geochemische Untersuchungen von Wattsedimenten waren bisher meist auf die obersten 40 cm begrenzt (Böttcher *et al.,* 1997, Llobet-Brossa *et al.,* 2002). Die Kenntnisse aus den Analysen mikrobieller Gemeinschaften in großen Sedimenttiefen (D´Hondt *et al.*, 2002, 2004, Parkes *et al.,* 2005) lassen erwarten, dass es in den tiefen Wattsedimenten noch erhebliche mikrobielle Aktivitäten gibt. Daher liegt das Hauptaugenmerk des Teilprojektes, dem diese Arbeit angegliedert ist, in der Untersuchung mikrobieller Prozesse in den tieferen Wattsedimenten und der Ermittlung ihres Beitrages zu Stoffflüssen.

Daraus ergaben sich die dieser Arbeit zugrunde liegenden zwei Themenkomplexe. Zum Einen bestand das Ziel dieser Arbeit in der Charakterisierung der mikrobiellen Gemeinschaften in Sedimenten des Wattenmeeres der südlichen Nordsee. Mit Hilfe verschiedener selektiver Medien sowie verschiedener Kultivierungsmethoden sollten Mikroorganismen unterschiedlicher physiologischer Gruppen isoliert werden. Außerdem sollte die Effektivität der angewendeten Methoden durch einen molekularbiologischen Vergleich zwischen Isolaten und Sediment eingeschätzt werden. Die entsprechenden Ergebnisse sind in Kapitel II dargestellt. Anschließend sollten die Isolate bezüglich ihrer Anpassung an ihren Lebensraum bzw. hinsichtlich ihrer Physiologie untersucht und phylogenetisch klassifiziert werden (Kapitel II und III), um sowohl die beiden Standorte als auch die Kultivierungsergebnisse mit denen der molekularbiologischen Studien (Wilms *et al.,* 2006a, b) zu vergleichen. (Kapitel II).

Zum Anderen spielte die Untersuchung der mikrobiellen Aktivitäten im Hinblick auf ihre vertikale Ausdehnung sowie der Einfluss mikrobieller Stoffumsetzungen auf chemische Gradienten im Sediment eine Rolle. Eine ausführliche Darstellung mikrobieller Aktivtitätsparameter (Exoenzymaktivitäten und Sulfatreduktionsraten) sowie Sedimentparameter (Lithologie, chemische Sediment- und Porenwasserdaten) findet sich Kapitel IV.

Kapitel II bis IV sind für Veröffentlichung in internationalen Zeitschriften gedacht, weshalb sie in Englisch verfasst wurden. Kapitel II wurde bereits veröffentlich, Kapitel III wurde eingereicht und Kapitel IV liegt als Manuskript vor. Tabellen und Abbildungen wurden in den laufenden Text der Artikel eingefügt und entsprechend nummeriert. Die Literaturverzeichnisse befinden sich immer am Ende des jeweiligen Kapitels.

Kapitel II

Microbial diversity in coastal subsurface sediments: a cultivation approach using various electron acceptors and substrate gradients

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Microbial Diversity in Coastal Subsurface Sediments: a Cultivation Approach Using Various Electron Acceptors and Substrate Gradients

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Microbial communities in coastal subsurface sediments are scarcely investigated and have escaped attention so far. But since they are likely to play an important role in biogeochemical cycles, knowledge of their composition and ecological adaptations is important. Microbial communities in tidal sediments were investigated along the geochemical gradients from the surface down to a depth of 5.5 m. Most-probable-number (MPN) series were prepared with a variety of different carbon substrates, each at a low concentration, in combination with different electron acceptors such as iron and manganese oxides. These achieved remarkably high cultivation efficiencies (up to 23% of the total cell counts) along the upper 200 cm. In the deeper sediment layers, MPN counts dropped significantly. Parallel to the liquid enrichment cultures in the MPN series, gradient cultures with embedded sediment subcores were prepared as an additional enrichment approach. In total, 112 pure cultures were isolated; they could be grouped into 53 different operational taxonomic units (OTU). The isolates belonged to the *Proteobacteria*, "*Bacteroidetes*," "*Fusobacteria*," *Actinobacteria*, and "*Firmicutes*." Each cultivation approach yielded a specific set of isolates that in general were restricted to this single isolation procedure. Analysis of the enrichment cultures by PCR and denaturing gradient gel electrophoresis revealed an even higher diversity in the primary enrichments that was only partially reflected by the culture collection. The majority of the isolates grew well under anoxic conditions, by fermentation, or by anaerobic respiration with nitrate, sulfate, ferrihydrite, or manganese oxides as electron acceptors.

The Wadden Sea, located on the southern shores of the North Sea, is a highly productive tidal flat ecosystem. It is characterized by a high nutrient input from the land as well as from the open sea. This nutrient supply stimulates intense benthic primary production, fuelling microbial activities in the upper sediment layers. As a result, oxygen is depleted within the uppermost few millimeters of the sediment. While nitrate, iron oxihydroxides, and manganese oxides are important electron acceptors for the degradation of organic matter within the upper anoxic horizons of the sediment, sulfate reduction becomes the predominant anaerobic degradation process in deeper layers.

To date, microbiological investigations dealing with intertidal sediments have generally been restricted to the uppermost 10 to 40 cm (20, 27, 28, 31, 55), the zone of the highest microbial activities. However, in the last decade, the discovery of the deep biosphere within marine sediments and its extension to several hundreds of meters below the surface has indicated that a major part of the microbial biosphere might be present in subsurface sediments (35, 51). Yet only a few investigations have dealt with coastal subsurface sediments, and these have focused on geochemical parameters and biogeochemical activities and have demonstrated microbial activities even at a depth of some meters (7, 23).

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Although in most cases cultivation-based methods failed to detect the most abundant members of microbial communities in situ (2, 18, 49), we have chosen a cultivation-based approach, because it offers the opportunity to isolate indigenous microorganisms in pure culture. In turn, microorganisms that are active in situ can be identified using molecular biological methods such as microautoradiography in combination with fluorescence in situ hybridization (12, 30) or stable isotope probing (5, 37). However, these methods cannot reveal the whole spectrum of physiological capacities that are necessary to understand the ecology of a single bacterial species. Hence, for the investigation of microbial adaptations to environmental conditions, pure cultures remain crucial (22).

Intertidal flats on the German North Sea coast were studied down to a depth of 5.5 m below surface in a combined and detailed microbiological (this study) and molecular biological (R. Wilms et al., submitted for publication) approach that was complemented by geochemical characterization of the sediments (E. Freese et al., submitted for publication). In the present study, two different cultivation approaches were chosen for their practicability. A most-probable-number (MPN) approach was used for the quantitative assessment of microbial communities using a defined medium with many different carbon compounds, each at a low concentration, in combination with different electron acceptors targeting different physiological groups. As an alternative enrichment technique, undisturbed sediment sections were placed in substrate gradients and were used for enrichment and isolation of microorganisms as well. This approach should help to avoid the potential substrate shock (46) and minimize disturbance during sampling.

MATERIALS AND METHODS

Study area and sampling. Sediment samples were taken from tidal flats located close to the island of Spiekeroog in the East Frisian Wadden Sea on the German North Sea coast (19). Two sites were analyzed: Neuharlingersieler Nacken (NSN) (53°43.270′N, 7°43.718′E) and Gröninger Plate (GP) (53°43.638′N, 07°45.960′E). At low tide, 6-m-long aluminum tubes were driven into the sediment using a vibrocorer provided by the Senckenberg Institute (Department for Marine Research, Wilhelmshaven, Germany). After retrieval, the aluminum liners were cut longitudinally, leaving behind a potentially contaminated surface. By use of a sterile spatula, the uppermost few millimeters of the freshly exposed sediment surface were removed. Sediment samples were taken aseptically from the undisturbed sediment underneath using sterile plastic syringes with cut-off tips.

Physical and chemical parameters. Temperature was measured at the sediment surface and at the bottom of the core immediately after sampling. Concentrations of oxygen in pore water were determined by means of needle electrodes (Microscale Measurements, The Hague, The Netherlands) (38). Sediment density and porosity were determined according to Bak (3). Pore water chloride and sulfate concentrations were measured by ion chromatography with conductivity detection (Sykam, Gilching, Germany) (40).

Total cell counts. Sediment samples were fixed by the addition of sterile glutaric dialdehyde (filtered with a 0.2-μm-pore-size filter; final concentration, 2%) and were stored at 4°C in the dark. Prior to analysis, sterile-filtered Tween 80 (final concentration, 0.01%) was added to the fixed sediment slurries and the samples were ultrasonicated (3 times, for 10 s each time). An aliquot of the fixed sediment slurry (5 to 10 μl) was diluted 1,000-fold in particle-free sterile PBS buffer (0.9 g NaCl, 15 mM sodium phosphate buffer, pH 7.4), thoroughly shaken, and filtered through a white polycarbonate membrane (pore size, 0.2 μm; diameter, 25 mm; Anodisc 25; Whatman, Maidstone, United Kingdom). Staining with 4′,6′-diamidino-2-phenylindole (DAPI) and counting were performed according to Süß et al. (48).

Growth media. For preparation of sediment slurries, MPN series, and gradient tubes and for the isolation of pure cultures, an artificial seawater medium was used. The anoxic medium used contained the following components: NaCl (24.3 $g \cdot liter^{-1}$), $MgCl_2 \cdot 6H_2O$ (10.0 $g \cdot liter^{-1}$), $CaCl_2 \cdot 2H_2O$ (1.5 $g \cdot liter^{-1}$), KCl (0.66 $g \cdot liter^{-1}$), Na_2SO_4 (4.0 $g \cdot liter^{-1}$), KBr (0.1 $g \cdot liter^{-1}$), H_3BO_3 (0.0025 $g \cdot liter^{-1}$), $SrCl_2 \cdot 6H_2O$ (0.04 $g \cdot liter^{-1}$), NH_4Cl (0.021 $g \cdot liter^{-1}$), KH_2PO_4 (0.0054 $g \cdot liter^{-1}$), and NaF (0.003 $g \cdot liter^{-1}$). The medium was supplemented with 1 $ml \cdot liter^{-1}$ of the trace element solution SL 10 (54) and 0.2 $ml \cdot liter^{-1}$ of a selenite and tungstate solution (53). After autoclaving, the medium was cooled under an atmosphere of N_2–CO_2 (80:20, vol/vol). Per liter of medium, 10 ml of a vitamin solution (4) and 30 ml of a CO_2-saturated sodium bicarbonate solution (1 $mol \cdot liter^{-1}$) were added from sterile stocks. Anoxic medium was reduced by addition of a sterile sodium sulfide solution (final concentration, 1.2 $mmol \cdot liter^{-1}$) and a sterile acid ferrous chloride solution (final concentration, 0.5 $mmol \cdot liter^{-1}$). If necessary, the pH was adjusted to 7.2 to 7.4 by addition of sterile HCl or Na_2CO_3.

For oxic incubations, a slightly modified medium was used. Instead of bicarbonate and CO_2, the medium was buffered with HEPES (2.38 $g \cdot liter^{-1}$) and the pH of the oxic medium was adjusted to 7.2 to 7.4 with NaOH prior to autoclaving. After autoclaving, the oxic medium was cooled down under air and supplemented with vitamins and sodium bicarbonate (0.2 $g \cdot liter^{-1}$) as described above.

For tests on aerobic growth and for the maintenance of pure cultures, a dilute yeast extract-peptone-glucose (YPG) medium was used. It consisted of the HEPES-buffered oxic seawater described above amended with yeast extract (0.03 $g \cdot liter^{-1}$), peptone (0.06 $g \cdot liter^{-1}$), sodium DL-lactate (1 $mmol \cdot liter^{-1}$), glucose (1 $mmol \cdot liter^{-1}$), vitamins, and sodium bicarbonate (0.2 $g \cdot liter^{-1}$).

Substrates for MPN series. As an electron donor and a carbon source for all MPN series, a mixture of monomeric compounds was used. This mixture contained the 20 common L-amino acids, the short-chain fatty acids formate, acetate, propionate, butyrate, valerate, and caproate, the alcohols methanol, ethanol, *n*-propanol, and *n*-butanol, and DL-malate, fumarate, succinate, DL-lactate, glycerol, and glucose (0.1 $mmol\ liter^{-1}$ each).

For the assessment of different physiological groups, the monomer mix was combined with different electron acceptors. (i) For aerobic microorganisms, the oxic medium was used. (ii) For anaerobic bacteria, including sulfate reducers, the anoxic sulfate-containing standard medium was used. (iii) For fermenting microorganisms, an anoxic but sulfate-free medium was used. (iv) For manganese-reducing bacteria, manganese oxide (5 mM) was added to the anoxic medium. (v) For iron-reducing bacteria, amorphous ferric hydroxide (5 mM) was added to the anoxic medium.

For the preparation of amorphous manganese oxides, 2 g H_2O_2 (35%) was added to 50 ml of a 1 M $MnCl_2$ solution and the reaction was started by the dropwise addition of 10 ml of 4 M NaOH. The assay mixture was centrifuged (20,000 × *g*) for 10 min. The supernatant was collected separately and again treated with H_2O_2 and NaOH. Finally, the particulate manganese oxides were washed twice, resuspended in distilled water to a final volume of 50 ml, and autoclaved. The resulting manganese oxides represented a mixture of MnOOH and MnO_2.

Amorphous ferrihydrite was prepared by careful titration of 8.0 g $FeCl_3 \cdot 6H_2O$ dissolved in 70 ml of water with 4 M NaOH to a final pH of 7.0. The precipitate was washed twice with distilled water and resuspended in distilled water to a final concentration of 400 $mmol \cdot liter^{-1}$.

Substrates for gradient tubes. For gradient cultures, two different substrate combinations were used: (i) TCA, a mixture of 20 common L-amino acids (final concentration, 0.25 mM each) and DL-malate, fumarate, succinate, and DL-lactate (final concentration, 2 mM each) and (ii) ALC, a mixture of the short-chain fatty acids formate, acetate, propionate, butyrate, valerate, and caproate (final concentration, 2 mM each) as well as the alcohols methanol, ethanol, *n*-propanol, and *n*-butanol (final concentration, 2 mM each). All concentrations were calculated by considering the total end volume of the gradients.

Preparation and incubation of MPN series. Viable counts were determined by the MPN method. Sediment slurries were prepared immediately after sampling by diluting in anoxic seawater and were used as an inoculum. Subsamples of these slurries were pasteurized (70°C, 15 min) and were used for the determination of viable counts of spores.

MPN series for anaerobic microorganisms were prepared in an anaerobic hood using polypropylene deep-well plates (Beckman, Fullerton, CA) containing 900 μl of medium per well. For the deeper layers, MPN series with three parallels and six dilution steps were prepared (48). For the surface layers, where higher numbers were expected, MPN series with five parallels and eight dilutions were made. As an inoculum, 100 μl of sediment slurry was added to each well of the first dilution and diluted in 10-fold steps. After inoculation, the plates were covered with sterile lids (CAPMAT; Beckman, Fullerton, CA) sealing each well separately. The MPN plates were put into gas-tight plastic bags equipped with a gas-generating and catalyst system for anoxic conditions (Anaerocult C mini; Merck, Darmstadt, Germany).

MPN series for aerobic microorganisms were prepared in microtiter plates (Corning, New York, NY) in a microbiological cabinet. Every well contained 180 μl of medium. MPN series for aerobes were inoculated with 20 μl of sediment slurry. After inoculation, the plates were covered with sterile lids (Corner Notch Lid; Corning, New York, NY) and wrapped with Parafilm to avoid water loss by evaporation.

On each plate four dilution series were left without inoculum as a control. All MPN series were incubated for 12 weeks at 20°C. Growth was checked by epifluorescence microscopy after staining with DAPI. MPN counts were calculated according to De Man (14). Ninety-five percent confidence levels obtained in the present study were in the range of three to five times and one-third to one-fifth of the MPN values.

Preparation of gradient cultures. We have established enrichment cultures using undisturbed sediment samples embedded in substrate gradients. These offer two main advantages. The microorganisms remain in their habitual surroundings, and due to the diffusion from the bottom of the tube, substrate concentrations increase only slowly. This helps to avoid a substrate shock (46) but still supplies enough substrate to sustain visible growth.

Substrate gradients were prepared as follows. The substrate was placed at the bottom of a glass tube, and 1 ml of 4% agar was added. After cooling and solidifying, this substrate reservoir was overlaid with 6 ml of anoxic substrate-free mineral medium mixed with 3 ml of 4% agar. This agar-solidified medium overlay served as a spacer between the substrate reservoir and the embedded sediment. The spacer was in turn overlaid with 5 ml anoxic substrate-free mineral medium. To this medium 2 ml of 4% agar was added, and the tubes were kept at 50°C. After a sample of sediment (1 cm^3, taken by use of syringes with cut-off tips) was added, the tube was immediately placed in ice-cold water in order to minimize a potential heat shock. The headspace of the tubes was flushed with N_2–CO_2 (80:20, vol/vol), and the tubes were sealed with butyl rubber stoppers.

Isolation of pure cultures. Pure cultures were isolated from the highest positive dilutions of the MPN series. All subculturing and isolation were performed using the same media. Agar plates were used for aerobes, and deep agar dilution series were used for anaerobes. Subculturing and isolation from gradient cultures were also done in gradient tubes; the agar-solidified top layer was replaced by liquid culture or deep agar dilution series, respectively. The purity of cultures was tested by microscopy and denaturing gradient gel electrophoresis (DGGE) as described by Süß et al. (48).

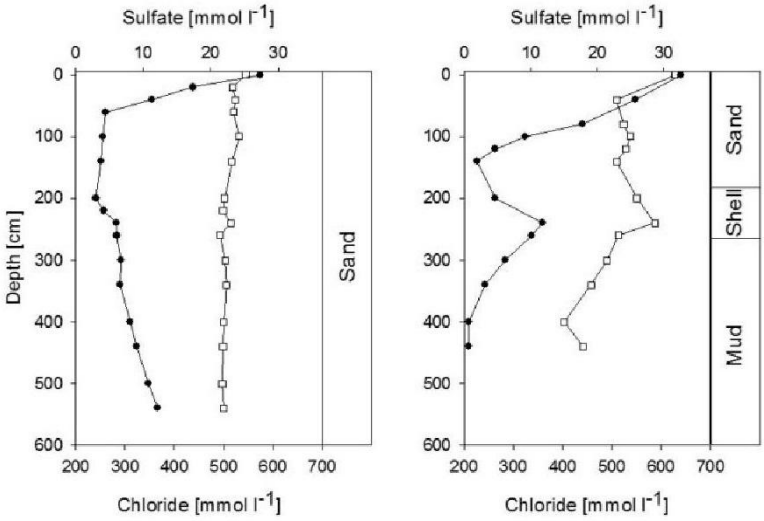

FIG. 1. Depth profiles of sulfate (●) and chloride (□) and major lithological facies in sediments from the Gröninger Plate (left) and Neuharlingersieler Nacken (right) sites.

Physiological characterization. At least one representative strain of each operational taxonomic unit (OTU) was characterized physiologically. For tests on anaerobic growth, a slightly modified sulfate-free anoxic seawater medium containing resazurin (0.25 mg · liter^{-1}) was used. After autoclaving, the medium was reduced by the addition of sterile sodium dithionite until the redox indicator turned colorless. Fermentative growth was tested with glucose (5 mmol · liter^{-1}), an amino acid mixture (final concentration, 2 mmol · liter^{-1}), and DL-lactate (10 mmol · liter^{-1}) as single substrates, but also with anoxic YPG and a sulfate-free anoxic monomer medium. Tests for anaerobic respiration were performed with either sodium acetate or lactate (10 mmol · liter^{-1}) as an electron donor in combination with one of the following electron acceptors: sulfate (28 mmol · liter^{-1}), nitrate (10 mmol · liter^{-1}), $Fe(OH)_3$ (20 mmol · liter^{-1}), or MnO_2 (10 mmol · liter^{-1}). Sulfide formation was checked as described by Widdel (52). Nitrite and ammonium were analyzed photometrically (24). Tests were prepared in an anaerobic cabinet as described for the MPN series. All assays were prepared in duplicate and were incubated at 20°C for at least 2 weeks in the dark. In some cases when the results were inconsistent, experiments were repeated in completely filled screw-cap tubes. Growth was checked by fluorimetry (33a). From each well 200 μl was transferred to a black microplate (Nunc 237108; VWR International, Darmstadt, Germany), and 50 μl of a Sybr Green I (Molecular Probes, Leiden, The Netherlands) solution (2,000-fold dilution in TE buffer [200 mM Tris and 50 mM EDTA, pH 8]) was added. Fluorescence was measured after a 12-h incubation in the dark at 4°C using a fluorescence microplate reader (BMG Labtechnologies, Offenburg, Germany). Growth was scored as positive if at least fivefold higher fluorescence than in substrate-free controls was detected.

Isolation of nucleic acids from pure cultures, PCR, and sequencing. For DNA extraction, cultures were centrifuged and resuspended in 50 μl sterile distilled water, and 100 μl Tris-HCl (10 mM, pH 8.0) and 100 μl lysozyme (0.8 mg · ml^{-1}) were added. The assay mixtures were incubated at room temperature for 10 min, and after addition of 40 μl of a sodium dodecyl sulfate (SDS) solution (9.6 ml of 20% SDS added to 2.4 ml of 0.5 M sodium acetate [pH 7.5] and 66.4 ml of distilled water) and 60 μl of 3 M sodium acetate, the suspension was incubated for 1 h on ice, followed by three freeze-thaw cycles. During each cycle the suspension was frozen at −80°C for 3 min and immediately boiled for 3 min. DNA was purified from the lysates using phenol-chloroform extraction followed by ethanol precipitation.

Almost-complete 16S rRNA gene fragments were amplified by PCR using oligonucleotide primers 8f (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492r (5′-GGT TAC CTT GTT ACG ACT T-3′). PCR products were purified using the QIAquick PCR purification kit (QIAGEN, Hilden, Germany). Sequencing was performed using the DYEnamic Direct Cycle Sequencing kit (Amersham Biosciences, Freiburg, Germany) as described previously (48).

DGGE analysis of sediment and enrichment cultures. DNA for DGGE analysis was extracted from original sediments, from sediment embedded in gradient tubes, and from liquid MPN cultures. For extraction, 500 mg sediment or 100 μl liquid culture plus 600 μl TE-buffer (10 mM Tris and 1 mM EDTA, pH 7.5) was transferred into 1.7-ml tubes (Sorenson Bioscience Inc., West Salt Lake City, UT) containing 500 mg of 0.1-mm zirconia-silica beads (Biospec Products, Bartlesville, OK) and 500 μl of an SDS mixture. The samples were homogenized twice, for 1 min each time, on a bead beater (Biospec Products, Bartlesville, OK) followed by centrifugation at 15,000 rpm for 15 min at 4°C in a microcentrifuge (Eppendorf, Hamburg, Germany). DNA was purified from the extracts using phenol-chloroform extraction followed by ethanol precipitation. DNA fragments were amplified using primers GC357f and 907r. DGGE was carried out as described by Süß et al. (48) using an INGENYphorU-2 system (Ingeny, Leiden, The Netherlands) and a 6% (wt/vol) polyacrylamide gel containing denaturant gradients of 50 to 70% for separation of PCR products. The gels were stained for 2 h with 1× SybrGold (Molecular Probes, Leiden, The Netherlands) in 1× Tris-acetate-EDTA buffer and washed for 20 min in distilled water prior to UV transillumination. Individual DNA bands were excised from the gel with sterile scalpels, and the DNA was eluted into 50 μl molecular-grade water (Eppendorf, Hamburg, Germany) by incubation at 4°C. For subsequent sequence analysis, PCR products of DGGE bands were purified using the QIAquick PCR purification kit (QIAGEN, Hilden, Germany) and sequenced as described above.

Phylogenetic analysis. The partial 16S rRNA sequences of the isolates and the DGGE bands were compared to those in GenBank using the BLAST function (1) and were aligned with each other and the closest relatives using ClustalX (50). Distance matrices were calculated using the dnadist program within the PHYLIP 3.6 package (J. Felsenstein, Department of Genome Science, University of Washington [http://evolution.genetics.washington.edu/phylip.html]) by applying the algorithm of Jukes and Cantor. Isolates with sequence similarities of at least 97% (44) were grouped into a single OTU.

Nucleotide sequence accession numbers. The sequences obtained in this study are available from EMBL under accession numbers AJ786027 to AJ786110 for the isolates and AJ889123 to AJ889184 for the DGGE bands.

RESULTS

Chemical and physical parameters. Sediment samples were taken from the two sampling sites NSN and GP in June 2002. Cores were 450 cm (NSN) and 550 cm (GP) long. Sediments at the two sampling sites differed with respect to their bulk parameters. At the NSN site, sand dominated along the upper

TABLE 1. MPN counts obtained with oxic and anoxic media supplemented with different electron acceptors of sediments from the Neuharlingersieler Nacken and Gröninger Plate sites

Site and sediment depth (cm)	Total cell count (g^{-1})[a]	MPN count (g of sediment^{-1})[a] obtained by:				
		Oxic incubation	Anoxic incubation			
			Sulfate free	Sulfate	Iron hydroxide	Manganese oxide
NSN						
0.5	8.06×10^8	3.96×10^5	3.60×10^6	3.60×10^5	7.21×10^7	7.21×10^7
5	6.52×10^8	3.27×10^4	4.16×10^4	2.97×10^4	1.49×10^8	5.95×10^7
50	1.93×10^8	1.38×10^5	1.60×10^4	6.07×10^4	3.04×10^7	3.04×10^7
100	2.50×10^8	6.37×10^2	3.19×10^5	1.27×10^4	3.51×10^4	6.69×10^4
200	ND	1.23×10^2	ND	ND	2.71×10^6	1.23×10^5
300	1.53×10^7	1.37×10^2	ND	ND	ND	ND
400	ND	8.76×10^1	ND	ND	2.63×10^3	4.38×10^2
450	6.46×10^7	9.19×10^1	ND	ND	ND	ND
GP						
0.5	1.39×10^9	1.25×10^6	5.49×10^4	5.49×10^6	2.75×10^8	4.99×10^6
5	5.85×10^8	3.14×10^5	2.23×10^2	1.71×10^5	2.00×10^7	2.00×10^5
50	8.27×10^7	4.00×10^5	1.53×10^4	3.64×10^5	1.82×10^5	7.27×10^5
100	1.27×10^8	6.10×10^4	3.05×10^4	1.04×10^3	4.57×10^4	4.57×10^5
200	ND	3.32×10^4	4.22×10^2	8.44×10^3	9.04×10^1	1.21×10^2
300	6.13×10^7	2.29×10^2	1.48×10^4	8.65×10^2	ND	ND
400	ND	2.33×10^3	3.62×10^3	5.69×10^3	ND	ND
500	6.46×10^7	1.22×10^3	3.04×10^4	1.03×10^3	5.70×10^2	1.14×10^3
550	4.65×10^7	4.27×10^2	2.85×10^3	9.69×10^2	ND	ND

[a] ND, not determined.

160 cm, followed by a porous layer of sand and remains of a buried mussel (*Mytilus edulis* Linné) bed between depths of 160 and 220 cm (Fig. 1). Downwards, the sediment consisted mainly of mud and clay (grain size, <63 μm). At the GP site, sediments were more homogenously composed with depth and were mainly dominated by a mixture of sand and mud (Freese et al., submitted). On the sampling date, temperatures were 20°C at the sediment surface and around 12°C at the bottom of the core at both sites.

Oxygen within the sediments was detected only within the uppermost 3 mm (data not shown). Pore water sulfate concentrations at the Neuharlingersieler Nacken and Gröninger Plate sites ranged between 27 and 32 mM at the sediment surface (Fig. 1) and decreased with depth. Minimum values were detected at 100 (GP) to 140 (NSN) cm below surface. At the NSN site, sulfate concentrations increased again below that level and reached a local maximum within the mussel shell-rich layers at a depth interval from 240 cm to 260 cm (Fig. 1). As indicated by the pore water chloride profiles, low sulfate concentrations were not the result of an influx of groundwater.

Total- and viable-cell counts. Total cell counts showed only minor differences between the two sites. At the sediment surface, around 1×10^9 cells $\cdot$ g^{-1} were detected (Table 1). An approximately 10-fold decrease was observed within the uppermost 50 cm of the sediments. Below that level, cell counts decreased less with depth and still reached values of around 1×10^7 to 4×10^7 cells $\cdot$ g^{-1} at the bottom of the core at both sites.

Viable counts were determined by the MPN method. At both sites, the highest counts were achieved at the surface or within a 5-cm depth (Table 1) with medium supplemented with $Fe(OH)_3$ as an electron acceptor. At the NSN site, 1.5×10^8 cells $\cdot$ g^{-1} were detected within a 5-cm depth, corresponding to 26% of the total cell count. At the GP site, the maximum cultivation success with $Fe(OH)_3$ as an electron acceptor was in a similar range (2.8×10^8 cells $\cdot$ g^{-1}, corresponding to 24% of the total cell count). Except for the layers near the surface at the GP site, MPN counts with manganese oxide as an electron acceptor were in a range similar to those with $Fe(OH)_3$.

Oxic and anoxic media without metal oxides yielded similar MPN counts in the uppermost two sediment layers at the two sites, which were 100- to 1,000-fold lower than those found when metal oxides were used as electron acceptors. Surprisingly, at both sites, MPN counts of aerobes were as high as or higher than those obtained with metal oxide-free anoxic medium.

At the NSN site, the MPN counts showed only minor variations within the upper 50 cm. Beneath that level, the numbers of aerobes declined, while with metal oxides high numbers were still achieved even for the mussel shell-rich layer. However, MPN counts decreased strongly in the mud-and-clay-dominated bottom layers irrespective of the medium used. At the GP site, MPN counts with $Fe(OH)_3$, sulfate, and oxygen showed a steady decrease with depth, while for the sulfate-free anoxic medium, almost no decrease with depth was found. The sulfate-free medium delivered the highest counts for the deepest layers from 300 cm downwards. At a depth of 500 cm, 30,400 cells per g of sediment were still detected.

Contribution of endospores to the viable community. In order to estimate the potential contribution of inactive endospores to the viable counts, parallel MPN series were inoculated with pasteurized sediment slurries. For surface sediments at the Neuharlingersieler Nacken site, the MPN counts obtained with pasteurized samples were approximately 1% of those obtained with untreated samples (Table 2). Absolute numbers of spores that could be stimulated to grow increased with depth and reached a maximum at a depth of 50 cm, where 1,500 to 5,500 per gram of sediment were retrieved. Beneath

TABLE 2. MPN counts of spores obtained after inoculation with pasteurized sediment slurry from the Neuharlingersieler Nacken site

Sediment depth (cm)	MPN count of spores g of sediment^{-1} (% of MPN counts of spores in untreated samples) after:	
	Oxic incubation[a]	Anoxic incubation[a, b]
0.5	3,170 (0.8)	1,080 (0.3)
5	1,190 (3.6)	1,200 (4.0)
50	5,520 (4.0)	1,530 (2.6)
100	130 (20.0)	250 (2.0)
200	120 (100.0)	ND
300	100 (75.0)	ND
400	90 (100.0)	ND
450	90 (100.0)	ND

[a] The sulfate-containing medium was used.
[b] ND, not determined.

that level, MPN counts with untreated and pasteurized slurries were in a similar range (Table 2), but absolute numbers of spores that were stimulated to germinate decreased.

Isolation of pure cultures. To identify the most abundant microorganisms growing in the various media, pure cultures were isolated from the highest positive MPN dilutions or from sediment embedded in gradient tubes. In total, 112 pure cultures were obtained, 64 from the Gröninger Plate site and 48 from the Neuharlingersieler Nacken site (Table 3). About one-third of these isolates were obtained from gradient cultures. The different isolates were affiliated with the major bacterial phyla on the basis of partial 16S rRNA gene sequences and could be grouped into 53 different OTUs within the *Alphaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria*, "*Bacteroidetes*," "*Fusobacteria*," *Actinobacteria*, and "*Firmicutes*." Of the different OTUs, 28 were represented only by a single strain, while 8 OTUs comprised two strains and 17 comprised three to eight strains (Table 3). This remarkably high diversity of different phylotypes was in part due to the investigation of two separate sampling sites obviously harboring different bacterial communities. For example, *Actinobacteria* and "*Fusobacteria*" were retrieved exclusively from the Neuharlingersieler Nacken site, while in turn some of the *Bacillus*-related strains were obtained only from the Gröninger Plate site.

In general, different phylotypes were isolated from gradient tubes and MPN series. Of the 17 OTUs comprising three strains or more, only 2 (those related to *Tenacibaculum mesophilum* and to *Eubacterium angustum*) were retrieved from MPN series as well as from gradient tubes. Sulfate-reducing bacteria were isolated almost exclusively from the gradient tubes.

Even more than by the kind of cultivation approach, the results of the isolation attempts were determined by the redox conditions of the enrichment procedure. Oxic and anoxic MPN series or anoxic gradient cultures never yielded strains belonging to the same OTU. However, this finding is surprising, since a large fraction of strains obtained under anoxic conditions turned out to be only facultatively anaerobic, e.g., *Shewanella*, *Vibrio*, or *Bacillus* strains. On the other hand, two-thirds of the isolates obtained from the oxic MPN series did not grow in any of the anaerobic growth tests at all and are therefore considered to be strictly aerobic (Table 3).

By the culture approach chosen here, a shift of phylogenetic groups along the sediment column was found. A higher number of different phylogenetic groups was retrieved from the surface layers (0.5 to 5 cm) than from the bottom layers below a depth of 100 cm. While predominantly *Proteobacteria* were obtained from the upper layers, "*Firmicutes*" dominated the strain collection for the deeper layers (Fig. 2). Only a few OTUs, comprising strains related to the genera *Desulfovibrio*, *Bacillus*, *Eubacterium*, and *Staphylococcus*, had representatives isolated from the surface as well as from the subsurface layers.

Physiological characterization of the pure cultures. To obtain information on the potential ecological niches of the isolates, physiological experiments were performed. The majority of the OTUs (20 out of 53) were found to comprise strictly anaerobic isolates, in particular those affiliated with the *Deltaproteobacteria* and the "*Fusobacteria*." But among the "*Firmicutes*" also, several strict anaerobes were found. Facultative anaerobes represented a similar number of phylotypes (17 of 53 OTUs). While most of the strictly aerobic bacteria were isolated from the sediment surface (e.g., some members of the *Alphaproteobacteria* and *Actinobacteria*), some were isolated even from the deepest sediment layers. The latter, however, were mostly *Bacillus*-related strains, most likely present as inactive endospores in situ.

Most of the anaerobic bacteria grew in mineral medium by fermentation of a single substrate (glucose) or a mixture of substrates (amino acids). However, some anaerobes (for example, the *Tepidibacter*-related strains) did require a more complex medium (monomer or YPG) to grow fermentatively. Nitrate was the electron acceptor most frequently used for anaerobic respiration. In most cases nitrate was reduced to nitrite, less frequently to N_2 or ammonium. Some fermenters also grew by iron or manganese reduction. However, strong differences among the strains were observed. While the *Shewanella*-related strains grew relatively fast on metal oxides, other strains needed at least 2 weeks to achieve slight turbidity and detectable metal reduction (e.g., the *Marinobacter*- and *Psychromonas*-related strains). On metal oxides, the sulfate-reducing bacteria generally grew very fast and to high cell densities. However, sulfate-reducing bacteria were scored positive for iron or manganese reduction only if they grew as fast as with sulfate as electron acceptor, in order to exclude growth by sulfur cycling (47).

Molecular comparison of the different enrichment methods. Molecular analysis of the original sediment samples, the gradient cultures, and the MPN series was performed using PCR and DGGE. The different bacterial types within the different samples were identified by excision and sequencing of the DGGE bands and compared to the pure cultures and the original sediment. The enrichment cultures obtained by the different cultivation approaches revealed a high diversity of microorganisms in total, but also within the single enrichments. In almost all samples, at least five but sometimes as many as nine different bands were detected (Fig. 3). Most of them could be assigned to the *Alpha*-, *Gamma*-, or *Deltaproteobacteria*, the "*Firmicutes*," the "*Bacteroidetes*," or the *Spirochaetes* (Table 4). However, several of these sequences (e.g., bands IE2, IIC2, and IIIA7) were only very distantly related to species already known (less than 10% sequence similarity).

At the sediment surface, the *Alphaproteobacteria* were the most frequently detected group in the enrichments. At a depth

TABLE 3. Phylogenetic grouping of pure cultures according to their partial 16S rRNA gene sequences

Closest relative (% sequence similarity)	No. of isolates obtained from the following:								Metabolic capacities[c]						Spore formation in stationary-phase cultures
	Origin depth[a] (cm)			Source of isolation					Aerobic growth	Fermentation	Growth by anaerobic respiration				
				MPN series[b]			Gradient								
	0–5	50–100	200–550	Oxic	Anox.	-Sulf.	TCA	ALC			NO_3^-	Mn^{4+}	Fe^{3+}	SO_4^{2-}	
Alphaproteobacteria															
Rhizobium rubi^T (97)	G1			1					++	−	−	−	−	−	−
Paracoccus carotinifaciens^T (99)			G1					1	+	Gl	−	−	−	−	−
Roseobacter gallaeciensis^T (96)	N1			1					+	−	−	−	−	−	−
Ruegeria atlantica^T (99)	N2			2					+	−	−	−	−	−	−
Roseovarius nubinhibens^T (96)	G1			1					+	−	−	−	−	−	−
Sphingobium yanoikuyae^T (98)			G1					1	−	+[d]	ND	ND	ND	ND	−
Gammaproteobacteria															
Glaciecola pallidula^T (96)	N1			1					++	−	−	−	−	−	−
Marinobacter lipolyticus^T (98)		N1		1					+	Gl, A	+[e]	(+)	(+)	−	−
Shewanella affinis^T (98)	G3					3			+	Gl, A	−	+	−	−	−
Shewanella violacea^T (97)		N2				2			+	A	+[e]	+	−	−	−
Halomonas ventosae^T (99)			G1	1					+	Gl, A	+[f]	−	−	−	−
Psychromonas profunda^T (96)					1				+	Gl, A	−	−	(+)	−	−
Photobacterium profundum^T (97)	G2	N1			1	2			+	Gl, A	+[e]	−	−	−	−
Vibrio splendidus^T (98)	G3, N2	N2				7			+	Gl, A	−	−	−	−	−
Deltaproteobacteria															
Desulfuromonas palmitatis^T (94)		G1			1				−	ND	ND	ND	ND	ND	−
Malonomonas rubra^T (97)	N1				1				−	Gl	+[e]	+	−	−	−
Desulfovibrio acrylicus^T (92)			G1					1	−	−	ND	ND	ND	+	−
Desulfovibrio acrylicus^T (99)			G1					1	−	−	+[g]	+	−	+	−
Desulfovibrio aespoeensis^T (94)	G3	G4					2	6	−	−	−	+	+	+	−
Desulfovibrio aespoeensis^T (92)		N1					1		−	A, L	+[g]	+	−	+	−
Desulfovibrio zosterae^T (97)	G2, N1						2	1	−	A	−	+	−	+	−
Desulfovibrio senezii^T (91)	G1						1		−	−	+[g]	−	+	+	−
Desulfobacterium catecholicum^T (96)	G1				1				−	−	−	+	−	+	−
"*Fusobacteria*," *Ilyobacter tartaricus*^{T} (93)	N2	N1			3				−	A	−	−	−	−	−
"*Bacteroidetes*"															
Salegentibacter salegens^T (93)		G1		1					+	Gl, A	+[g]	(+)	−	−	−
Tenacibaculum mesophilum^T (92)	G2	G1			1			2	+	Gl	−	−	−	−	−
Cytophaga fermentans^T (87)	N2				2				−	Gl	−	−	−	−	−
Actinobacteria															
Cellulomonas biazotea^T (96)		N2		2					+	−	−	−	−	−	−
Citricoccus muralis^T (98)	N2	N1		3					+	−	+[f]	+	−	−	−
Micrococcus luteus^T (99)	N1			1					+	−	−	−	−	−	−
"*Firmicutes*"															
Bacillus simplex^T (96)			G4		4				+	−	+[e]	−	−	−	−
Bacillus hwajinpoensis^T (99)	G1	G3	G2	6					+	Gl, A	−	−	−	−	+
Bacillus aquimaris^T (99)		G2	G1	3					++	−	−	−	−	−	+
Bacillus cohnii^T (98)			N2	2					+	Gl	−	−	−	−	+

Bacillus firmus[T] (98)			G1					1	+	+	+[e]	−	−	−	+
Bacillus novalis[T] (96)			G1				1		++	−	−	−	−	−	+
Bacillus infernus[T] (97)			G1, N1				1	1	+	Gl, A	−	−	−	−	+
Bacillus simplex[T] (99)	N1		G1	2					++	−	−	−	−	−	+
Halobacillus salinus[T] (99)			G1	1					++	−	−	−	−	−	+
Bacillus halodurans[T] (94)	G2	N3		5					++	−	−	−	−	−	−
Paenibacillus glucanilyticus[T] (98)	N1							1	+	Gl	ND	ND	ND	ND	+
Clostridium lentocellum[T] (95)			N2				2		−	+	ND	−	−	−	−
Tepidibacter thalassicus[T] (96)			G3		2	1			−	+	−	−	−	−	+
Tepidibacter formicigenes[T] (95)			G1			1			−	+	−	−	−	−	+
Clostridium novyi[T] (95)			N1				1		−	+	ND	ND	ND	ND	−
Alkaliphilus transvaalensis[T] (95)			G1				1		−	+	ND	ND	ND	ND	−
Alkaliphilus crotonatoxidans[T] (92)	N1					1			−	+	ND	ND	ND	ND	−
Eubacterium angustum[T] (91)	N1	N1	G2		1		3		−	Gl, A	−	+	−	−	−
Desulfosporosinus auripigmenti[T] (97)			G1, N3				2	2	−	L	(+)[g]	+	+	+	+
Sedimentibacter hydroxybenzoicus[T] (94)			G1				1		+	Gl	+[e]	(+)	(+)	−	+
Parasporobacterium paucivorans[T] (93)		G1						1	+	+	ND	ND	ND	ND	+
Staphylococcus equorum[T] (99)	G1		G1, N1	3					++	−	−	−	−	−	−
Staphylococcus succinus[T] (99)	G1	N2		3					++	−	−	−	−	−	−

[a] The prefix G before the number of isolates stands for the GP site; the prefix N stands for the NSN site.
[b] Anox., anoxic; -Sulf., anoxic sulfate-free MPN series.
[c] ++, strictly aerobic; +, facultatively anaerobic; (+), only weak growth, or growth not present in all strains tested; −, no growth; ND, not determined. Gl, glucose; A, amino acid mix; L, lactate.
[d] Substrate mixture.
[e] Reduction to nitrite.
[f] Reduction to dinitrogen.
[g] Reduction to ammonium.

of 50 cm, they were still present in high numbers, but they were not detected in enrichments from depths of 100 and 500 cm. "*Firmicutes*" were found at all depths but were absolutely dominant at depths of 100 cm and 500 cm. These results seemed to confirm the composition of the culture collection. However, direct comparison of the sequences derived from the DGGE bands with those of the isolates originating from the same enrichments revealed that only three of the phylotypes identified from the DGGE bands (IIE2, IIC7, IIC5) were obtained in pure culture. These DGGE bands matched isolates that were related to *Desulfovibrio aespoeensis*, *Bacillus aquimaris*, and *Bacillus hwajinpoensis*. In addition, one DGGE band (IIB4) obtained from anoxic MPN series corresponded to *Desulfovibrio aespoeensis*-related isolates, though none of the isolates was obtained from this particular MPN series. While 7 isolates were detected in the enrichment cultures, the remaining 105 strains were not retrieved in the respective enrichment cultures by PCR and DGGE. At least in some cases, this might be due to the fact that not all DGGE bands could be sequenced. Several bands, in particular from the original sediment sample, gave ambiguous sequences, in most cases due to the fact that several phylotypes were present in a single band and the sequences obtained revealed many ambiguities. However, the results indicate that subculturing during the isolation procedure led to a population shift from the types dominating the primary enrichment to less-dominant but presumably faster-growing types.

Most enrichment cultures were unique in their phylogenetic composition and showed little overlap with enrichments achieved by other methods or by use of different media. With a single exception (bands IIID6 and IIIE9), MPN series and gradient tubes from the same depth did not reveal matching DGGE bands, indicating that every enrichment approach was highly selective. This finding was supported by the fact that in some cases the same phylotype was enriched using the same approach but from two different depths (e.g., bands IID4 and IIID6 or bands IB2 and IIB3). Microscopic investigations of the enrichments revealed high morphological diversity and the presence of several unusually shaped bacteria, as, for example, large spirochetes or chains of vacuolated cells. In parallel to the shake procedure, an attempt was made to isolate some of these types directly by micromanipulation (21) and to transfer single cells into fresh medium. However, none of these mechanically separated cells grew in subculture.

DISCUSSION

This study presents the results of an analysis of microbial communities in intertidal sediments on the German North Sea coast from the surface down to the subsurface at a 550-cm depth. The use of improved media for MPN series resulted in high cultivation efficiency and, in combination with enrichment cultures in gradients, allowed the isolation of a highly diverse culture collection. Since the different media and enrichment strategies were selective for specific bacterial groups, the use of different approaches turned out to be a basic requirement for achieving high diversity.

Improving cultivation efficiency. In most previous investigations, MPN counts in marine sediments rarely exceeded 0.25% of the total cell count (2, 20, 27, 31). Only in a few cases could

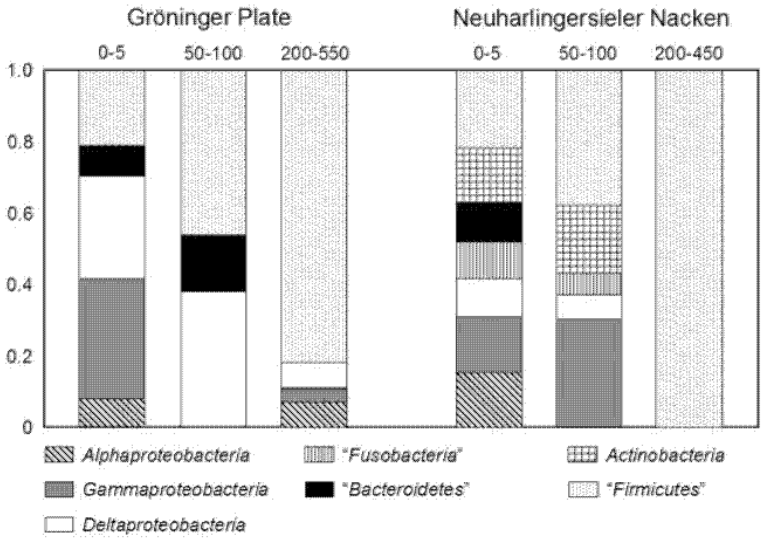

FIG. 2. Relative contributions of the different phylogenetic groups to the numbers of isolates present in the culture collection obtained from different depths at the Neuharlingersieler Nacken and Gröninger Plate sites. The numbers above the columns indicate depth in centimeters, and the numbers on the left indicate the relative contributions to the culture collection from the respective depths.

larger fractions of microbial communities be cultured from sediments (16, 55) or sediment-like habitats such as rice paddies (8). However, in the present study, consistently high cultivation efficiencies, as high as 23% of the total cell count, were achieved throughout the upper 50 cm of the sediment. One reason might be the use of a medium containing a wide range of different carbon sources, stimulating the growth of different microorganisms. A very similar medium was recently shown to increase cultivation efficiency even in several-thousand-year-old sediments of the Eastern Mediterranean Sea (48).

The highest MPN counts were obtained with oxidized metal species as electron acceptors. It is well documented that in marine sediments manganese or iron reduction can be as important as sulfate reduction (6), whereas little information on the numerical abundance of metal-reducing bacteria in marine sediments is available. Their numbers, estimated as viable counts, can be in a range similar to those reported for sulfate-reducing bacteria (27, 28). However, since a large variety of (fermentable) substrates was present in the medium, not all organisms enriched by this method are necessarily iron or manganese reducers. Interestingly, high numbers of Fe- and Mn-reducing bacteria were detected even in strictly anoxic sediment layers where no Fe(III) and Mn(IV) should be available. However, it is known that even some sulfate reducers, like the isolates presented here, can couple carbon oxidation to iron reduction (9). Some of the isolates presented here grew slowly under anoxic conditions with metal oxides but showed hardly any growth without an external electron acceptor (e.g., the isolates related to *Marinobacter* and *Salegentibacter*). These organisms might be active in situ in a syntrophic relationship and can use metal oxides as an unspecific electron sink (32), which would also explain their slow growth. We therefore have to assume that MPN counts of metal-reducing bacteria in anoxic sediment layers comprise a large metabolic variety of strict and facultative anaerobes.

Although oxic and anoxic isolation procedures resulted in completely different phylotypes, several of the anaerobic isolates turned out to be only facultatively anaerobic, e.g., the *Shewanella* isolates. Since these strains were obtained exclusively under anoxic conditions but were present in numbers similar to those of strains isolated under oxic conditions, as can

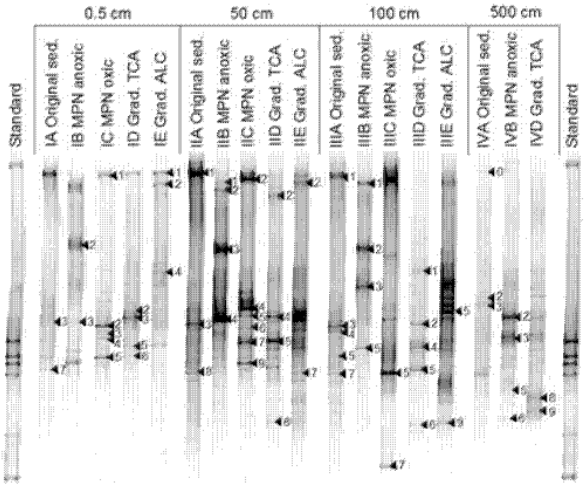

FIG. 3. Comparison of the different enrichment cultures and the microbial community in situ (original sediments [sed.]) by PCR and DGGE using primers specific to *Eubacteria*. Enrichment cultures in gradient tubes with carboxylic and amino acids are labeled "Grad. TCA"; those with alcohols and fatty acids are labeled "Grad. ALC." Bands that were excised for sequence analysis are numbered consecutively, beginning at the upper edge of the gel. Arrowheads indicate only those DGGE bands that gave reliable results after excision and sequence analysis.

TABLE 4. Phylogenetic affiliations of genotypes detected in the different enrichments inoculated with sediments from the Gröninger Plate site by PCR-DGGE and sequence analysis of DGGE bands and comparison with the original sediment samples

Depth (cm)	Sample[a]	Total no. of bands detected/seq.[b]	Band[c]	Closest relative sequence or species in GenBank (% sequence similarity)	Phylogenetic affiliation	Correspondence to other bands
0.5	Original	7/2	IA3	*Methylomicrobium buryatense*^{T} (90)	*Gammaproteobacteria*	
			IA7	*Anaeromyxobacter dehalogenans*^{T} (90)	*Deltaproteobacteria*	
	M anox	6/2	IB2	*Alkalibacterium olivapovliticus*^{T} (92)	*Firmicutes*	IIB3
			IB3	*Desulfovibrio aespoeensis*^{T} (94)	*Deltaproteobacteria*	
	M ox	5/5	IC1	*Psychroserpens burtonensis*^{T} (95)	*Bacteroidetes*	
			IC2	*Ruegeria atlantica*^{T} (96)	*Alphaproteobacteria*	IC3
			IC3	*Ruegeria atlantica*^{T} (98)	*Alphaproteobacteria*	IC2
			IC4	*Roseovarius tolerans*^{T} (96)	*Alphaproteobacteria*	IID5
			IC5	*Roseovarius tolerans*^{T} (98)	*Alphaproteobacteria*	
	GR TCA	7/4	ID2	*Sulfitobacter brevis*^{T} (97)	*Alphaproteobacteria*	
			ID3	*Alkaliphilus crotonatoxidans*^{T} (95)	*Firmicutes*	
			ID5	*Dethiosulfovibrio acidaminovorans*^{T} (92)	*Firmicutes*	
			ID6	Uncultured marine alphaproteobacterium BY-95 (97)	*Alphaproteobacteria*	
	GR ALC	5/3	IE1	Uncultured *Cytophagales* bacterium, clone Hyd24-40 (97)	*Bacteroidetes*	
			IE2	*Riemerella columbina*^{T} (87)	*Bacteroidetes*	
			IE4	Uncultured spirochete clone CD5C5 (97)	*Spirochaeta*	
50	Original	9/4	IIA1	Chloroplast *Thalassiosira eccentrica* (99)		
			IIA3	Uncultured gammaproteobacterial isolate, DGGE band GWS-SE-4 (97)	*Gammaproteobacteria*	
			IIA6	*Desulfonema magnum*^{T} (90)	*Deltaproteobacteria*	
			IIA8	*Anaeromyxobacter dehalogenans*^{T} (90)	*Deltaproteobacteria*	
	M anox	6/4	IIB1	*Cytophaga fermentans*^{T} (89)	*Bacteroidetes*	
			IIB2	*Alkalibacterium olivapovliticus*^{T} (90)	*Firmicutes*	
			IIB3	*Alkalibacterium olivapovliticus*^{T} (92)	*Firmicutes*	IB2
			IIB4	*Desulfovibrio aespoeensis*^{T} (94)	*Deltaproteobacteria*	
	M ox	9/6	IIC2	*Cytophaga* sp. 0sq516 (84)	*Bacteroidetes*	
			IIC4	*Roseobacter gallaeciensis*^{T} (96)	*Alphaproteobacteria*	
			IIC5	*Sulfitobacter mediterraneus*^{T} (97)	*Alphaproteobacteria*	
			IIC6	*Sulfitobacter dubius*^{T} (96)	*Alphaproteobacteria*	
			IIC7	*Bacillus aquimaris*^{T} (96)	*Firmicutes*	
			IIC9	*Bacillus indicus*^{T} (97)	*Firmicutes*	
	GR TCA	8/4	IID2	*Cytophaga fermentans*^{T} (89)	*Bacteroidetes*	
			IID4	*Clostridium populeti*^{T} (90)	*Firmicutes*	IIID2
			IID5	*Roseovarius tolerans*^{T} (95)	*Alphaproteobacteria*	IC4
			IID8	*Thermanaerovibrio acidaminovorans*^{T} (89)	*Firmicutes*	IIID6, IIIE9
	GR ALC	9/3	IIE2	*Desulfovibrio aespoeensis*^{T} (94)	*Deltaproteobacteria*	
			IIE7	*Lamprocystis purpurea*^{T} (90)	*Gammaproteobacteria*	
100	Original	7/5	IIIA1	*Tenacibaculum mesophilum*^{T} (89)	*Bacteroidetes*	
			IIIA3	*Thiorhodovibrio winogradskyi*^{T} (91)	*Gammaproteobacteria*	
			IIIA4	Uncultured gammaproteobacterium GWS-SE-5 (95)	*Gammaproteobacteria*	
			IIIA5	Uncultured deltaproteobacterium GWS-SE-6 (87)	*Deltaproteobacteria*	
			IIIA7	*Anaeromyxobacter dehalogenans*^{T} (88)	*Deltaproteobacteria*	
	M anox	5/4	IIIB1	*Cytophaga fermentans*^{T} (90)	*Bacteroidetes*	
			IIIB2	*Alkalibacterium olivapovliticus*^{T} (92)	*Firmicutes*	
			IIIB3	Uncultured bacterium BURTON-31 (95)	*Bacteroidetes*	
			IIIB5	*Desulfovibrio gabonensis*^{T} (95)	*Deltaproteobacteria*	
	M ox	7/3	IIIC5	*Bacillus hwajinpoensis*^{T} (97)	*Firmicutes*	
			IIIC7	*Cellulomonas fimi*^{T} (95)	*Firmicutes*	
	GR TCA	6/5	IIID1	*Cytophaga fermentans*^{T} (89)	*Bacteroidetes*	
			IIID2	*Clostridium populeti*^{T} (92)	*Firmicutes*	IID4
			IIID4	*Aminobacterium colombiense*^{T} (91)	*Firmicutes*	
			IIID5	*Dethiosulfovibrio acidaminovorans*^{T} (89)	*Firmicutes*	
			IIID6	*Thermanaerovibrio acidaminovorans*^{T} (89)	*Firmicutes*	IID8, IIIE9
	GR ALC	9/2	IIIE5	*Cytophaga fermentans*^{T} (92)	*Bacteroidetes*	
			IIIE9	*Thermanaerovibrio acidaminovorans*^{T} (89)	*Firmicutes*	IID8, IIID6
500	Original	7/4	IVA0	*Thalassiosira eccentrica* chloroplast (99)		
			IVA2	Uncultured deltaproteobacterium clone PI_6RA04 (82)	*Deltaproteobacteria*	
			IVA3	Uncultured bacterium Seq13(S5) (88)	*Chloroflexi*	
			IVA6	*Thermobacillus xylanilyticus*^{T} (94)	*Firmicutes*	
	M anox	6/5	IVB2	*Clostridium homopropionicum*^{T} (86)	*Firmicutes*	
			IVB3	*Tepidibacter formicigenes*^{T} (94)	*Firmicutes*	
			IVB5	*Desulfofaba hansenii*^{T} (85)	*Deltaproteobacteria*	
			IVB6	*Desulfotomaculum* sp. strain 175 (89)	*Firmicutes*	

Continued on following page

TABLE 4—*Continued*

Depth (cm)	Sample[a]	Total no. of bands detected/seq.[b]	Band[c]	Closest relative sequence or species in GenBank (% sequence similarity)	Phylogenetic affiliation	Correspondence to other bands
	M ox	0/0				
	GR TCA	9/2	IVD8	*Sporotomaculum syntrophicum*^{T} (96)	*Firmicutes*	
			IVD9	*Desulfotomaculum gibsoniae*^{T} (95)	*Firmicutes*	
	GR ALC	0/0				

[a] Original, original sediment sample. The following enrichments were investigated: MPN series under anoxic (M anox) or oxic (M ox) conditions as well as gradient tubes supplemented with carboxylic and amino acids (GR TCA) or with *n*-alcohols and fatty acids (GR ALC).
[b] seq., number of bands that could be unambiguously identified by sequence analysis.
[c] Individual bands are labeled for the origin depth (I to IV), for the sample (A to E), and with a consecutive number (1 to 9) within the lane in the DGGE gel (see Fig. 3).

be inferred from the MPN counts, it seems likely that the immediate exposure to air severely harms these cells. Similarly, a decreased oxygen partial pressure was shown to facilitate the enrichment and isolation of typical aerobic soil bacteria (45). It can be assumed that with the metal oxides a relatively oxidized electron acceptor was offered that circumvented the harmful effects of molecular oxygen (34). It might be advantageous for sediment bacteria to be able to cope with oxygen. Bioturbation can generate oxic microenvironments to depths of as much as 50 cm (29). Furthermore, the upper layers of tidal flats represent a rather unstable ecosystem. During storm events, large patches of sediment can be resuspended. Strict anaerobes, in turn, can be expected to be dependent on reducing conditions. Free pore water sulfide, however, was not detected along the upper 20 cm of the sediment (data not shown), indicating that several centimeters deep in the sediment, anoxic but still quite oxidized conditions prevail.

While high cultivation efficiencies were achieved along the uppermost 50 cm of the sediment, there was a decrease within deeper sediment layers. Oxidative stress as discussed above does not apply for the anoxic medium and therefore seems to be an unlikely cause. Small inoculum sizes, as in our study, have even been shown to increase viable counts (13). Substrate shock caused by high substrate concentrations (42, 46) has often been blamed for poor culturability. In fact, low substrate concentrations have been shown to improve cultivation success (10, 13, 25, 43, 45, 56). Although we used a substrate mixture containing different carbon sources, each of them was present in submillimolar concentrations, and a medium similar to that used in the present study achieved high MPN counts even in Mediterranean sediments up to 100,000 years old (48). Incubation time was shown to have an impact on viable counts as well, since some of the organisms that are abundant in situ might grow only slowly. However, 12 weeks, as in this study, should be long enough, as found for soil microbial communities (13). Molecular analysis of the same sediments (Wilms et al., submitted) revealed that bacteria belonging to a subgroup of the phylum *Chloroflexi* that is typically found in the marine subsurface (11, 36) were also dominant in the deep layers of the tidal flats investigated here. But since no representative of these presumably typical subsurface bacteria is available as a pure culture, we can only speculate about their nutritional demands.

Relatively low cultivation efficiencies could also be caused by overestimation of the number of potentially viable cells, particularly in deep sediments. DAPI stains living cells, irrespective of the metabolic state. However, it was estimated that the fraction of dead cells that can be stained might represent up to 70% of the total cell counts in marine sediments (33). On the other hand, the use of media with low substrate concentrations can possibly lead to problems with growth detection due to the low level of biomass formed. But even if a bacterium grows on only one of the substrates provided in the monomer mixture in the present study, e.g., lactate, and the growth yield reported for *Acetobacterium woodii* (41) is considered, theoretically enough biomass is formed for microscopic detection.

Microbial diversity detected by the different enrichment approaches. In the present study a remarkable high diversity in the culture collection was achieved, outranging similar investigations on deep-sea pelagic environments (40) or sediments (15, 48). During the other studies almost all bacteria that could be cultured by the methods applied were obtained in pure culture as indicated by rarefaction analyses. Obviously, in the present study the number of OTUs detected is at least three times higher, although a similarly high number of isolates was obtained. One reason is likely to be the use of different cultivation and isolation procedures. As discussed above, almost no group was retrieved by more than a single method (oxic or anoxic, MPN or gradient culture). This result supports the view that no single method or medium is suitable to reveal the whole diversity within a single sample. It is likely that an even higher diversity could have been achieved if more medium variations had been used. On the other hand, tidal surface sediments are patchy, e.g., due to bioturbation, and offer a number of different microhabitats and redox horizons suitable for different physiological groups (29). Furthermore, the tidal sediments investigated in the present study revealed layers of different grain sizes such as sand and mud. At least for the deeper layers, the different sedimentological parameters of the two sampling sites might be one major reason for the different composition of the culture collection. As shown also by molecular methods, every sedimentological facies harbors its own specific microbial community (Wilms et al., submitted).

Phylogenetic analysis of the enrichment cultures. DGGE analysis of the enrichment cultures revealed an even higher diversity than that present in the culture collection. Nevertheless, little correspondence between the enrichment cultures and the natural community was found. One of the main reasons was probably that only some of the bands yielded reliable sequences, in most cases due to the presence of several phylotypes in one band. It was expected that some of the types that are dominant in situ would be retrieved at least in the gradient

tubes (26). Obviously, however, even the addition of low substrate concentrations or the application of substrate gradients seemed to stimulate less abundant types in particular. That community shifts can be achieved even by less thorough disturbance was shown by Eilers et al. (17), who found a dominance of easily culturable bacterial genera even after short-term incubation. On the other hand, we do not know which genotypes were present in the ambiguous bands. It can be assumed that at least some of our isolates remained undetected in the natural community or the primary enrichments.

Improved classical approaches such as agar plates have been applied successfully to soil microbial communities and led to the isolation of many yet uncultured groups of bacteria (25, 39). However, a basic requirement for the isolation of pure cultures, as performed in the present study, was the ability of the microorganisms to form colonies in deep agar or on agar plates. It can be imagined that several of the types observed in the enrichment cultures were unable to form colonies (43) and therefore were lost during isolation. The use of micromanipulation of single cells (21) as an alternative isolation strategy was unsuccessful. This might be due to cell damage during handling or to inability of these organisms to grow independently in the absence of syntrophic partners (26). For these organisms, alternative strategies such as dialysis cultures, dilution series, or growth on filters (43) will have to be used in the future.

Microbial communities in the subsurface of a tidal flat. During the last decade the discovery of the "deep biosphere" has drawn much attention to deep-sea subsurface environments (15, 35, 36, 51), and it was suggested that the majority of prokaryotic life on earth is found in the subsurface (51). However, most of these investigations dealt with very deep layers, and so far it is not clear at which depth the deep biosphere starts. Whitman et al. (51) defined the subsurface in sediment as layers below a depth of 10 cm. This definition might fit sediments underlying oceans with low primary production and sedimentation. As an example, the 8,500-year-old sapropels in the Eastern Mediterranean Sea can be found at shallow depths of 17 to 35 cm below the sea floor (48). Coastal seas, however, show a much higher sedimentation rate. The estimated age of the buried mussel bed at a depth of approximately 200 cm at the Neuharlingersieler Nacken site was about 650 years (J. Rullkötter, personal communication). However, after this duration of burial, it is to be expected that only very recalcitrant organic material is present, and these layers can clearly be seen as a subsurface environment. This is also reflected by the composition of the culture collection. Almost no overlap between the surface layers (0 to 0.5 cm) and the deep layers (200 cm and below) was found (Table 3). This result is supported by a parallel analysis by PCR and DGGE using primers specific to *Bacteria* (Wilms et al., submitted). The finding that the deep layers (200 cm and below) are dominated by a subgroup of *Chloroflexi*, typically found in the subsurface, provides a link to the microbial communities within the "deep biosphere" that is present down to some hundred meters below the sea floor (36).

It is less clear whether the sediment layers at depths of 50 and 100 cm can be seen as part of the subsurface. However, these layers are already beneath the zone of bioturbation (29), are characterized by sulfidic conditions, and are unlikely to provide easily degradable organic matter to the microbial communities. Clear differences were found in the bacterial types isolated from these intermediate layers and those from the surface layers, for example, the lack of *Alphaproteobacteria* in the intermediate layers. However, these differences were less pronounced than the differences between the surface layers on the one hand and the shell and mud layers on the other. For this reason, it is suggested that these layers can be seen as subsurface habitats, but to clearly distinguish them from the deeper layers, the term "shallow subsurface" seems to be appropriate.

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Kapitel III

Roseobacter pelophilus sp. nov. and *Roseovarius pelophilus* sp. nov., facultatively anaerobic bacteria isolated from tidal flat sediments

Roseobacter pelophilus sp. nov. and *Roseovarius pelophilus* sp. nov., facultatively anaerobic bacteria isolated from tidal flat sediments

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Running title: *Roseobacter pelophilus* sp. nov.

The EMBL accession numbers for the 16S rRNA gene sequences of *Roseobacter pelophilus* SAM4^T and *Roseovarius pelophilus* G5II^T are AJ968651 and AJ968650, respectively.

Abstract

Five bacterial strains were isolated from the highest positive dilutions of most probable number (MPN) series inoculated with tidal flat sediments from the German North Sea coast. The isolates were identified as members of the *Roseobacter* clade within the *Alphaproteobacteria*. They were nutritionally versatile growing on a variety of carbon compounds including carbohydrates, amino acids and carboxylic acids like lactate or fumarate. All strains grew under anoxic conditions on complex media like marine broth, indicating fermentation. Four of the strains also grew anaerobically by use of the alternative electron acceptors dimethyl sulfoxide (DMSO) or trimethylamine-N-oxide (TMAO). The isolates grew in a temperature range from 4 to 35°C. Three of the strains showed constant growth yields from the lowest growth temperature to some °C below t_{max}, what is typical for psychrotrophic microorganisms. Bacteriochlorophyll *a* was detected only in strain SAM4. Phylogenetic analysis placed the five isolates into four different lineages within the *Roseobacter* clade. Based on the phylogenetic analysis and comparison of phenotypic and physiological traits two of the strains, N5III and N5IV, were identified as new strains of *Ruegeria atlantica*, while strain N05I could be identified as *Phaeobacter gallaeciensis*. However, two of the strains did not match closely validly described species. Therefore, two new species are proposed: *Roseobacter pelophilus* sp. nov., with strain SAM4^T (= DSM 17270^T, = LMG 23018^T) as type strain and *Roseovarius pelophilus* sp. nov. with strain G5II^T (= DSM 17271^T, = LMG 23019^T) as type strain.

Introduction

During the last two decades, molecular methods helped to vastly expand our knowledge on microbial diversity in the environment and to identify the dominating bacterial phyla. Most of these new phyla were not represented in the culture collections and no isolates are available so far (Hugenholtz *et al*., 1998). A notable exception from this discrepancy between the results of cultivation-based and molecular methods are bacteria belonging to the *Roseobacter* clade within the *Alphaproteobacteria*. *Roseobacter* clade bacteria are globally distributed and form one of the dominant groups of marine bacterioplankton (Mullins *et al*., 1995; Gonzalez & Moran, 1997; Rappé *et al*., 1997; Giovannoni & Rappé, 2000). Selje *et al*. (2004) found up to 2×10^5 cells $\cdot$ ml^{-1} affiliating with the *Roseobacter* clade in North Sea water, corresponding up to 20 % of total DAPI cell count.

Based on physiological investigations on the cultured isolates, *Roseobacter* clade bacteria were considered as strict aerobes, with *Roseobacter denitrificans* (Shiba, 1991) and *Roseovarius crassostreae* (Boettcher *et al*., 2005) as exception, being able to use nitrate as terminal electron acceptor. However, members of the *Roseobacter* clade have also been repeatedly detected in marine sediments by molecular as well as by cultivation methods (Johnson & Hill, 2003; Süß *et al*., 2004; Buchan *et al*., 2005). During a quantitative cultivation approach performed with tidal flat sediments *Roseobacter* clade bacteria were among the dominant culturable types and were obtained even from the permanently anoxic sediment layers (Köpke *et al*., 2005). But since isolation was generally achieved using oxic media one can only speculate about the *in situ* metabolism. In the present work the *Roseobacter* clade isolates from the tidal sediments were investigated in detail with a main focus on their potential anaerobic metabolism.

Materials and Methods

Sampling, enrichment and cultivation

Sediment samples were taken from three tidal flats (site Neuharlingersiel [NSL], site Neuharlingersieler Nacken [NSN] and site Gröninger Plate [GP]) close to the island of Spiekeroog on the German North Sea coast (Rütters *et al*., 2002; Köpke *et al*., 2005). The oxic/anoxic interface within the sediments was found at depths between 2 and 3 mm. Samples from the surface (0-0.5 cm) and from 5 cm depth were used for the preparation of MPN series in October 1998 (site NSL, Sass *et al.*, unpublished) and June 2002 (Köpke *et al*., 2005) that served as source for the isolation of pure cultures.

For the preparation of MPN series, isolation and growth of pure cultures an artificial oxic seawater medium was used. The oxic medium contained the following components (in $g \cdot l^{-1}$): NaCl (24.3), $MgCl_2 \cdot 2\ H_2O$ (10), $CaCl_2 \cdot 2\ H_2O$ (1.5), KCl (0.66), Na_2SO_4 (4), KBr (0.1), H_3BO_3 (0.0025), $SrCl_2 \cdot 6\ H_2O$ (0.04), NH_4Cl (0.021), KH_2PO_4 (0.0054), NaF (0.003). The medium was buffered with HEPES ($2.38\ g \cdot l^{-1}$) and supplemented with $1\ ml \cdot l^{-1}$ of trace element solution SL 10 (Widdel *et al*., 1983) and $0.2\ ml \cdot l^{-1}$ of selenite and tungstate solution (Widdel & Bak, 1992). The pH of the oxic medium was adjusted to 7.2 – 7.4 with NaOH prior to autoclaving. After autoclaving, the oxic medium was cooled down under air and the cold medium was supplemented with 10 ml of a vitamin solution (Balch *et al*., 1979) and sodium bicarbonate ($0.2\ g \cdot l^{-1}$) from sterile stocks.

For tests on anaerobic growth a slightly modified anoxic medium was used. Instead of HEPES, the medium was buffered with bicarbonate and CO_2. After autoclaving, the anoxic medium was cooled under an atmosphere of N_2/CO_2 (80/20, v/v) and 30 ml of a CO_2-saturated sodium bicarbonate solution ($1\ mol \cdot l^{-1}$) were added to the cold medium. The anoxic medium was amended with resazurin ($0.25\ mg \cdot l^{-1}$) as a redox indicator and was reduced by the addition of few crystals of sterile sodium dithionite until the redox indicator turned colourless. If necessary, the pH was adjusted to 7.2 – 7.4 by addition of sterile HCl or Na_2CO_3.

For strain maintenance and growth tests an oxic, marine broth (MB, Difco 2216, Becton-Dickinson, Heidelberg, Germany) or a dilute yeast extract-peptone-glucose medium was used (YPG). The latter was based on the oxic seawater medium described above, supplemented with yeast extract ($0.03\ g \cdot l^{-1}$), peptone ($0.06\ g \cdot l^{-1}$), sodium lactate ($5\ mmol \cdot l^{-1}$), glucose ($1\ mmol \cdot l^{-1}$).

MPN series prepared in 1998 were supplemented with a mixture of amino acids (2 mM alanine and cystine, arginine, asparagine, 1 mM each), those prepared in June 2002 with a monomer mixture containing the common 20 L-amino acids, short-chain fatty acids (C_1 to C_5), *n*-alcohols (C_1 to C_4), malate, fumarate, succinate, lactate, glycerol, and glucose (each at 0.1 mmol · l^{-1} ; Köpke *et al*., 2005).

Phenotypical characterization

For phase contrast micrographs agar-coated microscope slides (Pfennig & Wagener, 1986) were used. Cell dimensions were determined using a Leitz DMRB microscope (Wetzlar, Germany) equipped with a digital image analysis system (H & K, Berlin, Germany). Flagella were stained according to Heimbrook *et al*. (1989). Microbiological routine tests (Gram staining, oxidase, catalase) were performed with exponential phase cells according to standard procedures (Gerhardt *et al*., 1994).

Bacteriochlorophyll was determined spectrophotometrically (Cohen-Bazire *et al*., 1957). The strains were grown on Marine Broth 2216 in the dark or under diurnal light-dark cycles. Cells were harvested, washed and transferred into a 2 ml tube and 1.5 ml of ice-cold (-20°C) acetone/methanol (7:2, vol/vol) were added. The assay was mixed well and incubated at room temperature in the dark for 12 h. Cell debris was removed by centrifugation. Absorption spectra were recorded from 400 to 900 nm on an UV/VIS spectrophotometer (Lambda2S, Perkin Elmer, Überlingen, Germany).

The G + C content of the DNA was determined by HPLC analysis (Mesbah *et al*., 1989) at the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Whole cell fatty acid patterns were analyzed in cells grown aerobically with acetate as sole electron and carbon source. Cells were harvested at the end of the exponential phase, washed with PBS buffer (130 mmol · l^{-1} NaCl, 10 mmol · l^{-1} sodium phosphate buffer, pH 7.2). Fatty acids were analyzed after alkaline extraction of freeze-dried cells as described by Sass *et al*. (2002).

Physiological tests

Substrate utilization was tested in miniaturized assays in microtiter plates (Corning, New York, USA) using the oxic mineral medium. For each substrate an inoculum-free control and for each strain a substrate-free blank were prepared. Inoculated plates were incubated for four weeks in the dark at 20°C. Because the growth yield on some of the substrates was poor, growth was checked by fluorimetry (Köpke *et al*., 2005; Martens-Habbena & Sass,

2006). From each well, 50 µl of culture were transferred into a black fluorescence plate (NUNC, Wiesbaden, Germany) and 50 µl of a Sybr Green I solution (2000 fold dilution in TE buffer, 200 mM Tris and 50 mM EDTA, pH 8) were added. Fluorescence was measured after incubation for 2 h in the dark at room temperature using a fluorescence microplate reader (BMG Labtechnologies, Offenburg, Germany).

Fermentative growth was tested in anoxic sulfate-free medium with glucose (5 mM), casamino acids (0.1 %), or in anoxic marine broth (Difco 2216). Tests for anaerobic respiration were performed with the sulfate-free anoxic mineral medium with either sodium acetate or lactate (10 mM each) in combination with the following electron acceptors: nitrate (10 mM), nitrite (2 mM), thiosulfate (10 mM), sulfite (5 mM), ferrihydrite (20 mM), manganese oxides (10 mM), elemental sulfur, trimethylamine-N-oxide (TMAO, 5 mM), dimethylsulfoxide (DMSO, 5 mM), anthrachinone-2,6-disulfonic acid (AQDS, 0.5 mM), or humic acids (0.1 mg $\cdot$ ml^{-1}, Sigma-Aldrich).

Acid-soluble iron-free humic acid suspensions were prepared according to Benz *et al*. (1998). The use of AQDS as electron acceptor was indicated by a colour change, measured at a wavelength of 450 nm (Lovley & Coates, 1996). The formation of sulfide in assays with thiosulfate, sulfite or elemental sulfur was checked as described by Widdel (1980). Nitrite or ammonium were analysed photometrically (Grasshoff *et al*., 1999).

Na^+ requirement was checked in YPG medium with Na^+ replaced by K^+. Growth rates were determined in batch cultures in duplicates by measuring the turbidity at 436 nm (Spectronic 70, Bausch and Lomb, Rochester, NY).

Determination of growth yields

For the determination of growth yields, cells were grown under oxic conditions in 200 ml of artificial seawater supplemented with acetate (final concentration 10 mM). At the end of the exponential phase the OD_{436} of each batch culture was determined. Cells were washed twice with carbonate-free PBS buffer and freeze-dried (Alpha 2-4 Christ, Osterode, Germany). Total carbon contents were determined by combustion analysis on a CHNSO elemental analyzer (EA 1108, Carlo Erba Instruments, Rodena, Italy). Acetate consumption was determined by HPLC with UV detection according to Sass *et al*. (2002). For the calculation of the bacterial dry mass from carbon contents an overall composition of cellular biomass of $C_4H_7O_3$ (Widdel & Pfennig, 1981) was assumed.

Isolation of nucleic acids, PCR and sequencing

DNA was extracted and almost complete rRNA genes were amplified as described previously (Overmann & Tuschak, 1997) using the Bacteria-specific primers 8f and 1492r. The QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) was used to purify the PCR amplification products. Sequencing was performed as described by Süß *et al*. (2004) using the *DYEnamic Direct Cycle Sequencing Kit* (Amersham Biosciences, Germany) and an automated infrared laser fluorescence sequencer (*LiCor DNA Sequencing System 4200*, LiCor, USA).

Phylogenetic analysis

Sequences most closely related to the 16S rRNA gene sequences of the strains described in this study were searched for in GenBank using the BLAST program (Altschul *et al*., 1997). Sequence alignment and phylogenetic analyses applying different treeing methods (maximum likelihood and neighbour-joining) were performed using the ARB software package [http://www.mikro.biologie.tu-muenchen.de] (Ludwig *et al*., 2004). Alignment positions that differed in more than 50 % of all sequences were excluded from analysis to prevent ambiguous tree topologies. The 16S rRNA gene sequences of the strains SAM4^T, G5II^T, N05I, N5III and N5IV were deposited in EMBL under the accession numbers: AJ968647 to AJ968651.

Results

Isolation of bacteria

Among the isolates obtained from the highest positive dilutions of MPN series five strains turned out to be members of the *Roseobacter* clade. All of the isolates were characterized in detail. Strain SAM4^T was isolated from a MPN series (6×10^7 cells g^{-1} sediment) inoculated under oxic conditions with sediment from 5 cm depth at site Neuharlingersiel in October 1998. The other four strains originated from MPN series prepared in June 2002 (Köpke *et al.*, 2005). Strain N05I originated from the sediment surface (site Neuharlingersieler Nacken, MPN count 3×10^5 g^{-1}), strain N5III and strain N5IV from 5 cm depth (site NSN, MPN count 3×10^4 g^{-1}). Strain G5II^T was isolated from site Gröninger Plate (5 cm depth, MPN count 3×10^5 g^{-1}).

Morphology and motility

Strains G5II^T, N05I, N5III and N5IV formed smooth, convex and beige colonies when grown on marine broth or YPG agar. Older colonies of SAM4^T became pinkish on MB medium, but remained beige on YPG agar. All five strains were shown to be Gram-negative rods measuring between 0.5 x 1.5 and 0.9 x 3.0 µm (Tab. 1), with strain N05I being the only one forming rosettes (Fig. 1). Cells of strains N05I, G5II and SAM4 were motile by means of monotrichous flagella. Endospore formation was never observed. All isolates exhibited catalase activity and, with exception of strains N05I and N5IV, oxidase activity as well.

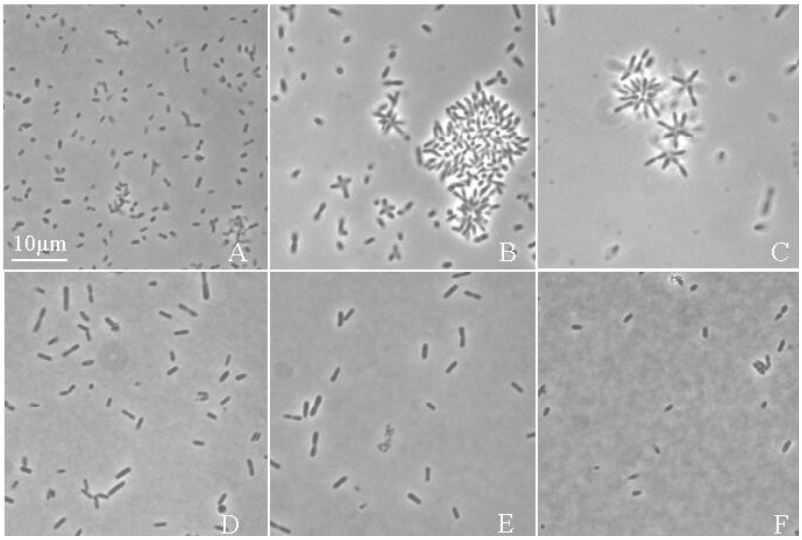

Fig. 1: Phase-contrast light micrographs of *Roseovarius pelophilus* G5II^T (A), strain N05I (B+C), N5III (D), N5IV (E) and *Roseobacter pelophilus* SAM4^T (F).

Bacteriochlorophyll *a* was detected only in older cells of strain SAM4^T grown in the dark or under diurnal light cycles. Spectra revealed characteristic peaks at 768 - 770 nm, 477 - 481 nm and a smaller one at 594 - 595 nm. A very similar spectrum was obtained with *Roseobacter denitrificans*^{T} as reference strain.

Growth characteristics

All five strains turned out to be typical marine bacteria growing at salinities from 0.3 to 0.7 and up to 7.2 to 10.2 % (Tab. 1). None of the strains grew in medium without added Na^+. With the exception of strain G5II^T which required at least pH 6.5 for growth, the strains grew from pH 6.0 to 9.0 (Tab. 1). All strains grew in a temperature range from 4°C to 30 or 35°C (Tab. 1). Maximal growth rates were in a range from 1.62 – 2.17 d^{-1} (Tab. 1). With strains SAM4^T and N05I fastest growth was obtained at 25°C, with strain G5II^T at 20°C, and with strains N5III and N5IV at 15°C, respectively. The strains strongly differed in respect to their growth rate over temperature profiles. For strain SAM4^T, N05I and G5II^T 'regular' curves were obtained with the growth rate slowly increasing with rising temperatures and rapidly dropping above T_{opt}. Strains N5III and N5IV, in contrast, showed in all investigations very untypical profiles (Fig. 2). Plotting log μ against the inverse temperature in the Arrhenius plot revealed a linear increase between 4 and 15 (strains G5II, N05I, N5III, N5IV) or 20°C (strain SAM4). At higher temperatures the increase in growth rate ceased indicating harmful effects at elevated temperatures (data not shown).

Strain SAM4^T showed more or less constant growth yields with values around 140 mg dry weight per l YPG medium at temperatures between 4 and 30°C (Fig. 2). At higher temperatures growth yield was significantly lower. Strains G5II^T and N05I showed a similar growth yield versus temperature relationship, but with reduced yields already above 25°C, and maximum yields around 160 and 210 mg dry weight per l YPG, respectively. Due to their temperature range for growth and their growth yield to temperature relationship, strain G5II^T, N05I and SAM4^T can be characterized as psychrotrophs (Morita, 1975; Isaksen & Jørgensen, 1996).

Strains N5III and N5IV showed relatively constant growth yields only between 15 and 30°C, with maximum values around 180 and 270 mg dry weight per l YPG, respectively. At temperatures below and above growth yield strongly declined. This growth yield to temperature relationship is typical for mesophilic microorganisms (Isaksen & Jørgensen, 1996). But since the two strains are growing at low temperatures, they can be characterized as psychrotolerant mesophiles (Sass *et al*., 1998).

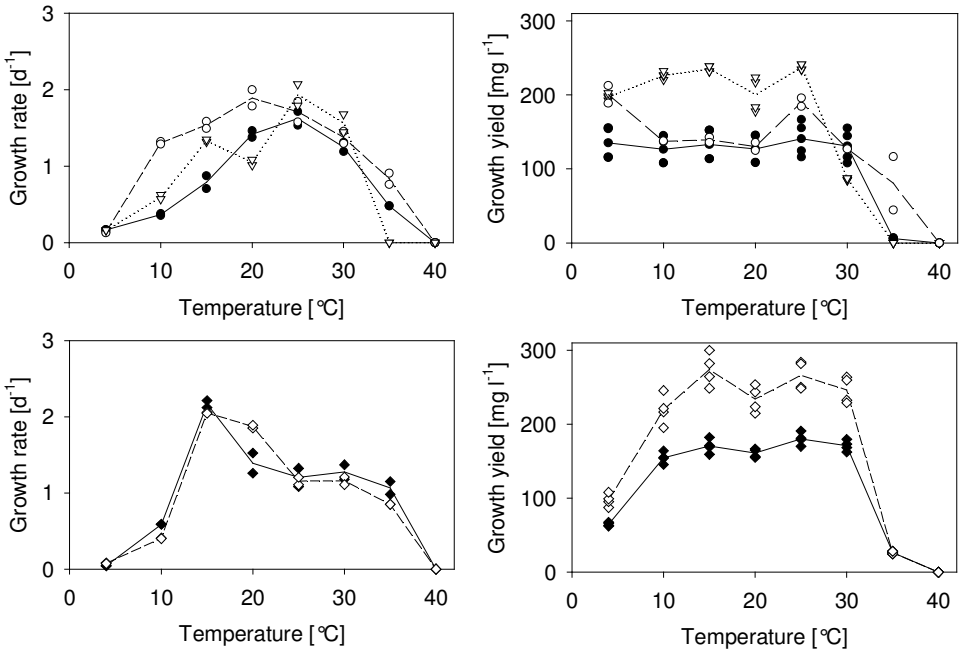

Fig. 2: Growth rates and growth yields over temperature for strains SAM4^T (filled circles), G5II^T (open circles), N05I (inverted triangles), N5III (filled diamonds) and N5IV (open diamonds. Symbols represent the average of two parallels.)

Physiological characteristics

All strains turned out to be nutritionally versatile, utilizing a wide range of substrates like peptone, carbohydrates, amino acids, carboxylic acids like lactate or other compounds like betaine (Tab. 1), while primary alcohols or aromatic compounds were not utilized. Among the polysaccharides tested, only xylan and laminarin were generally used (with the exception of strain G5II^T), while chitin and starch supported growth only in strain SAM4^T and cellulose was never utilized. None of the strains hydrolysed gelatine. While strains N5III and N5IV exhibited exactly the same substrate utilization pattern, pronounced differences were observed to and among the other isolates. For example gluconate, asparagine and glycolate were utilized only by strain SAM4^T, while strain N05I was the only one growing on isoleucine, tryptophane or nicotinate. None of the strains grew with the C_1-compounds formate and methanol.

Table 1: Phenotypical and physiological properties of the new isolates. None of the strains grew on cellulose, arabinose, methanol, ethanol, propanol, butanol, ethylene glycol, Tween 80, formate, crotonate, malonate, benzoate or salicylate.

Substrates	**SAM4^T**	**G5II^T**	**N05I**	**N5III**	**N5IV**
Cell size [µm]	0.7-1.5 x 1.4-3.0	0.6-1.2 x 1.5-2.0	0.8-1.5 x 1.5-2.3	0.7-1.5 x 1.4-2.4	0.5-0.9 x 1.3-1.9
Motility	+	+	+	-	-
G+C content [mol%]	56.4	57.7	54.7	ND	ND
pH range	6 - 9	6.5 - 9	6 - 9	6 - 9	6 – 9
Salinity range [%]	0.3 – 10.2	0.3 – 8.2	0.7 – 8.2	0.7 – 7.2	0.7 – 7.2
Growth substrates:					
Peptone	+	+	+	+	+
Casein hydrolysate	+	+	+	+	+
Yeast extract	+	-	+	+	+
Starch	(+)	-	-	-	-
Chitin	+	-	-	-	-
Xylan	+	-	+	+	+
Laminarin	(+)	-	+	+	+
Glucose	+	+	+	+	+
Fructose	+	+	-	+	+
Rhamnose	+	+	-	+	+
Mannose	+	+	+	+	+
Xylose	+	-	+	+	+
Mannitol	+	+	+	+	+
Gluconate	+	-	-	-	-
Glucosamine	+	+	+	-	-
Cellobiose	+	+	-	+	+
Maltose	+	+	+	+	+
Saccharose	+	+	-	+	+
Trehalose	+	+	-	+	+
Glycerine	+	+	+	+	+
Glycolate	+	-	-	-	-
Pyruvate	+	-	+	+	+
Lactate	+	-	+	+	+
2-Ketoglutarate	-	+	+	+	+
Citrate	+	-	+	+	+
Acetate	+	+	+	+	+
Propionate	+	-	-	-	-
Butyrate	-	-	+	+	+
Valerate	+	-	-	+	+
Hexanoate	+	-	+	+	+
Octanoate	+	-	+	+	+
Succinate	+	+	+	+	+
Fumarate	-	-	+	+	+
Malate	+	+	+	+	+
Tartrate	-	-	-	-	-
Alanine	+	-	+	+	+
Arginine	+	+	+	+	+
Cysteine	-	-	-	+	+
Glutamine	+	-	+	+	+
Isoleucine	+	-	+	-	-
Phenylalanine	-	+	+	+	+
Tryptophan	-	-	+	-	-
Proline	+	-	+	+	+
Betaine	+	+	+	+	+
Nicotinate	-	-	+	-	-

Growth under anoxic conditions

None of the strains turned out to be strictly aerobic. Anaerobic growth with acetate as electron donor was obtained with a single or in case of strains N05I, N5III and N5IV with two different electron acceptors. The electron acceptor most frequently reduced was trimethylamine-N-oxide (TMAO) used by strains SAM4^T, N05I, N5III and N5IV. Strains N05I, N5III and N5IV grew also with dimethyl sulfoxide (DMSO) as terminal electron acceptor. None of the strains grew with nitrate, nitrite, sulfite, elemental sulfur, thiosulfate or soluble ferric citrate, iron hydroxide, manganese oxides, AQDS or humic acids. Fermentative growth on glucose or casamino acids was not observed. All five strains grew on sulfide-reduced anaerobic marine broth agar. Generally, anaerobic growth on mineral medium ceased after one to two transfers, but persisted slightly longer on anaerobic marine broth. An exception was strain SAM4^T that showed more sustained anaerobic growth. However, as indicated by the colourless redox indicator resazurine, the positive assays were indeed oxygen-free.

Cellular fatty acid profiles

The principal fatty acid in whole cell extracts of all five strains was *n*-18:1ω7c (80 to 92 %). Other characteristic (>1 %) fatty acids were *n*-16:0, *n*-16:1ω7 and *n*-18:0.

Molecular phylogenetic analysis

Phylogenetic analyses of almost complete 16S rRNA genes revealed that all five strains affiliate with four distinct clusters belonging to the *Roseobacter* clade within the Alphaproteobacteria. Strain SAM4^T turned out to be most closely related to *Roseobacter litoralis*^{T} (96.6 % similarity), *Rsb. denitrificans*^{T} and *Sulfitobacter mediterraneus*^{T} (96.3%). Strain G5II^T clustered within the genus *Roseovarius* with *Rsv. crassostreae*^{T} (97.8 % similarity) as closest relative. The 16S rRNA gene sequences of N5III and N5IV were almost identical and shared highest similarity to *Ruegeria atlantica*^{T} (99.7 %), while the phylogenetic affiliation of strain N05I was less obvious. Closest relatives of strain N05I were *Phaeobacter inhibens* (97.5 % similarity), *Roseobacter gallaeciensis* and *Leisingera methylohalidivorans* (97.3 %).

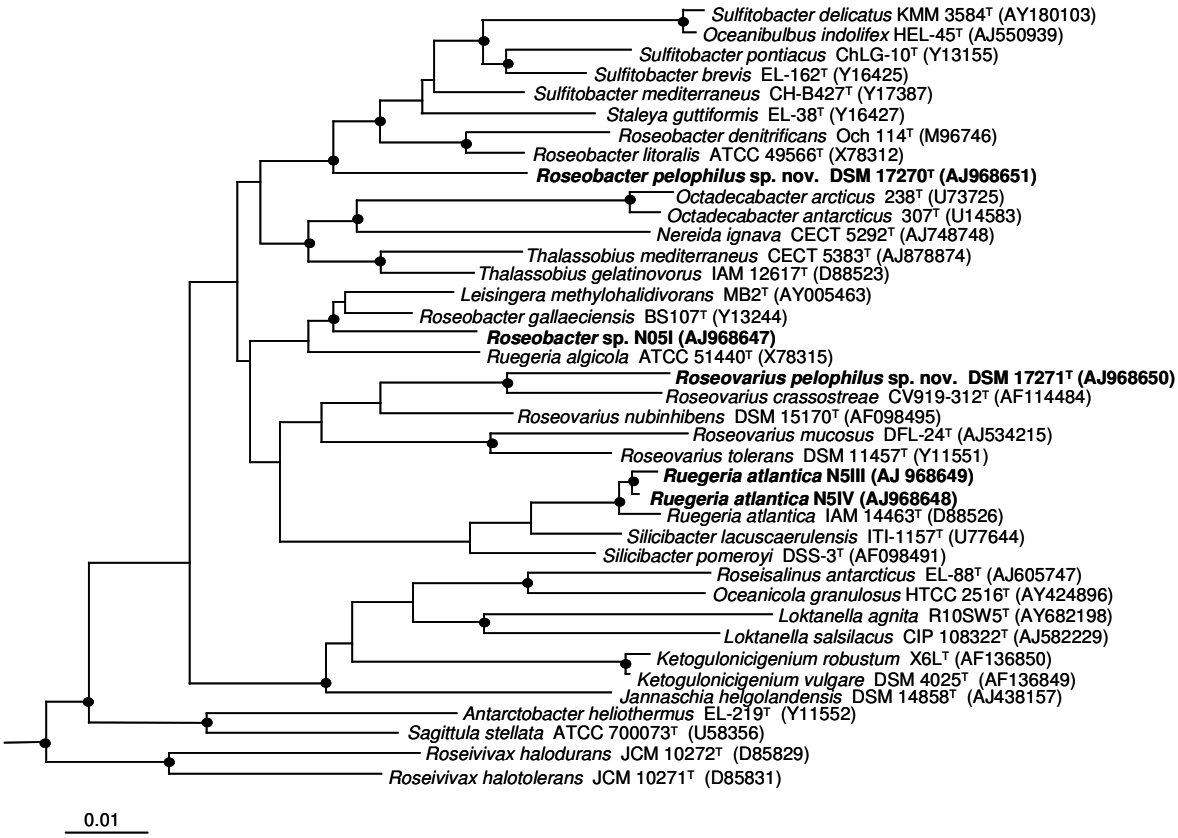

Fig. 3: Neighbour-joining tree based on 16S rRNA sequences showing the phylogenetic position of the two new species *Roseobacter pelophilus* SAM4^T, *Roseovarius pelophilus* G5II^T and of the strains N05I, N5III and N5IV within the *Roseobacter* clade of the *Alphaproteobacteria*. Solid circles indicate that the corresponding nodes were also present in the maximum-likelihood tree. The tree was rooted by using several *Gammaproteobacteria* as outgroup. Accession numbers are given in parentheses.

The phylogenetic tree presented in Fig. 3 represents a maximum-likelihood tree representative for all phylogenetic trees obtained. The arrangement of the different subclusters and the position of the single strains did hardly change regardless of the method (maximum-likelihood, neighbour-joining) used.

Discussion

In the present work five bacterial strains isolated from surface sediments of a tidal flat and belonging to the *Roseobacter* clade were investigated for their phylogenetic affiliation and their metabolic capacities. The strains grouped with four subclusters of the *Roseobacter* clade and affiliated with the genera *Roseobacter*, *Roseovarius* and *Ruegeria*. All strains grew under anoxic conditions either by use of terminal electron acceptors like TMAO or DMSO, or by fermentation.

Anaerobic metabolism in *Roseobacter* clade bacteria

Although the *Roseobacter* clade is known as one of the dominant groups of marine bacterioplankton (Eilers *et al*., 2000; Selje *et al*., 2004), they were repeatedly detected in sediments as well (Süß *et al*., 2004; Köpke *et al*., 2005). Gonzalez *et al*. (1999) estimated that members of the *Roseobacter* clade can account for even up to 11 % of the 16S rRNA gene copies in coastal sediment communities. This numerical predominance cannot be explained by the assumption that strictly aerobic bacteria survive during burial or that oxic microlayers are present around the burrows of the macrozoobenthos.

Members of the *Roseobacter* clade have been shown to be metabolically versatile. They were suggested to be involved into the degradation of aromatic compounds (Buchan *et al*., 2000) or hydrocarbons (Johnson & Hill, 2003). They have been shown to oxidize manganese (Hansel & Francis, 2006) or carbon monoxide (Buchan *et al*., 2000) and by degrading dimethylsulfoniopropionate (DSMP) to play an important role in the marine organic sulfur cycle (González *et al*., 2003; Kiene *et al*., 1999). But since *Roseobacter* clade bacteria are still considered as strict aerobes, the strains described here expand the range of metabolic capacities found within this group. The strains grew by fermentation on complex media and used TMAO and DMSO as terminal electron acceptors, which are common degradation products of biomass in the marine environment.

However, although visible colonies were formed under clearly anoxic conditions, these generally became smaller in subcultures until subculturing under anoxic conditions ceased at all. On complex marine broth anaerobic growth was more sustainable than in mineral medium. At the moment we can only speculate what is the reason for this growth behaviour, but it might indicate the failure of an assimilatory pathway under anoxic conditions and that it partly can be at least partly compensated by the use of complex media.

Phylogenetic affiliation of the isolates

Strain SAM4^T shared high similarities in 16S rRNA gene sequences with members of two different genera: *Roseobacter* and *Sulfitobacterium*. However, based on phenotypic and physiological straits, strain SAM4^T resembles more *R. litoralis* and *R. denitrificans*. While *Sulfitobacter mediterraneus* does not produce bacteriochlorophyll *a* (Pukall *et al*., 1999), *Roseobacter* spp. were described as aerobic, bacteriochlorophyll *a*-containing anoxygenic phototrophic bacteria (Shiba, 1991). Colony morphology and pigmentation, as well as the molar DNA G+C content of strain SAM4^T is more similar to that of *Roseobacter litoralis* (Shiba, 1991) than to that of *Sulfitobacter mediterraneus* (Pukall *et al*., 1999). However, the ability to grow under anoxic conditions separates strain SAM4^T from *R. litoralis*. Therefore, strain SAM4^T is proposed to be the type strain of a new species, *Roseobacter pelophilus* sp. nov.

Phylogenetic analysis of the 16S rRNA gene sequence of strain G5II^T clearly revealed its affiliation with the genus *Roseovarius*, what is supported by several phenotypic features like colony pigmentation, requirement for Na^+ and the lack of bacteriochlorophyll *a*. However, despite its high similarity (97.8 % in the 16S rRNA gene) to *Roseovarius crassostreae*, and its common capacity to use alternative electron acceptors, there is a range of physiological capacities which clearly separate strain G5II^T from this species (Boettcher *et al*., 2005). Strain G5II^T can be classified as a psychrotroph, while *R. crassostreae* is a clearly mesophilic bacterium not growing at 4˚C. In contrast to *R. crassostreae*, which exhibits a very limited number of growth substrates, strain G5II^T grows on monosaccharides, some short-chain fatty acids and on compounds like citrate, lactate or succinate. Based on these metabolic differences, we propose to establish a new species *Roseovarius pelophilus* sp. nov. with strain G5II^T as the type strain.

Based on their 16S rRNA gene sequences, strains N5III und strain N5IV clearly affiliated with *Ruegeria atlantica*, what is supported by very similar substrate utilization patterns (Rüger & Höfle, 1992; Uchino *et al*., 1998). Strain N05I showed similarly high similarities (>97.3 %) on the 16S rRNA gene level to the genera *Phaeobacter* and *Leisingera*. However, due to its phenotypic features and its physiological capacities, strain N05I strongly resembles *P. gallaeciensis* and *P. inhibens* (Martens *et al*., 2006), but showed almost no overlap with *L. methylhalidovorans* which is characterized by a very restricted substrate spectrum (Schaefer *et al*., 2002). Because strain N05I shares this high similarities with the already described *Phaeobacter* species, it obviously does not represent a new species, but can be seen as a strain of *P. gallaeciensis*.

Description of *Roseobacter pelophilus* sp. nov. (SAM4)

Roseobacter pelophilus (pe.lo'hi.lus. Gr. n. *pelos* mud; Gr. adj. *philus* loving; M.L. adj. *pelophilus* mud-loving).

Gram-negative rods, 0.7-1.5 µm wide and 1.4-3.0 µm long. Motile by means of a polar flagellum. Colonies on marine broth agar plates are beige to pinkish. The molar G+C content is 56.4 mol%. Temperature range for growth 4 to 35°C with optimum growth at 25°C. Growth was observed between pH 6 to 9 and at salinities from 0.3 to 10.2 %. Requires Na^+. Substrates used aerobically are: peptone, casamino acids, yeast extract, starch, chitin, xylan, laminarin, saccharose, cellobiose, maltose, trehalose, rhamnose, xylose, fructose, glucose, mannose, mannitol, gluconate, glucosamine, acetate, propionate, valerate, hexanoate, octanoate, succinate, malate, glycolate, pyruvate, lactate, citrate, glycerine, alanine, arginine, glutamine, isoleucine, proline and betaine. Under anoxic conditions trimethylamine-N-oxide (TMAO) serves as an electron acceptor. Cells exhibit oxidase and catalase activity and do contain bacteriochlorophyll *a*. Major cellular fatty acids are *n*-18:1ω7 and *n*-16:0.

The type strain of the species is strain SAM4^T (= DSM 17270^T, = LMG 23018^T) was isolated from tidal flat sediment on the German North Sea coast.

Description of *Roseovarius pelophilus* sp. nov. (G5II)

Rsv. pelophilus (pe.lo'hi.lus. Gr. n. *pelos* mud; Gr. adj. *philus* loving; M.L. adj. *pelophilus* mud-loving).

Cells are Gram-negative rods, 0.6-1.2 µm wide and 1.5-2.0 µm long possessing a polar flagellum. Forms beige colonies on agar. The molar G+C content is 57.7 mol%. Temperature range for growth is 4 to 35°C with optimum growth at 20°C. Grows between pH 6.5 and 9, and at salinities from 0.3 to 8.2 %. Requires Na^+. Substrates used for aerobic growth are: peptone, casamino acids, saccharose, cellobiose, maltose, trehalose, rhamnose, fructose, glucose, mannose, mannitol, gluocsamine, acetate, succinate, malate, 2-ketoglutarate, glycerine, arginine, phenylalanine and betaine. Cells exhibit oxidase and catalase activity. Bacteriochlorophyll *a* was not detected. Major cellular fatty acids are *n*-18:1ω7 and *n*-16:0.

The type strain of the species is strain G5II^T (= DSM 17271^T, = LMG 23019^T) was isolated from tidal flat sediment on the German North Sea coast.

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Kapitel IV

Presence and activity of microbial populations in the subsurface of a tidal flat

Presence and activity of microbial populations in the subsurface of a tidal flat

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Key words: tidal sediment, sulfate reduction, exoenzymes, subterranean estuaries

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Abstract

Microbiological and biogeochemical investigations of tidal flats focussed on the upper tens of centimetres, the assumed zone of highest activities, whereas deeper layers remained more or less neglected. To overcome this lack of data, an integrated study, combining microbiological, geochemical, and sedimentological methods was carried out with up to 550 cm long sediment cores taken in summer 2002, autumn 2003 and winter 2004 from two sites in the Wadden Sea (southern North Sea). The two sampling sites were similar with respect to their lithological settings along the upper 200 cm but clearly differed beneath. While the bottom layers at site Gröninger Plate (GP) consistent of sand, at site Neuharlingersieler Nacken (NSN) a layer of mussel shells and mud-dominated sediments were found. These muddy layers were highly enriched in total organic carbon (up to 2.2 %), total sulfur (up to 1.8 %), reactive iron and manganese. At both sites, sulfate was depleted within 100 to 150 cm, but showed non-standard gradients a greater depth. At site NSN the shell layer acted as an aquifer supplying a deep sulfate peak, while at site GP sulfate was steadily increasing from about 300 cmbsf downwards. The isotopic composition of organic matter revealed a significant contribution of marine sources to the TOC in surface-near layers ($\delta^{13}C$ around –20 ‰) and the presence of predominantly terrigenous material in the deeper layers. Highest sulfate reduction rates (up to 680 nmol cm^{-3} d^{-1}) were measured close to the sediment surface. While sulfate reduction rates dropped significantly, potential exoenzyme activities remained often in the same order of magnitude along the uppermost 100 cm. This discrepancy between absolute and potential rates indicates the presence of a potentially active but substrate-limited microbial community.

Introduction

Tidal flats are in general highly productive ecosystems characterized by intense primary production in the water column and the benthos (Gillespie and MacKenzie, 1981, Poremba *et al.,* 1999). Microbial processes in the sediment show a vertical sequence of terminal electron acceptors used that follows the decreasing redox potentials (Froelich *et al.,* 1979). Oxygen is generally depleted within the uppermost few millimeters, followed by iron and manganese reduction that possibly can reach rates comparable to sulfate reduction (Thamdrup *et al.* 1994) but are confined to surface-near layers. In the reduced layers, the microbial degradation of organic matter proceeds mostly via dissimilatory sulfate reduction produced hydrogen sulfide (Jørgensen, 1982, Skyring, 1987, Llobet-Brossa *et al*, 2002). Most of these reduced sulfur is removed from porewater by reoxidation (up to 90 % of the produced hydrogen sulfide), precipitation as iron sulfide and to sometimes by the release into the overlying water body or the atmosphere (Aller and Rude 1988, Jørgensen, 1982, Kristensen *et al.*, 2000). The activity of sulfate-reducing bacteria is generally controlled by temperature but mostly by the availability of organic matter (Koretsky, 2003, Kristensen *et al.*, 1998, Schubert *et al*, 2000, Vosjan, 1974). While microbial communities inhabiting surface near sediment layers are more or less constantly supplied with easily degradable organic matter by sedimentation, communities within the deeper layers have to deal with more recalcitrant organic material. Accordingly, microbial activities strongly decrease already within the uppermost tens of centimetres and most studies so far focused on the upper 30 to 50 cm (Böttcher *et al.,* 1998b, Freitag *et al.,* 2003), while only a few biogeochemical investigations extended to greater depths in coastal sediments (Goldhaber *et al.*, 1977, Chambers *et al.*, 2000, Thomsen *et al.* 2001).

However, during the last years the discovery of the deep biosphere and its extension to several hundreds of meters below the seafloor (Parkes *et al.*, 1994) showed that subsurface sediments inhabit microbial communities that are more active than expected. The extent of the activities was shown to depend highly on the lithological settings and the depositional history (D'Hondt *et al.*, 2004, Parkes *et al.*, 2005). Highest activities were often found at lithological or geochemical interfaces. Tidal areas are highly dynamic systems with resuspension, small-scale and short-time changes in depositional and non-depositional areas (Hild, 1999) and can be expected to show some of the features observed in the deep biosphere on a smaller scale. These aspects lead to a strong patchiness with respect to lithological parameters. To reveal how this affects microbial communities and their activities from the surface to the subsurface layers of tidal sediments an integrated

investigation of up to 550 cm long sediment cores was performed, combining microbiological (total and viable cell counts), biogeochemical (sulfate reduction and potential exoenzyme activities), as well as sedimentological and geochemical (TOC, $\delta^{13}C$) methods. Since this study was accompanied by a detailed investigation of the composition of the microbial communities (Köpke *et al.*, 2005, Wilms *et al.*, 2006a, b, c), it gives a good overview of the presence and performance of microbial communities in subsurface sediments of a tidal flat.

Materials and methods

Study area and sampling

Sediment samples were taken from the backbarrier area of the island Spiekeroog in the Wadden Sea, on the German North Sea coast. Sediment cores were taken at low tide from sites Neuharlingersieler Nacken (53°43.270´N, 7°43.718´E) and Gröninger Plate (53°43.638´N, 07°45.960´E) in June 2002, September 2003 and February 2004 (Fig. 1). Six-meter long aluminum tubes were driven into the sediment using a vibro corer. After retrieval, the aluminum liners were cut longitudinally. The freshly exposed and potentially contaminated surface was carefully removed with sterile spatula and samples were taken from beneath using sterile plastic syringes with cut-off tips. For depth profiles the sediment surface was defined as zero.

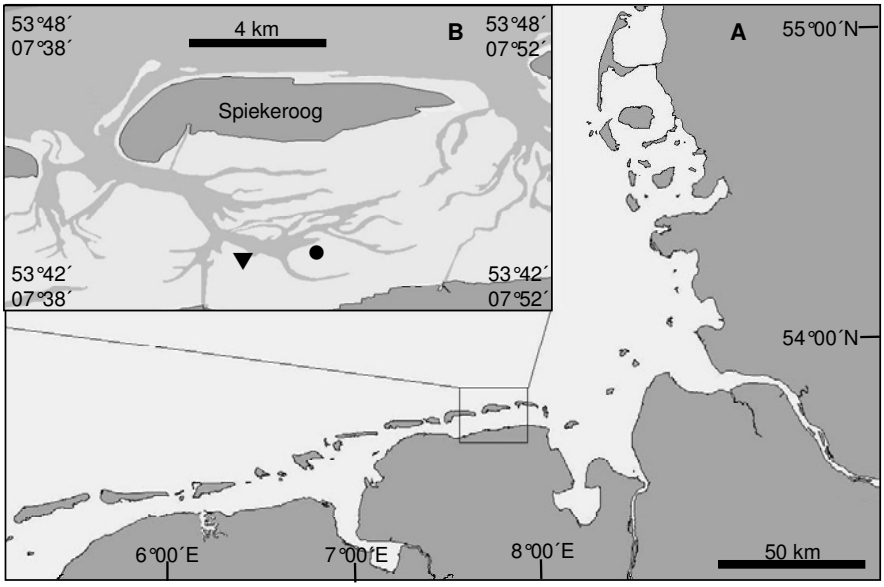

Fig. 1. Map of the southern North Sea [A] and the backbarrier area of the island Spiekeroog [B]with the sampling sites Neuharlingersieler Nacken [▼] and Gröninger Plate [●].

Physicochemical parameters

Temperature was measured at the sediment surface and at the bottom of the core immediately after sampling by use of a temperature probe. Porewater was gained as described by Rütters *et al.* (2002). Porewater chloride and sulfate concentrations were analyzed by ion chromatography with conductivity detection (Sykam, Gilching, Germany; Bak *et al.,* 1991). Samples were diluted with eluent prior to analyses (Sass *et al.*, 2001).

Porewater ammonium concentrations were determined photometrically according to Grasshoff *et al.* (1999).

Total carbon and sulfur were analyzed by combustion in an element analyzer (Leco CS-444, St. Joseph, MI). The total organic carbon content (TOC) was determined as the difference between total carbon and inorganic carbon measured with a CO_2-coulometer (UIC, Joliet, IL).

Dissolved organic carbon (DOC) was analyzed with a Multi N/C3000 instrument (Jena AG, Germany). 400 µl of porewater was diluted with water, acidified with 2N HCl and purged with synthetic air to remove inorganic carbon. The samples were combusted at 850°C in synthetic air with CeO_2 as catalyst. CO_2 was detected by a non-dispersive infrared detector. For external calibration a dilution series of a DOC standard (CertiPUR®, Merck, Germany) was treated and analyzed in the same way as the porewater samples. The isotopic composition of bulk organic matter ($\delta^{13}C$) was analyzed on an elementar analyzer (Carbo Erba EA 1108, Milan, Italy) coupled to an isotope-ratio monitoring mass spectrometer (Finnigan MAT 252, ThermoFinnigan, Waltham, MA) via a Conflo II split interface after removal of carbonate by acid treatment.

Grain-size distributions were determined using a laser particle sizer (Fritsch Analysette 22, Idar-Oberstein, Germany). Proportions of different grain size fractions are given as vol%.

Reactive iron and manganese pools were quantified by extraction using buffered Na-dithionite solution (Thamdrup *et al.,* 1994). Concentrations of reactive iron were determined spectrophotometrically using a ferrozine-HEPES solution (modified after Stookey, 1970) after reduction with hydroxylaminehydrochloride. Concentrations of reactive manganese were analyzed by atomic absorption spectrometry (PE 4100, Perkin Elmer, Wellesley, MA).

Total cell counts

Total cell counts were determined by epifluorescence microscopy. Samples were fixed by the addition of sterile glutaric dialdehyde (0.2 µm filtered, 2 % final concentration) and were stored at 4°C in the dark. After the addition of sterile-filtered Tween 80 (0.01 % final concentration) to the fixed sediment slurries, samples were ultrasonicated (3 x 10 s). Staining of sediment samples with DAPI (4,6-Diamidino-2-phenylindol) and counting were performed according to Köpke *et al.* (2005).

Viable counts

Viable counts for heterotrophic aerobes and anaerobes were determined by the most-probable-number (MPN) method. For the preparation of sediment slurries and MPN series an artificial seawater medium was used. The oxic medium contained the following components: NaCl (24.3 $g \cdot l^{-1}$), $MgCl_2 \cdot 2\ H_2O$ (10 $g \cdot l^{-1}$), $CaCl_2 \cdot 2\ H_2O$ (1.5 $g \cdot l^{-1}$), KCl (0.66 $g \cdot l^{-1}$), Na_2SO_4 (4 $g \cdot l^{-1}$), KBr (0.1 $g \cdot l^{-1}$), H_3BO_3 (0.0025 $g \cdot l^{-1}$), $SrCl_2 \cdot 6\ H_2O$ (0.04 $g \cdot l^{-1}$), NH_4Cl (0.021 $g \cdot l^{-1}$), KH_2PO_4 (0.0054 $g \cdot l^{-1}$), NaF (0.003 $g \cdot l^{-1}$). The medium was buffered by HEPES (2.38 $g \cdot l^{-1}$) and supplemented with 1 $ml \cdot l^{-1}$ of trace element solution SL 10 and 0.2 $ml \cdot l^{-1}$ of a selenite and tungstate solution (Widdel and Bak, 1992). The pH of the oxic medium was adjusted to 7.2 – 7.4 with NaOH prior to autoclaving. After autoclaving, the oxic medium was cooled down under air and finally supplemented with 10 ml of a vitamin solution (Balch *et al.,* 1979) and sodium bicarbonate (0.2 $g \cdot l^{-1}$) from sterile stocks.

The anoxic medium differed in the way that sodium bicarbonate and CO_2 replaced HEPES as the buffer system. After autoclaving, the medium was cooled down under an atmosphere of N_2/CO_2 (80/20, v/v). Per liter of medium 10 ml of a vitamine solution and 30 ml of a CO_2-saturated sodium bicarbonate solution (1 $mol \cdot l^{-1}$) were added from sterile stocks. The anoxic medium was reduced by the addition of sodium sulfide (1.2 $mmol \cdot l^{-1}$ final concentration) and ferrous chloride (0.5 $mmol \cdot l^{-1}$ final concentration) from sterile stocks. If necessary, the pH was adjusted to 7.2 – 7.4 by addition of sterile HCl or Na_2CO_3.

A monomer mixture served as electron donor and carbon source for all MPN series (Köpke *et al.* 2005). It contained the 20 common L-amino acids, the short-chain fatty acids formate, acetate, propionate, butyrate, valerate, caproate, the alcohols methanol, ethanol, *n*-propanol, *n*-butanol, as well as malate, fumarate, succinate, lactate, glycerol, and glucose (0.1 $mmol\ l^{-1}$, each).

MPN series were inoculated with sediment slurries. These were prepared immediately after sampling by addition of approximately 2 cm^3 of sediment to 18 ml of anoxic artificial seawater. Subsamples of these slurries were pasteurised (70°C, 15 min) and were used for determination of viable counts of bacteria present as endospores.

MPN series for anaerobic microorganisms were prepared in an anaerobic chamber using polypropylene deep well plates (Beckman, Fullerton, CA) containing 900 µl of medium per well. As inoculum, 100 µl of sediment slurry were added to each well of the first dilution, and diluted in tenfold steps. After inoculation, the plates were covered with sterile lids (CAPMAT, Beckman, Fullerton, CA) sealing each well separately. The MPN

plates were put into gas-tight plastic bags equipped with a gas generating and catalyst system for anoxic conditions (Anaerocult C mini, Merck, Germany).

MPN series for aerobic microorganisms were prepared within a microbiological cabinet using polypropylene deep well plates as well, but were equipped with sterile cover plates allowing oxygen diffusion into the single wells. Finally the plates were wrapped with gas-permeable film to avoid water loss by evaporation.

MPN series were set up with five parallels each, and with two non-inoculated dilution series per plate that served as control. The incubation temperatures were chosen to reflect the *in situ* conditions. MPN series prepared in June 2002 were incubated for 12 weeks at 20°C, those prepared in October 2003 for 12 weeks at 15°C. Some MPN series prepared in February 2004 were subjected to a temperature shift. MPN series that were inoculated with samples from the surface to 300 cm depth were incubated at 4°C for the first eight weeks to select for psychrophilic bacteria and then for another eight weeks at 10°C to support growth of mesophilic bacteria. Series inoculated with samples from layers beneath were incubated at 10°C for the whole period. Growth in MPN series from February 2004 was checked first after eight weeks and a second time after 16 weeks.

MPN series were analyzed for growth by microscopy as well as by staining with Sybr Green I and subsequent analysis on a microtiter plate reader (BMG Labtechnologies, Offenburg, Germany) as described by Martens-Habbena and Sass (2006). For analyses, 200 µl from each well of the MPN plates were transferred into a black plate (NUNC, Wiesbaden, Germany) and 50 µl of a Sybr Green I solution (2000 fold dilution in 200 mM Tris and 50 mM EDTA, pH 8) were added. Fluorescence was measured after 12 h incubation in the dark at 4°C.

Measurement of potential exoenzyme activities

Potential exoenzyme activities were measured using substrate analogues labeled with 4-methylumbelliferone (MUF) or 7-amino-4-methylcoumarin (MCA) (Hoppe, 1983). The exoenzymes N-acteyl-glucosaminidase, β-glucosidase and alkaline phosphatase were assayed by use of MUF-N-Acetyl-β-D-glucosaminide, MUF-β-D-glucoside and MUF-phosphate (SIGMA, Deisenhofen, Germany), respectively. Aminopeptidase activity was analyzed using MCA-labelled L-leucine (SIGMA). For the preparation of slurries, 50 g of sediment were homogenized in 50 ml anoxic artificial seawater. Sediment slurry (1.4 ml) was transferred into autoclaved 10 ml vials containing stirring bars. The enzymatic reaction was started by the addition of 100 µl substrate analogue solution, yielding final

concentrations of 50 µM MUF-N-Acetyl-β-D-glucosaminide, 550 µM MUF-β-D-glucoside 550 µM MUF-phosphate or 10 µM MCA-labelled L-leucine (Coolen and Overmann, 2000). All analyses were carried out in triplicate. The vials were sealed with sterile butyl rubber stoppers and flushed with nitrogen. Samples were incubated at 20°C on a stirrer (250 rpm) for 0.5 h (MUF-phosphate), 1 h (MUF-N-Acetyl-β-D-glucosaminide), 2 h (MCA-labelled L-leucine) or 4 h (MUF-β-D-glucoside), respectively. Reaction in assays containing MUF-labelled substrate analogues were stopped by the addition of 100 µl NaOH (final concentration 40 mM) and 100 µl Na_4EDTA (final concentration 100 mM), the latter to prevent precipitation of carbonates. Samples with MCA-labelled substrate analogues in contrast, received 160 µl of acetone. Slurry subsamples were transferred into 2 ml tubes and centrifuged for 5 min at 10,000 × g. Aliquots (200 µl) of the supernatant were transferred into a black microtiter plate and the concentrations of free fluorophors were determined on a microtiter plate reader (excitation 360 nm, emission 460 nm, BMG Labtechnologies, Offenburg, Germany). For calibration, MUF and MCA standards in artificial seawater were added to sediment samples in concentrations from 0.025 to 2.5 µM. The amount of fluorophor adsorbed to the sediment matrix was calculated as the difference between the total amount added and the amount retrieved in the supernatant. Quenching of fluorescence by substances released from the sediment samples was determined in blanks spiked with MUF (5 mM) or MCA (5 mM).

Three different blanks were prepared to test background fluorescence and non-enzymatic hydrolysis (Coolen and Overmann 2000). Blank B1 received no substrate analogue and was targeting fluorescent compounds extracted from the sediment without boiling. Non-enzymatic hydrolysis of substrate analogues was analyzed in blank B2 by boiling for 30 minutes before incubation in order to inactivate enzymes. The release of fluorescent compounds by the sediment during boiling was determined in blank B3 that was incubated without addition of substrate analogue.

Determination of sulfate reduction rates

Sulfate reduction rates were determined by the use of radiotracers and the application of the core injection method (Jørgensen, 1978). Sediment subcores, usually in triplicates, were taken aseptically using sterile 5 ml plastic syringes with cut-off tips. Syringes were sealed with butyl-rubber stoppers and stored for transport in gas-tight plastic bags equipped with a gas generating and catalyst system for anoxic conditions (Anaerocult C mini, Merck, Germany). After preincubation for 24 h at *in situ* temperature, $^{35}SO_4^{2-}$ was injected

into the subcores (23 MBq per 20 µl, Amersham International, Braunschweig, Germany). The subcores were incubated for four days at *in situ* temperature. The incubation temperatures were chosen to reflect the in situ conditions. Samples taken in October 2003 were incubated at 15°C. Due to the temperature gradient observed in February 2004, samples from the surface down to 300 cm depth were incubated at 4°C, while for the deeper samples 10°C was chosen. Incubation was terminated by transfer into 10 ml of 20% zinc acetate solution.

The total amount of ^{35}S reduced was quantified by a single-step chromium reduction and destillation method as described by Kallmeyer *et al.* (2004). For this, samples were centrifuged for 10 min at 5000 x g and the supernatant was transferred into 25 ml tubes. Up to 4 g sediment were transferred into a 3-neck round-bottom glass flask that was placed on a magnetic stirrer and 20 ml dimethylformamide (DMF) and 500 µl of a 50 mM ZnS suspension were added. The headspace was flushed with nitrogen for 10 min. Subsequently, 8 ml of 6 M HCl were injected, followed by 16 ml of 1 M $CrCl_2$ solution. The samples were incubated for 2 h while sparged with nitrogen and continuously stirred. The liberated sulfide was trapped as zinc sulfide in 7 ml of 5 % (w/v) ZnAc-solution. Radioactivity was measured using a liquid scintillation counter (1415, Wallac, Turku, Finland) with a counting window of 4 to 167 keV and without luminescence correction. The scinitillation cocktail used was Lumasafe Plus® (Lumac, Landgraaf, The Netherlands) that was mixed with the trapped ZnS 2:1 (v/v).

Modelling of porewater sulfate variations

Depth profiles of porewater sulfate concentrations were interpreted using the PROFILE model (Berg *et al.,* 1998), considering steady-state conditions:

$$d/dx\left(\phi D_S\, d\left[SO_4^{2-}\right]/dx\right)+\phi\alpha\left(\left[SO_4^{2-}\right]_0-\left[SO_4^{2-}\right]\right)+SRR=0 \qquad [1]$$

with $[SO_4^{2-}]$ being the sulfate concentration in the porewater, $[SO_4^{2-}]_0$ is the bottom water concentration, x is the depth, ϕ is the porosity, and SRR is the net rate of sulfate consumption per unit volume of the sediment. The irrigation coefficient α was neglected in the calculation. D_S is the molecular diffusivity in seawater corrected for tortuosity according to Iversen and Jørgensen (1993):

$$D_S = D_{SW}/\left(1+3\left(1-\phi\right)\right) \qquad [2]$$

Values for diffusion coefficients (D_{SW}) at *in situ* temperatures, are given by Schulz (2000).

Statistical analysis

Potential correlation between enzyme activities, sulfate reduction rates and physicochemical settings was estimated by calculating the spearman rank correlation coefficient using the software package R-2.2.1 (http://cran.r-project.org/mirrors.html). This methode was used because it allows analysing data sets with a relatively low number of samples and that are not follow a normal curve of distribution.

Results

Sampling campaign and physical data

Sediment cores were taken in June 2002, October 2003, and February 2004. From both sampling sites up to 550 cm long cores were recovered. Generally, cores from a single site were similar with respect to their lithological depth sequence. Minor depth differences of the vertical sequence of sediment layers may result from small-scale patchiness, the uncertainty of the GPS site coordinates, or changes in sedimentation during the years. Along the uppermost 200 cm of the sediment, similar lithological features and geochemical gradients were observed among both sampling sites, while they clearly differed with respect to the layers beneath.

At both sites, surface temperature was 20°C in summer 2002 and 15°C in autumn 2003, with 10°C at the bottom of each core. In winter 2004 the two cores exhibited a surface temperature of 4°C, but differed with respect to their bottom temperature being 4°C at site NSN and 10°C at site GP, respectively. Daily changes of up to 10°C can be expected for the upper 10 cmbsf (cm below surface) during day-night cycles (Bosselmann *et al.,* 2003). The sediment surface was permanently oxidized, with oxygen penetrating between 2 and 4 mm into the sediment (data not shown).

Site Neuharlingersieler Nacken

Solid phase analysis

Visual examination of the sediment cores revealed three main zones that differed with respect to their lithological characteristics. This division was confirmed by detailed analyses of grain size distribution that was carried out for the cores taken in June 2002 and October 2003.

The top 175 to 200 cm of the sediment cores were sand-dominated with a few thin muddy intercalations and were suggested to be deposited in sand to mixed flat environments (Chang *et al.,* 2003). In 2002, within the upper sandy layers the so-called mud fraction (<63 µm) represented less than 27 %. In 2003 the upper sediment layers were less uniform with few narrow layers containing up to 73 % mud. Beneath this sandy layers a highly porous zone, dominated by sand and mussel shells, extended down to 250 cmbsf. This section presumably represents a part of an old tidal channel deposit. However, within this layer, the mud content slowly started to increase with further depth. The bottom layers, in turn, consisted almost entirely of mud and clay (Fig. 2). The presence of shell debris and

fibrous plant residues indicates that this muddy sediment was deposited in a salt marsh environment (Reineck, 1982, Chang *et al.*, 2003). Water content of the sand-dominated and shell layers ranged from 20.3 % to 30.1 %, while in the bottom mud layers up to 37.1 % was detected.

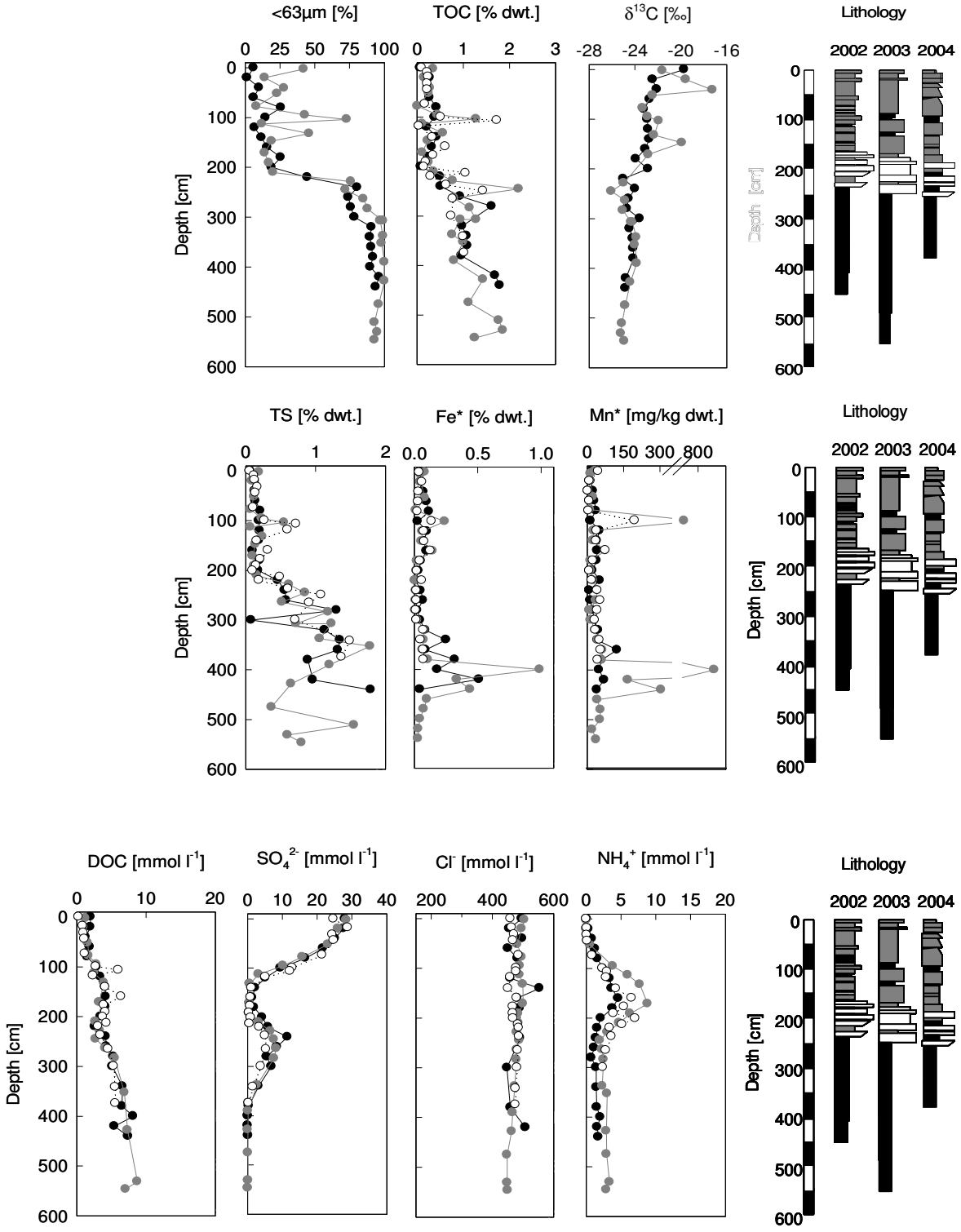

Fig. 2: Depth distribution of solid phase and porewater parameters (left) and plots of sedimentary structures (right) at site NSN. Values are plotted June 2002 (black dots), October 2003 (gray dots) and February 2004 (white dots). Sedimentary structures were divided into sand dominated layers (gray), shell-containing layers (white), and mud dominated layers (black).

Similar, to the lithological settings, depth profiles of TOC content and stable carbon isotope ratio showed only minor variations between the cores taken during the different sampling campaigns. TOC contents followed the lithological settings and were lowest at the sediment surface and in about 200 cmbsf. Within the sand dominated layers down to the upper shell layers, TOC values generally ranged from 0.17 to 0.44 % and were elevated only within some thin mud intercalations. Correspondingly, TOC contents strongly increased within the deep mud layers and reached values up to 2.2 % (Fig. 2). Parallel to the mud fraction, stable carbon isotope ratios of TOC were measured for samples taken in 2002 and 2003. TOC was heaviest at the sediment surface with values close to –20 ‰ vs. V-PDB. Except for a few local sections, $\delta^{13}C$ values showed a general decrease with depth within the upper sand and shell layers but remained more or less constant within the muddy bottom layers, with $\delta^{13}C$ values between about –24 to –25 ‰.

Total sulfur (TS = SO_4 + FeS_x + FeS_2 + S^0 + HS + $S_{org.}$) contents showed a similar depth distribution like TOC. The sand and shell-dominated layers were characterized by low TS contents (0.1 to 0.3 %) except for some local maxima observed in the mud sections (Fig. 2). TS values started to increase with depth within the shell layers. The highest values with maxima up to 1.8 % were reached in the muddy bottom layers.

The still reactive iron and manganese fractions (Fe* = FeOOH, FeS and $Fe^{2+}_{,aq}$; Mn* = MnO_2 + Mn^{2+}) were only enriched in the mud sections (Fig. 2). Due to the sampling intervals chosen in the present study, no enrichment of solid phase Fe(III) and Mn(IV) was found in the upper sediment layers (Fig.2), what can be expected for intertidal sediments with oxidized surfaces like they are present in the study area (Bosselmann *et al.,* 2006; Dellwig *et al.,* 2006). The downcore variations indicate the importance of solid phase sources compared to porewater derived metals (see below). Maximum contents for Fe* (0.99 %) and Mn* (450 to 1212 mg kg^{-1} sediment) were found in discrete mud layers, in zones were no persisting MnO_2 can be expected (see porewater discussion below). This may indicate the formation of Mn(II) rich carbonates under anoxic conditions (Böttcher, 1998a) that have been found to form in experimental incubations with intertidal mixed flat surface sediments (Peters *et al.,* 2006). The covariation with the Fe* fraction may indicate the coexistence with FeS.

Porewater analysis

Concentrations of dissolved organic carbon (DOC) were the lowest in the uppermost sediment layers, with the highest values in summer (June 2002, 1.2 mmol C l^{-1}) and lower

values in winter (February 2004, 0.3 mmol C l^{-1}). DOC depth profiles showed only minor variations among the cores with maximum values around 9 mmol C l^{-1} in the bottom layers (Fig. 2)

Porewater sulfate concentrations were around 28 mmol·l^{-1} at the sediment surface and showed only a little decline within the uppermost 40 cm of the sediment, but strongly decreased beneath and were reaching almost depletion between 120 to 200 cm depth. An unusual second sulfate maximum was detected between 200 to 360 cm depth (Fig. 2). This peak was located just above the interface between the shell and the mud layers. Because porewater chloride concentrations were constant along the entire sediment column, it can be ruled out that either the sulfate minimum or the deep maximum were caused by an inflow of low saline groundwater.

Ammonium concentrations were the lowest at the surface, increased with depth and showed a maximum in the sulfate minimum zone with concentrations up to 9 mmol l^{-1} (Fig. 2). Beneath, ammonium decreased again to 2 to 3 mmol l^{-1} within the shell layer and remained almost constant towards the bottom of the core.

Site Gröninger Plate

Solid phase analysis

Sediment cores from site GP were largely dominated by sand with a few thin mud or shell layers (Fig. 3). It was suggested that the sediment was deposited in sand to mixed flat environments (Chang *et al.*, 2003).

In June 2002, the contribution of mud fraction was relatively constant along the whole sediment core, with single local maxima reaching values of up to 34 % (Fig. 3). In October 2003, a similar depth distribution was observed for the upper 150 cm of the sediment. The deeper layers, however, were characterized by larger mud intrusions, with the fraction of fine-grained sediment material increasing to up to 95%.

TOC contents generally ranged between 0.02 to 0.67 % (Fig. 3), except for two zones (170-230 cm and 320-440 cm) that were clearly enriched in TOC with values up to 2.8 %. The highest $\delta^{13}C$ values of around –20 ‰ were observed at the sediment surface. The organic matter became isotopically lighter with depth. Below 250 cm sediment depth, $\delta^{13}C$ values ranged between –23.5 to –25 ‰.

Depth profiles of total sulfur showed the lowest values of about 0.03 % in surface-near layers (Fig. 3). Total sulfur contents increased only slightly with depth, and generally

did not exceed 0.55 %. However, few local maxima with values up to 1.1 % were observed in some sandy and mud-rich layers and coincided with elevated TOC contents (Fig. 3).

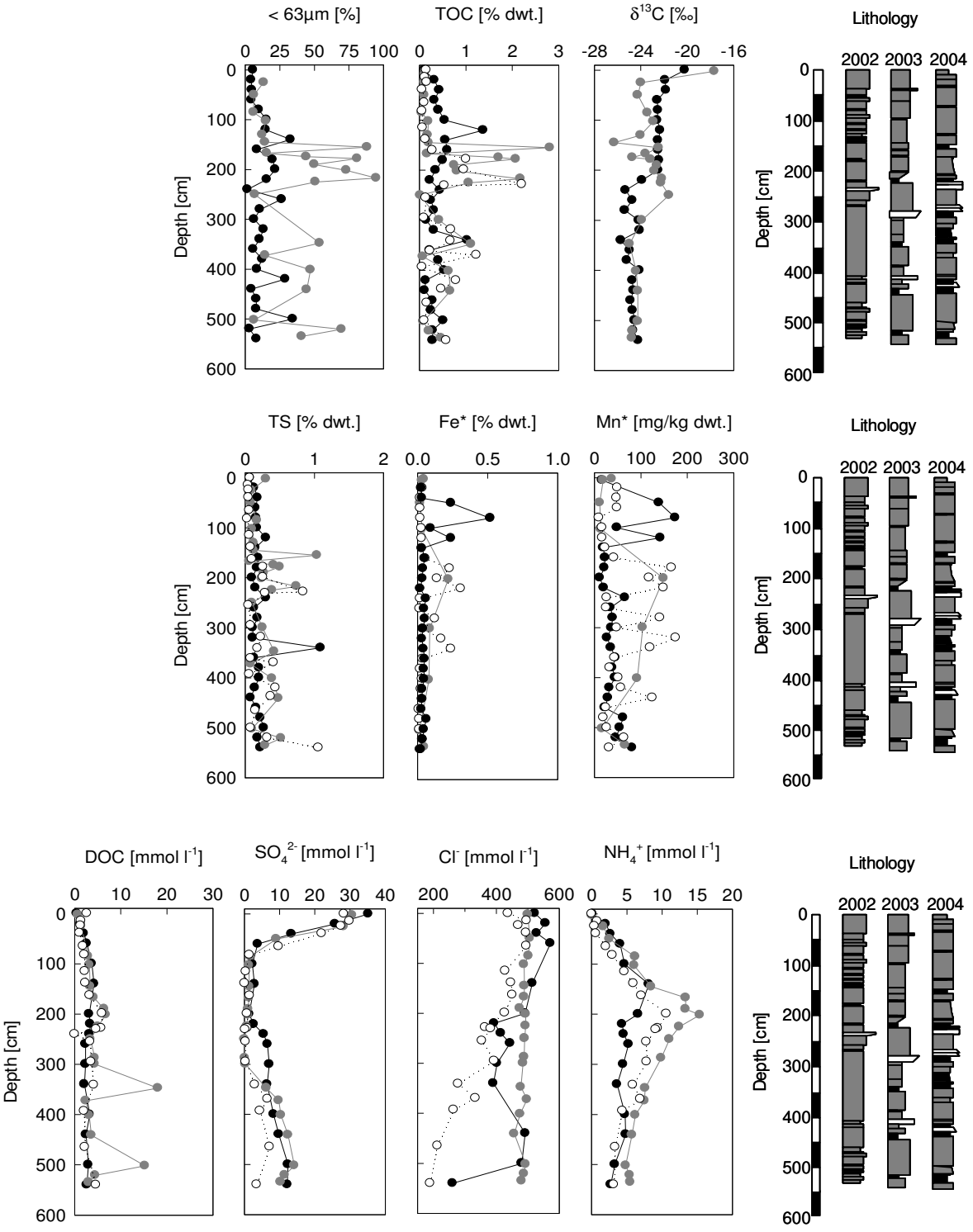

Fig. 3: Depth distribution of solid phase and porewater parameters (left) and plots of sedimentary structures (right) at site GP. Values are plotted for June 2002 (black dots), October 2003 (gray dots) and February 2004 (white dots). Sedimentary structures were divided into sand dominated layers (black), shell layers (white) and mud dominated layers (gray).

No enrichment of solid phase Fe(III) and Mn(IV) in the uppermost 40 cm was observed. Still reactive iron and manganese were generally enriched in the mud layers. Manganese profiles significantly differed among the individual cores taken from site GP (Fig. 3). In 2002, highest reactive manganese contents (up to 200 mg kg^{-1} dwt.) were found in the upper 150 cm of the core, but were three to five times lower in the layers beneath. In 2003 and 2004 almost reverse gradients of reactive manganese were found, with low amounts in the upper layers and high amounts in the bottom layers. Still reactive iron contents were in a range of 0.01 to 0.09 % (Fig. 3), except from a few enriched sand and mud layers with values up to 0.52 %.

Porewater analysis

At the sediment surface, porewater concentrations of dissolved organic carbon were in a range from 0.4 mmol C l^{-1} (June 2002) to 2.6 mmol C l^{-1} (February 2004). DOC concentrations increased along the upper 200 cm to values around 6.8 mmol C l^{-1} and showed a slight decrease in the layers beneath.

Porewater sulfate concentration at the sediment surface were around 30 mmol l^{-1} and even higher in June 2002, probably due to evaporation as it can be inferred from the concomitantly elevated chloride concentration (Fig. 3). Sulfate concentrations were more or less constant along the uppermost 20 to 30 cm of the sediment and strongly declined beneath reaching almost depletion at about 90 cm depth. Beneath 200 cm depth, sulfate concentrations increased steadily with depth again. In February 2004, sulfate concentrations in the deep layers were only half as high as in the other cores. These low sulfate concentrations were accompanied by lowered chloride concentrations and elevated temperatures, what might indicate a lateral inflow of low-saline groundwater.

Ammonium concentrations were lowest at the surface and increased with depth. Like at site NSN, the highest ammonium concentrations were detected in the sulfate minimum zone with maximum values of 8 to 15 mmol l^{-1} (Fig. 3). Beneath, ammonium concentrations decreased again, but were still high at the bottom of the cores with concentrations around 5 mmol l^{-1}.

Relationships between solid phase parameter and between porewater parameter

A positive correlation of TOC content and mud fraction (Fig. 4) was observed at site NSN and site GP. This relationship is in agreement with previous measurements in intertidal

flats and indicates the co-transport of organic matter and the clay-silt fraction (Mayer, 1995, Delafontaine, *et al.,* 1996, Böttcher *et al.,* 1998b, 2000a).

At site NSN, the sum of pyretic iron ($Fe[S_2]$ = TRS/32/2*55.85) and FeOOH contents was positively correlated to the mud fraction (Fig. 4). This relationship was also observed at site GP, but only for sediment samples taken in 2003.

The contents of the total reduced sulfur fraction (TRS = TS – SO_4^{2-}) were positively correlated with TOC contents (Fig. 4). Based on the results obtained for the surface-near layers of a nearby mud flat it can be expected that organic sulfur (S_{org}) does not contribute significantly to the TRS pool (Böttcher *et al.,* 2000a), that actually mainly consists of pyrite-sulfur.

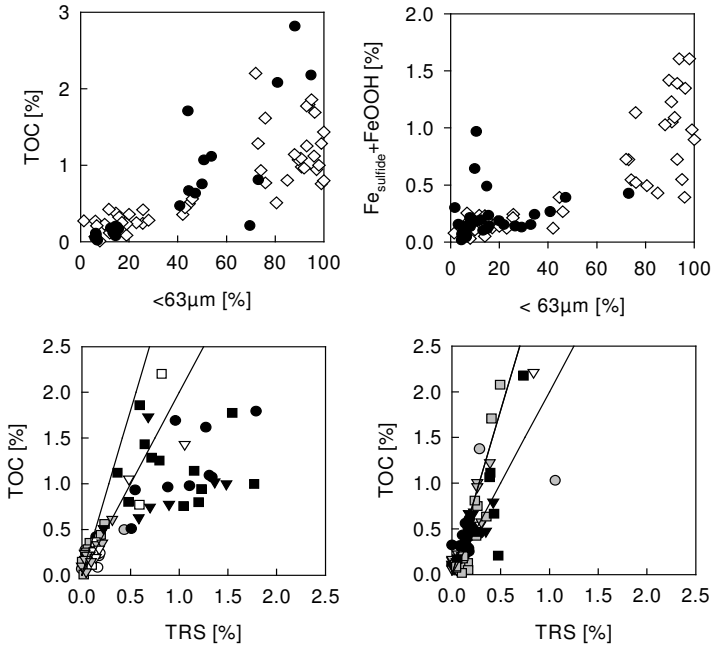

Fig. 4: Top: Correlation of total organic carbon (TOC) content (left) and the sum of pyretic iron and FeOOH with the sediment fraction smaller than 63 µm. Sediment samples were taken in June 2002 and September 2003 at site NSN (white diamonds) and site GP (black circles). Bottom: Variation of TOC content with total reduced sulfur (TRS) contents at site NSN (left) and site GP (right). Sediment samples were taken in June 2002 (circles), October 2003 (squares) and February 2004 (triangles). Samples of differing in their lithological composition are colour-coded. Gray: sand dominated layers, white: shell layers, black: mud dominated layers. Lines mark the range found for normal marine sediments (Berner and Raiswell, 1983).

The majority samples from the sand-dominated layers of both sites were within the TOC:TRS range found for other marine sediments (2.8 ± 0.8; Berner and Raiswell, 1983), with exception of a few sandy samples from site GP exhibiting elevated values like they are typically found in limnic systems. In contrast to this some sediment layers, in particular the lower shell layers and muddy sediments at site NSN but also at site GP, were enriched

in TRS sulfur and TOC:TRS ratios shifted towards lower values like they are typical for euxinic systems.

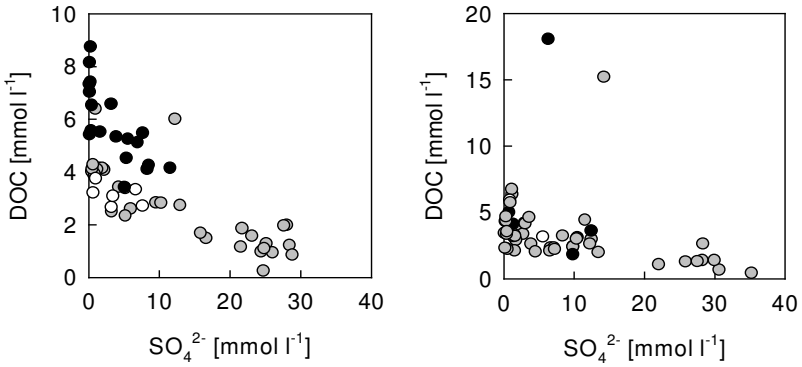

Fig. 5: Variation of DOC concentrations with residual sulfate concentrations in the porewater at site NSN (left panel) and site GP (right panel). Sedimentary structures were divided into sand dominated layers (gray), shell layers (white) and mud dominated layers (black).

In the sand and shell-dominated layers of site NSN, sulfate concentrations were apparently depending significantly on DOC concentrations. In the deeper mud layers DOC concentrations were more scattered at low sulfate concentrations. At site GP, DOC and sulfate showed only a weak negative correlation (Fig. 5), although highest DOC concentrations were measured in layers with sulfate minima and vice versa.

Total cell counts

Total cell counts obtained by epifluorescence microscopy after staining sediment samples with DAPI were highest at the sediment surface. At site GP, between 5×10^8 to 1.4×10^9 cells g^{-1} sediment were determined, with the lowest numbers observed in February 2004. At site NSN, cell counts at the sediment surface were about half as high as compared to site GP in summer 2002 and autumn 2003, but were in the same order of magnitude in winter 2004. Generally, cell counts strongly decreased within the uppermost 50 cm of the sediment. At site GP, a roughly 20-fold decrease was observed, while at site NSN cells counts declined only by a factor of four. Beneath half a meter depth, this decrease slowed down so that at the bottom of the core still between 2.1×10^7 and 10^8 cells g^{-1} sediment were detected. In February 2004, a very unusual situation was observed with the highest counts (about 10^9 cell g^{-1}) not at the surface but in 5 cm depth.

Viable counts

Viable counts of heterotrophic bacteria were highest at the sediment surface and generally decreased 10 to 1000-fold with depth along the upper 100 cm of the sediment. Interestingly, viable counts for aerobes often clearly exceeded numbers obtained after anoxic incubation. Highest values at site GP were 2 x 10^7 g^{-1} for aerobes and 1.2 x 10^6 g^{-1} for anaerobes, at site NSN 1.3 x 10^6 g^{-1} (aerobes) and 5.5 x 10^5 g^{-1} (anaerobes), respectively. From 100 cm downwards counts hardly changed with depth and rarely exceeded 1000 g^{-1} (Tab. 1). In these deep layers similar values were obtained for aerobes and anaerobes.

With respect to the maximum viable counts remarkable differences of up to two orders of magnitude were found between the different sampling campaigns. Counts at site NSN were highest in February 2004 and lowest in June 2002, while for site GP an inverse situation was found. These differences, however, appeared to be relatively erratic and could hardly be related to seasonal changes. The shift in incubation temperatures for series prepared in winter 2004 did not results in higher numbers than incubation exclusively at 4°C.

Tab. 1: Total cell counts [$g^{-1}_{sediment}$] and MPN counts [$g^{-1}_{sediment}$] obtained with oxic or anoxic media supplemented with a monomer mixture of sediment cores from sites Neuharlingersieler Nacken and Gröninger Plate. Total cell counts and MPN counts of site NSN are listed together for bottom layers although the core was 450 cm long in 2002 and 550 cm in 2003. It is the same for layers above, when values given for 370 cmbsf (2004) were summarized with those observed at 400 cmbsf in 2002.

	Depth [cm]		Oxic incubation	Anoxic incubation		Oxic incubation	Anoxic incubation		Oxic incubation	Anoxic incubation
		TCC	MPN	MPN	TCC	MPN	MPN	TCC	MPN	MPN
Neuharlingersieler Nacken	0.5	6.94×10^8	3.96×10^5	3.60×10^5	8.06×10^8	1.09×10^7	1.21×10^6	6.12×10^8	1.99×10^7	3.08×10^4
	5	5.79×10^8	3.27×10^4	2.97×10^4	6.52×10^8	1.37×10^6	4.11×10^5	8.48×10^8	3.37×10^5	1.08×10^5
	50	1.69×10^8	1.38×10^5	6.07×10^4	1.93×10^8	1.83×10^5	5.48×10^3	8.35×10^8	5.65×10^4	1.38×10^3
	100	n.d	6.37×10^2	1.27×10^4	2.50×10^8	2.38×10^4	2.38×10^4	7.04×10^8	2.12×10^3	1.55×10^2
	300	n.d	1.37×10^2	n.d.	1.53×10^7	5.47×10^1	1.80×10^2	1.63×10^8	1.60×10^1	$>1.60 \times 10^1$
	400	n.d	8.76×10^1	n.d.	n.d	n.d.	n.d.	1.02×10^8	$>1.13 \times 10^1$	$>1.13 \times 10^1$
	bottom	6.46×10^7	9.19×10^1	n.d	2.11×10^7	1.80×10^2	1.80×10^2	n.d	n.d	n.d
Gröninger Plate	0.5	1.12×10^9	1.25×10^6	5.49×10^6	1.39×10^9	n.d.	9.68×10^3	4.98×10^8	1.02×10^5	2.35×10^4
	5	9.75×10^8	3.14×10^5	1.71×10^5	5.85×10^8	n.d.	7.81×10^4	8.15×10^8	1.02×10^5	5.75×10^3
	50	6.03×10^7	4.00×10^5	3.64×10^5	8.27×10^7	n.d.	1.54×10^2	4.28×10^8	9.97×10^3	8.73×10^1
	100	n.d.	6.10×10^4	1.04×10^3	1.27×10^8	n.d.	1.59×10^3	9.24×10^7	8.43×10^2	7.14×10^1
	300	n.d.	2.29×10^2	8.65×10^2	6.13×10^7	n.d.	6.68×10^1	5.24×10^7	1.96×10^2	5.22×10^1
	400	n.d.	2.33×10^3	5.69×10^3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	540	6.46×10^7	4.27×10^2	9.69×10^2	4.65×10^7	n.d.	1.58×10^2	1.41×10^8	1.71×10^2	1.01×10^2

Viable counts of endospores increased with depth (data not shown) and reached maximum numbers (up to 24,000 g^{-1}) at 50 to 100 cmbsf, before slowly decreasing with depth again. Along the upper 50 cm of the sediment endospore numbers were clearly lower (less than 5 %) than those of vegetative cells. From 100 cm downwards the relative abundance of endospores strongly increased with numbers mostly equalling or even exceeding those of vegetative cells.

Vertical distribution of exoenzyme activities

Potential activities of four different microbial extracellular enzymes, alkaline phosphatase, β-glucosidase, N-acetyl-glucosaminidase and L-leucine-aminopeptidase, were measured during the sampling campaigns in October 2003 and February 2004. In surface sediments (10 cm depth) activities were generally highest and were in the same order of magnitude at both sites with values of up to 1000 µmol cm^{-3} h^{-1} for phosphatase and values between 10 and 75 µmol cm^{-3} h^{-1} for N-acetyl glucosaminidase, β-glucosidase and aminopeptidase, respectively (Fig. 6). However, pronounced differences were found for the subsurface layers of the two sites. At site NSN, at 100 cm depth activities of the enzymes were often almost as high as in the surface layer, in the case of phosphatase even higher. In the mud-dominated layers (300-550 cm depth), however, activities were 10 to 20-fold lower or in the case of glucosaminidase and aminopeptidase even often below the detection limit. At site GP, exoenzyme activities at 100 cm depth were already clearly lower than at the surface or at the respective depth of site NSN. Towards the deeper layers, activities decreased only slightly but were significantly higher than in the muddy layers of site NSN.

Interestingly, surface-near potential activities measured in February 2004 were in most cases higher than those observed in October 2003, although it should be kept in mind that all assays were incubated at 20°C, hence above the *in situ* temperatures. Nonetheless, this effect was more pronounced at site GP compared to site NSN. In the deeper layers, in contrast, variations could not be correlated to seasonal effects.

While the measured absolute activities generally decreased with depth, cell specific activities, normalized to total cell counts, remained more or less constant. The specific activities of alkaline phosphatase activities at site NSN and site GP were in the range of $1.7 \cdot 10^{-13}$ to $5.9 \cdot 10^{-12}$ mol·$cell^{-1}$·h^{-1}, whereas activities of N-acetyl-glucosaminidase and aminopeptidase ranged from $9.2 \cdot 10^{-15}$ to $3.7 \cdot 10^{-14}$ mol·$cell^{-1}$·h^{-1}. In February 2004 cell-specific activities were in a similar range at both sites, but were two to 10 fold higher at site NSN in October 2003. However, cell-specific activities found at sites GP and NSN

exceeded those observed in other tidal sediments (King, 1986) by up to 8 orders of magnitude but were comparable to those detected in other North Sea tidal flats (Coolen and Overmann 2000).

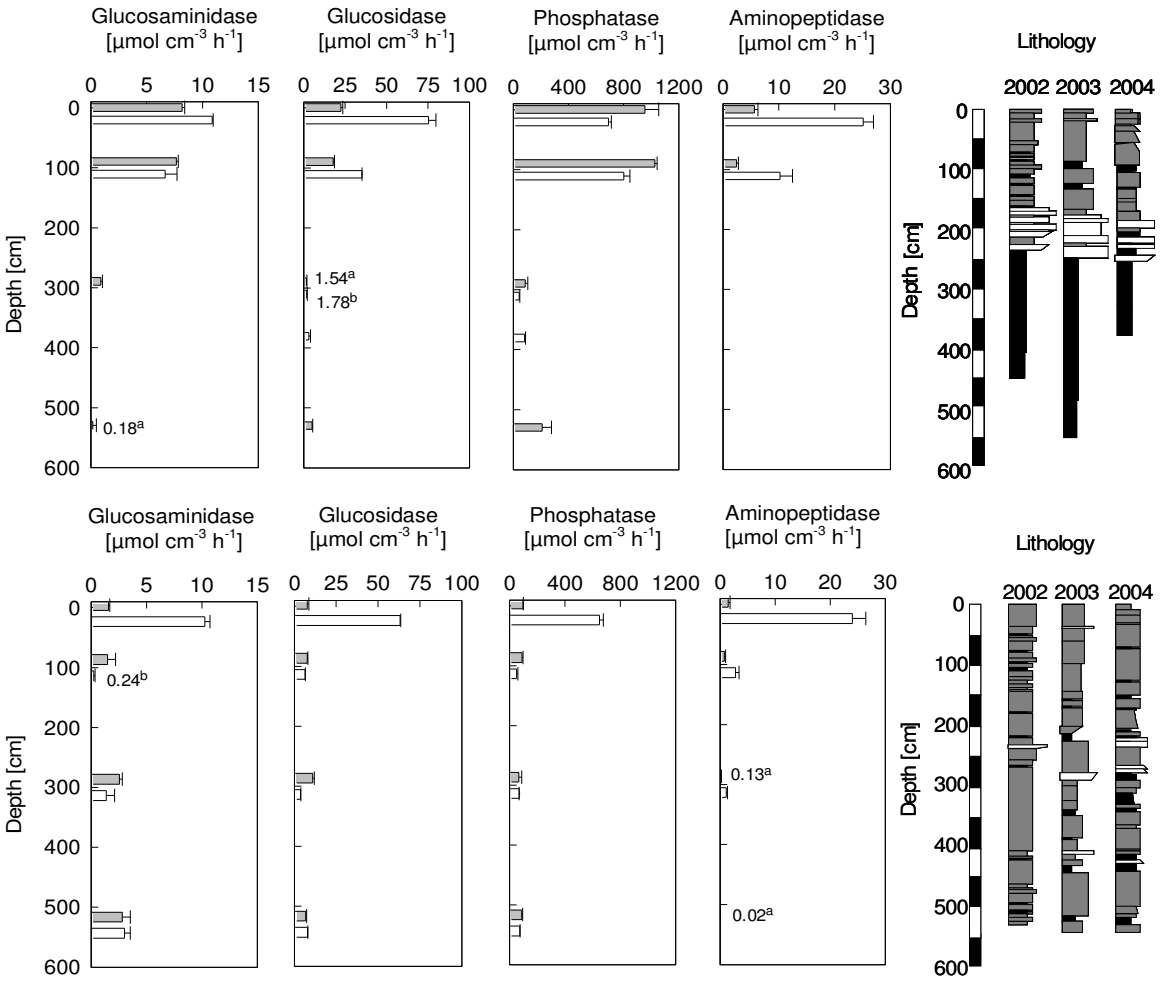

Fig. 6: Depth profiles of enzyme activities of site NSN (top) and site GP (bottom). Values are plotted for October 2003 (gray) and February 2004 (white). The error bars of the vertical bars indicate standard deviations. Data belonging to sampling campaign of October 2003 are marked by ′a′, of February are marked by ′b′.

To find out which environmental settings influence enzyme activities most, a correlation analysis of exoenzyme activities and organic matter as well as total cell counts was carried out. Temperature could not be included in this analysis, since all assays were incubated irrespective of the *in situ* temperatures at 20°C.

The Spearman rank correlation coefficient indicated that at site NSN, except for phosphatase, all exoenzyme activities depended significantly on sediment depth (Tab. 2) but were also correlated to DOC concentrations. A tight correlation, positive or negative, between enzyme activities and TOC content was not found. Both, DOC concentrations and

the TOC contents, increased significantly with depth and were positively correlated to the mud fraction (TOC: $S = 0.78$, $p < 0.005$, $n = 40$, DOC: $S = 0.90$, $p < 0.005$, $n = 40$).

At site GP, a completely different situation was found with exoenzyme activities, except for aminopeptidase ($S = -0.88$, $p < 0.005$, $n = 8$), being not positively correlated with depth (data not shown). A correlation with organic matter contents was found only for aminopeptidase and N-acetyl-glucosaminidase. While aminopeptidase activity was negatively correlated to the TOC content ($S = -0.83$, $p < 0.05$, $n = 8$), a positive correlation was found for N-acetyl-glucosaminidase ($S = 0.71$, $p < 0.05$, $n = 8$). Both, the TOC content and the DOC concentration depend on the mud fraction (see above) but were, like the mud fraction, not significantly correlated to the sediment depth.

Tab. 2: Spearman rank correlation coefficient (S) of exoenzyme activities at site NSN with other variables. Number of samples n = 8. Dissolved organic carbon = DOC, total organic carbon = TOC, total cell counts = TCC. A significance level of $\alpha = 0.05$ was used. Statistical significance: ns = $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$.

	Depth [cm]	β-Glucosidase [µmol cm^{-3} h^{-1}]	N-acetyl-glucosaminidase [µmol cm^{-3} h^{-1}]	Phosphatase [µmol cm^{-3} h^{-1}]	Amino-peptidase [µmol cm^{-3} h^{-1}]	DOC [mmol l^{-1}]	TOC [%]	TCC [cm^{-3}]
Depth [cm]	-	-0.72*	-0.88***	ns	-0.88***	0.95***	0.85**	-0.84**
β-Glucosidase		-	0.78*	ns	0.94***	-0.81*	ns	0.91**
N-acetylglc			-	0.80*	0.87**	-0.78*	-0.73*	Ns
Phosphatase				-	ns	ns	ns	Ns
Aminopeptidase					-	-0.91**	-0.76*	0.94***
DOC						-	0.86**	-0.95***
TOC							-	-0.81*
TCC								-

Sulfate reduction rates

Sulfate reduction rates measured using the radiotracer method were highest at or close to (5 cmbsf) the sediment surface. At site Neuharlingersieler Nacken, maximum rates were 680 and 50 nmol cm^{-3} d^{-1} in October 2003 and February 2004, respectively. In 50 and 100 cm depth, sulfate reduction rates showed minor variation between the sampling campaigns and were 10 to 20-fold lower than at the surface. Despite the deep sulfate maximum, sulfate reduction rates in the shell layers were even lower than in the sulfate minimum zone. The lowest rates were obtained with samples from the deep mud-dominated layers and were four orders of magnitude lower than at the sediment surface.

At site Gröninger Plate a slightly different situation was found. In the sediment-near (0 and 5 cmbsf) layers, maximum rates were between 120 and 220 nmol cm^{-3} d^{-1} (Fig. 7), with exception for February 2004 when sulfate reduction at the surface was below the detection limit. Sulfate reduction rates were already about 100-fold lower at 50 cm than at 5 cm depth, but showed a less pronounced decline with increasing depth, and reached still up to 1 nmol cm^{-3} d^{-1} at the bottom of the core. Along the upper 100 cm of the core sulfate reduction rates at site GP were generally lower, while in the deeper layers rates were one to two orders of magnitude higher than at site NSN.

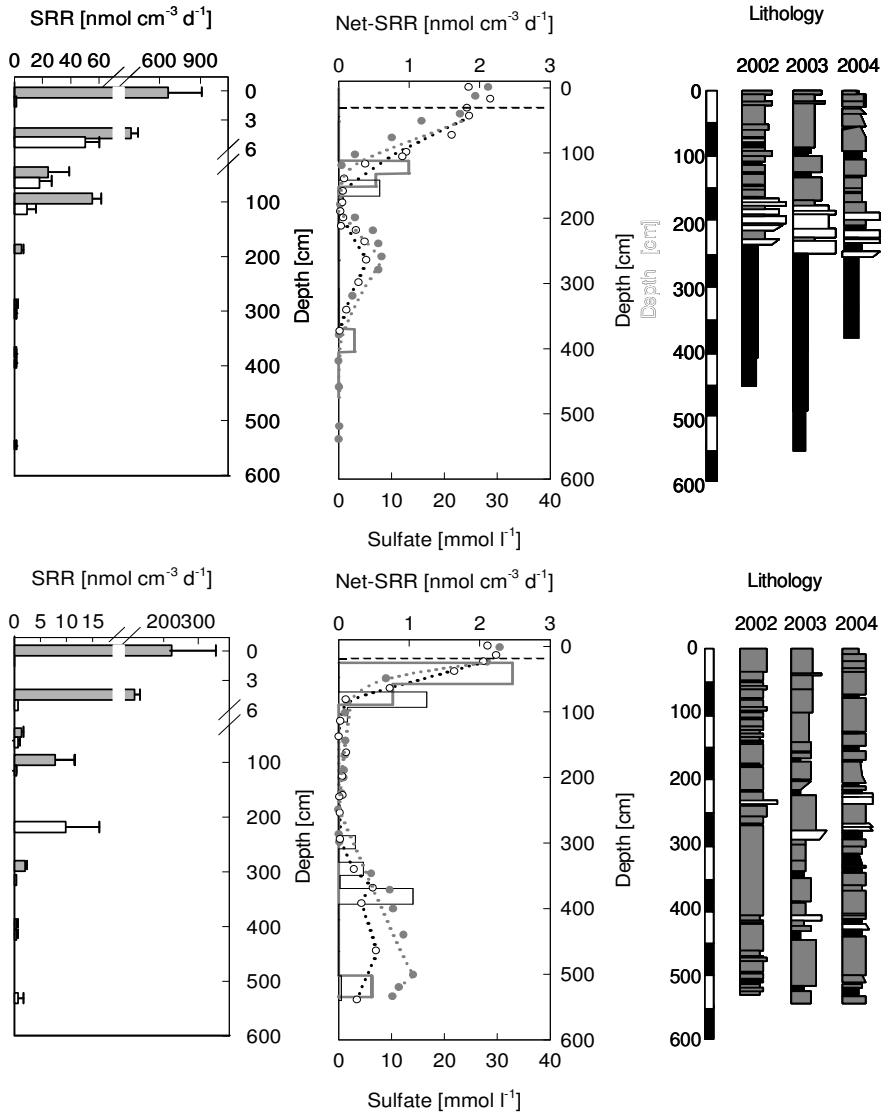

Fig. 7: Depth profiles of sulfate reduction rates at site NSN (top) and site GP (bottom). Sulfate reduction rates were determined by the radioactive tracer method (bars). Net-SRR (solid lines) were calculated from porewater sulfate profiles (circles) using the PROFILE model of Berg *et al.,* (1998). Dottes lines simulate the sulfate data that were calculated by the PROFILE model. Values are plotted for October 2003 (gray) and February 2004 (white). From the sediment surface down to the depth marked by dashed lines, porewater sulfate concentrations were more or less constant and were not considered for modeling.

In general, sulfate reduction rates measured in October 2003 were higher than those in February 2004. This effect was more pronounced in the upper layers, where the difference could be even one to two order of magnitude and therefore apparently was not only due to temperature changes.

The modeled depth-integrated microbial net sulfate reduction rates were 138.6 mmol m^{-2} a^{-1} at site NSN and 435.3 mmol m^{-2} a^{-1} at site GP in September 2003 and were up to 2.7 times lower in February 2004. Modeling resulted in higher net sulfate reduction rates especially in the transition zones where sulfate concentration decreased and apparently methane concentrations increased (Methane profile for site NSN is given in Wilms *et al.,* 2006c).

The Spearman rank correlation coefficient indicated that at site NSN sulfate reduction ($S = -0.63$, $n = 14$, $p < 0.05$), like the number of *dsr*-gene targets ($S = -0.98$, $n = 14$, $p < 0.005$; data from Wilms *et al.* 2006c) or the total cell counts ($S = -0.81$, $n = 14$, $p < 0.005$), significantly correlated with sediment depth. On the other hand, SRR were positively correlated with porewater sulfate ($S = 0.70$, $n = 14$, $p < 0.005$) concentrations but did not depend significantly on organic carbon, neither DOC nor TOC. The number of *dsr*-genes targets, in contrast, was negatively correlated to DOC ($S = 0.96$, $n = 14$, $p < 0.005$) and TOC ($S = -0.79$, $n = 14$, $p < 0.005$). Neither SRR nor *dsr*-genes targets depended significantly on the mud-faction (< 63 µm). Therefore, 'depth' seems to be a more complex composite variable with a strong influence of the lithological settings.

At site GP, no significant correlation between SRR and organic matter or porewater sulfate concentrations was observed. Total cell counts, in turn, were positively correlated to dissolved sulfate ($S = 0.60$, $n = 14$, $p < 0.05$) and negatively correlated to DOC ($S = -0.54$, $n = 14$, $p < 0.05$).

Discussion

In the present study several meter long sediment cores from two adjacent tidal flats were investigated applying microbiological and geochemical methods. Active microbial communities were detected along the whole cores down to a depth of five to six metres below the surface. Although cell numbers and absolute microbial activities (sulfate reduction) decreased with depth, potential cell-specific activities (exoenzymes) showed only minor changes indicating the presence of a potentially active but presumably substrate-limited microbial community in the deeper layers. The geochemical gradients were not only controlled by microbial activities, but were also affected by the sedimentological settings, in particular by grain size. This lithological control did not only lead to unusual sediment profiles (e.g. sulfate) but in turn influenced microbial activities for example by providing a deep sulfate supply.

Lithological and geochemical settings

At both sites sediment cores exhibited unusual porewater gradients. In undisturbed marine sediments sulfate is expected to decrease steadily with depth (Thomsen *et al.*, 2001, Jørgensen *et al.*, 2000). In contrast, in many coastal sediments (Chambers *et al.*, 2000), and in particular tidal flats (Böttcher *et al.,* 1998b) like that investigated here, sulfate remains more or less constant along the uppermost 50 cm. This is due to advective porewater flows caused by sediment surface roughness (Huettel *et al.*, 1998) and intense bioturbation or bioirrigation (Kristensen 2000).

However, a more unexpected finding was the presence of a sulfate peak around 240 cm depth at site NSN. Different aspects indicate a sulfate supply in the deeper sediment layers due to lateral porewater transport. First, the lithological settings at site NSN suggest this assumption since the porous shell layers facilitate porewater transport that is more likely horizontal due to the fine grained sediments below, that are known to act as aquitard due to their low permeability (Robinson *et al.,* 1998). On the other hand the shell layers at this depth were estimated to be buried 600 to 800 years ago (Ziehe, pers. comm.) and therefore this peak cannot be just a remnant. Secondly, the shape of the sulfate maximum indicates diffusion into the sulfate-depleted layers above and beneath and points to the shell layers as a sulfate source. Finally, the TRS enrichment at site NSN, that mostly likely results from dissimilatory sulfate reduction, may also indicate an additional sulfate supply.

Maximum concentrations in the deep sulfate peak appear to change seasonally, they were about two-fold higher during the summer than the winter sampling campaign. This cannot sufficiently be explained by tidal pumping (Riedl, 1972) or dispersion (Chooper, 1959), what would lead to relatively constant sulfate concentrations. Microbial activities, in turn, can be expected to consume more sulfate during summer.

Freshwater flow across the sediment-water interface (known as submarine groundwater discharge, SGWD), which is driven by an inland hydraulic head, is known to play a role in the export of nutrients from subterranean aquifers into the near-shore marine environments (Robinson *et al.,* 1998, Slomp and Van Capellen, 2004) but can also be attributed to a decrease in salinity. However, investigations on an annual cycle of SGWD in a temperate coastal area indicated a reversal of the ground water discharge between winter and summer with a net influx of saline water during summer (Michael *et al.*, 2005, Simmons, 1992). A possible explanation for this might be a negative water balance due to evapotranspiration being greater than precipitation inlands of the sampling site. Indications for this process can also be seen in the gradients obtained at site GP, where sulfate increases with depth and indicates an aquifer beneath. Like at site NSN, sulfate concentrations at the bottom of the core were lower in winter than in June or September. However, the simultaneous decrease in chloride indicates a higher freshwater discharge towards the tidal creek in winter.

Organic matter, stable carbon isotopes and grain size

Previous studies in the area revealed $\delta^{13}C$ signatures similar to those in the present study in surface sediments (Böttcher *et al.,* 2000a, Rusch *et al.,* 1998). Variations in the carbon isotope ratios of organic matter are indicative for changes in the relative contribution of marine and terrestrial sources. Values in the range of –19 to –22 ‰ were reported for marine plankton (Fry and Sherr, 1984, Hedges and Mann, 1979), while –16 to –18 ‰ was found characteristic for benthic diatoms (Haines and Montague, 1979). Böttcher *et al.* (1997, 1998b) measured $\delta^{13}C$ values between –10 to –18 ‰ for selected macroalgae and mussels from the study area (Gröninger Plate). The $\delta^{13}C$ range for potential OM sources indicates that most of the organic matter buried is of marine origin. However, TOC becomes enriched in the lighter isotope further downcore, what can be explained by the preferred degradation of easily degradable organic matter of marine origin compared to the more recalcitrant terrigenous organic matter. In near-bottom layers at both study sites, carbon isotope ratios of TOC were in a range generally found for terrestrial sources. The

isotopic composition of terrigenous sources range from –26 to – 29 ‰ for vascular plant detritus or Holocene peats (Ertel and Hegdes, 1985, Böttcher *et al.,* 1998b) whereas –24 to – 28 ‰ was reported for soils dominantly covered by C_3 vegetation (Dickens *et al.,* 2006). These values are well reflected by terrestrial particulate organic matter ($^{13}C/^{12}C$ ~ –27 ‰) transported by river discharge into the North Sea (Salomons and Mook, 1981).

The percentage of marine organic carbon can be estimated from the measured isotopic composition applying a binary mixing model (Böttcher *et al.,* 1998b, 2000a, Volkman *et al.,* 2000) using average values of –27 ‰ for terrestrial and of –19 ‰ for marine sources. In this estimate possible minor diagenetic changes upon OM degradation are not considered. The results of this calculation indicate that marine organic matter at site NSN comprises of 46 to 93 % of the total OM amount in the sand-dominated zones with the highest values in near surface layers. The muddy layers, that were deposited in a salt marsh environment, obviously contain less marine organic matter (12 – 52 %). Likewise, at site GP the surface sediments contained the highest content of marine organic matter (64 to nearly 100 %), while in the layers beneath the marine fraction decreased with depth to values between 7 and 59 %.

From downcore profiles of carbon isotope ratios decreasing bioavailability of organic matter with depth can be inferred, what is not reflected by absolute TOC contents. The observed increase in TOC contents is more likely the result of the accumulation of refractory (e.g. peat-derived) organic material in the deeper sediment sections (Volkman *et al.,* 2000). TOC values found for the uppermost layers are in accordance to previous reports (Böttcher *et al.*, 1998b, Volkman *et al*., 2000), whereas the maximum values of 2.2 % (site NSN) and 2.8 % TOC (site GP), found in this study, clearly exceed those found in many offshore sediments which can be assumed to receive less terrigenous input (Mayer, 1994). However, these values are in a range found for suspended particles (Hild, 1997) or in surface-near sediments of a nearby mud flat (Böttcher *et al*., 2000a).

TOC-TRS relationship

Sulfate reduction is the dominant anaerobic mineralization pathway in intertidal sediments of the southern North Sea (Böttcher *et al.*, 2000a; Llobet-Brossa *et al.*, 2002) and it can be expected that its geochemical signature is reflected in the accumulation of biogenic reduced sulfur (mostly in form of iron sulfides). The downcore increase of total reduced sulfur at site NSN generally corresponds with rising mud contents (Fig.2) and hence to the organic matter content. Although the local maximum in TRS just beneath 100 cm depth

coincides with elevated mud contents, it is also situated at a sulfate-methane interface (Wilms *et al.,* 2006b) and it is likely that it resulted from the anaerobic oxidation of methane. A similar enrichment of iron sulfides in the zone of anaerobic methane oxidation was found in other marine sediments (Böttcher *et al.*, 2000b, Jorgensen *et al.*, 2004).

While Berner and Raiswell (1983) reported TOC/TRS ratios around 2.8 ± 0.8 for 'typical' coastal to hemipelagic sediments with overlying oxic water column, in intertidal mud flats of the southern North Sea, ratios in order of magnitude were found in a section where sulfate reduction starts to exceed oxic and suboxic diagenetic processes (Böttcher *et al.*, 2000a). However, the sand-dominated and some of the mud-rich sediments at sites GP and NSN nonetheless agreed more or less well to the TOC/TRS ratio of Berner and Raiswell (1983). Surprisingly, the muddy deeper layers at site NSN showed much lower ratios, in spite of the elevated TOC contents. At first sight this strong accumulation of TRS might correlate to the content of silt, what however did not apply to the mud-rich sections at site GP. Therefore, the enrichment in total sulfur might rather be due to a steady supply in sulfate via the aquifer in the shell layer.

Extractable Iron and Manganese

Extractable iron and manganese contents (Fe*, Mn*) consist of iron(III)oxihydroxides, manganese oxides, iron monosulfides (that mostly were oxidized upon sample handling) and to a lesser extent reduced iron and manganese species in the porewater (e.g., Kostka and Luther, 1994). The presence of oxidized iron and manganese species, however, can be expected only for the layers above the sulfate reduction zone (Froelich *et al.*, 1979, Aller and Rude 1988). At both sites, at greater depths distinct enrichment of Fe* and Mn* was observed and it is unlikely that these point to elevated porewater contents of iron and manganese. In contrast, these enrichments were found at depths where sulfate started to get depleted (site NSN: about 100 and 400 cmbsf, site GP: about 80 and 200 cmbsf) and sulfate-methane interfaces can be found (Wilms *et al.*, 2006b). At these distinct zones the anaerobic oxidation of methane can be expected to take place, which produces CO_2 and hydrogen sulfide and generates alkalinity (e.g., Boetius *et al.*, 2000). Under these conditions the enhanced formation of authigenic iron monosulfides (Jorgensen *et al.*, 2004,) and Mn(II)carbonates (Böttcher *et al.*, 2000b, Böttcher, 1998a) has been described. At site NSN, the decrease in Fe* beneath about 430 cmbsf appears unrelated to variations of TRS and might indicate pyrite formation due to sulfidization of metastable iron sulfides (mackinawite). The variability in depth profiles among the different cores taken at site GP

suggests at least some lithological control on the distribution of Fe* and Mn*. Extractable metals were enriched in the muddy section and indicate that besides authigenic solid phase contents, metals extracted from clay minerals may also contribute to the Fe* and Mn* pools.

Vertical changes in microbial activities

At both sites, microbial activities were detected along the whole sediment column investigated, down to several meters of depth. Highest microbial activities, for example sulfate reduction, were measured in the surface layers what appears to be a general feature of shallow, near-shore sediments (Kristensen, *et al.*, 1998, Llobet-Brossa *et al.* 2002). This can be explained by the supply of easily degradable organic matter by sedimentation. By the action of burrowing macrozoobenthos, part of this material can be transported even to depths of 30 to 50 cmbsf (Kristensen 2000). As discussed above, in the deeper layers mostly highly recalcitrant material is present and accordingly microbial activities can be expected to decrease with depth. This was found for sulfate reduction rates, but at site NSN potential exoenzyme activities at 100 cm depth were in the same order of magnitude as at the surface. But it has to be kept in mind that the use of fluorescently labeled substrate analogues delivers only potential activities. The addition of the $^{35}SO_4^{2-}$ does generally not significantly alter the *in situ* concentrations of sulfate and therefore the estimated sulfate reduction rates can be expected to reflect the actual activities. The natural substrates (cellulose, chitin, peptides) for the targeted extracellular enzymes, in turn, are unlikely to be still present in the deeper (tens of centimeters to meters) layers of the sediment and hence the addition of substrate analogues increases the *in situ* concentration of potential substrates by up to some orders of magnitude. Therefore, the measured enzyme activities almost certainly overestimate the actual *in situ* values, but since enzyme activities are often elevated under oligotrophic conditions, this indicates the presence of an active but substrate-limited microbial community. Substrate limitation in spite of an increase of TOC with depth emphasizes that microbial communities are depending rather on the quality than the quantity of organic material.

It could be argued that some of the activity was due to enzymes released from decaying cells. It has been reported that enzymes could be adsorbed by particles and thus protected against proteolysis for several days (Nannipieri *et al.*, 1982). A survival of released enzymes for up to tens of years, what would be necessary to cause the activities in the present case, however, can be ruled out. In turn, the presence of active communities in

the uppermost 100 cm of the sediment is supported by the results of the MPN series, since metabolically active bacteria were found to be easier to cultivate (Kell *et al.*, 1998). Towards the deeper layers, cultivation success strongly decreased. This decrease cannot sufficiently be explained by cells being in an inactive state or a low-energy survival mode (Price and Sowers 2004). Molecular studies have shown a shift from *Proteobacteria* dominating in the upper 100 to 200 cm to *Chloroflexi*, which are supposed to be highly specified deep biosphere bacteria able to cope with highly recalcitrant organic material (Coolen *et al.*, 2002, Wilms *et al.*, 2006b). Since, none of these bacteria was successfully cultivated so far, the low viable counts obtained for the deeper layers reflect rather the specialized needs of these bacteria that were not met by the media used in the present study (Köpke *et al.*, 2005).

At site NSN where the upper layers generally tend to exhibit higher activities than site GP, the deep muddy layers in contrast show very lowed activities. On the one site, the sorption of organic matter onto mud particles (Keil *et al.*, 1994) supports the preservation of organic matter (Böttcher *et al.*, 1998b, Volkman *et al.*, 2000) and hence reduces its bioavailability (Mayer, 1994). On the other hand, the small diameter of the pores hampers the diffusion of substrates and the chemotactical behaviour of the microorganisms towards potential substrate sources. Hence lower activities compared to more coarse-grained sediments are found, even if cell numbers are in the same range (Chapelle and Lovley, 1990, Phelps *et al.,* 1989). But while activities in the mud layers are low, they can nevertheless fuel microbial communities in the adjacent layers by supplying organic material (Detmers *et al.*, 2001, Parkes *et al.*, 2005). Therefore, microbial activities are not only influenced by the quality of organic material, but in particular by the lithological settings of the sediments.

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Kapitel V

Diskussion und Ausblick

1. Diskussion

Im Folgenden werden die einzelnen Ergebnisse zusammenhängend diskutiert, wobei auf eine Wiederholung der in den Artikeln ausführlich dargelegten Aspekte verzichtet wird.

Um das Ökosystem „Wattsediment“ verstehen zu können, ist es notwendig, die mikrobiellen Gemeinschaften dieser zu charakterisieren, da die Bakterien durch ihre Aktivitäten Einfluss auf die chemische Zusammensetzung der Sedimente nehmen. Bisher beschränkten sich die Untersuchungen von Wattsedimenten zumeist auf die oberen 50 cm (Böttcher *et al.,* 2000, Freitag *et al.,* 2003, Llobet-Brossa *et al.,* 1998) oder waren auf bestimmte Organismengruppen begrenzt (Brinkhoff *et al.,* 1998, Llobet-Brossa *et al.,* 2002, Mussmann *et al.,* 2003, Wieringa *et al.,* 2000).

In der hier vorliegenden Studie wurden die mikrobiellen Gemeinschaften in Wattsedimenten zweier verschiedener Standorte bis zu 550 cm Tiefe mittels mikrobiologischer Methoden charakterisiert. Mit den dazu parallel durchgeführten molekularbiologischen Untersuchungen (Wilms *et al.,* 2006a, b, c) ergab sich ein umfassenderes Bild der mikrobiellen Gemeinschaften. Dieses wurde in der vorliegenden Arbeit außerdem um den Aspekt der mikrobiellen Aktivitäten erweitert sowie zu den geochemischen Eigenschaften der Sedimente in Bezug gesetzt. Durch diese Vielzahl von Methoden, die hier zum Einsatz kamen, unterscheidet sich diese Arbeit von vorhergehenden Untersuchungen der Wattsedimente.

1.1 Zusammensetzung mikrobieller Gemeinschaften in Wattsedimenten

Die Charakterisierung der mikrobiellen Gemeinschaft der Wattsedimente vom Neuharlingersieler Nacken (NSN) und der Gröninger Plate (GP) mit Kultivierungsmethoden zeigte, dass die oberflächennahen Sedimentschichten (0-5 cm) vor allem von α-, γ- und δ-*Proteobacteria* dominiert wurden. Allerdings spiegelten sich diese Ergebnisse nur zum Teil in den molekularbiologischen Untersuchungen wieder. Diese ergaben eine Dominanz von γ- und δ-*Proteobacteria* innerhalb der oberen 160 cm des Sediments (Wilms *et al.,* 2006b). Die meisten der Isolate, die von Stevens *et al.* (2004) aus dem gleichen Habitat isoliert wurden, konnten ebenfalls α- und γ-*Proteobacteria* sowie *Actinobacteria* zugeordnet werden. Sowohl α- als auch γ-*Proteobacteria* stellen bedeutende Gruppen in marinen Systemen dar (Gonzalez und Moran, 1997, Crump *et al.,* 1999). Sie wurden im gleichen oder angrenzenden Habitaten in der Wassersäule und auch

im Sediment mittels kultivierungsabhängiger (Stevens *et al.*, 2005, Eilers *et al.*, 2000) als auch -unabhängiger Methoden nachgewiesen (Glöckner *et al.,* 1999, Stevens *et al.*, 2005).

Während die mikrobiologischen Untersuchungen eine Zunahme des Anteils der *Firmicutes* auf bis zu 100 % mit der Sedimenttiefe erkennen ließen, wurden mittels molekularbiologischer Methoden keine *Firmicutes*-Verwandten detektiert. Stattdessen wurden in allen untersuchten Sedimentabschnitten Phylotypen der *Chloroflexi*-Gruppe nachgewiesen, die in den tiefen Sedimentschichten sogar dominierten (Wilms *et al.,* 2006b). Vertreter dieses Phylums, die früher als grüne Nicht-Schwefel-Bakterien (GNSB) bezeichnet wurden (Garrity und Holt, 2001), konnten jedoch mit den in dieser Studie angewendeten Kultivierungsbedingungen nicht in Reinkultur gewonnen werden. Eine vergleichbare Problematik trat bei der Untersuchung von Mittelmeersedimenten auf. Trotz nachgewiesener Dominanz von GNSBs in Sapropelen mittels molekularbiologischer Methoden (Coolen *et al.,* 2002) wurden diese nicht erfolgreich isoliert (Süß *et al.,* 2004). Allerdings war man bisher nur bedingt in der Lage, Bakterien dieses Phylums zu isolieren, so dass sich dieses hauptsächlich aus unkultivierten Vertretern zusammensetzt (Hanada *et al.,* 1993).

Sowohl die molekularbiologischen als auch mikrobiologischen Untersuchungen der Wattsedimente ergab eine vertikale Veränderung in der Zusammensetzung der mikrobiellen Gemeinschaft. Bis auf wenige Ausnahmen gab es keine Überlappung zwischen den Isolaten der oberflächennahen oxischen Schichten (0.5 bis 5 cm) und den tieferen anoxischen Sedimenten (ab 200 cm Tiefe). Die molekularbiologische Analyse resultierte in einer Aufspaltung der standortbezogenen Bakteriengemeinschaft in drei große Gruppen (Wilms *et al.*, 2006a). In diesen spiegelten sich physiko-chemische Eigenschaften, wie die Sedimentstruktur (Standort NSN) oder chemische Parameter, wie der Sauerstoffgehalt oder die Sulfatkonzentration (Standort GP; Wilms *et al.*, 2006a) wider.

Als Einfluss nehmender Faktor auf die Zusammensetzung der Bakteriengemeinschaft wurde die Verfügbarkeit verwertbaren organischen Materials beschrieben. Im Gegensatz zu den tieferen Sedimentlagen werden die oberflächennahen Sedimente immer wieder mit leicht abbaubaren, organischen Substraten durch Microphytoplankton, sedimentierendes Phytoplankton und abgestorbene benthische Organismen versorgt (Villbrandt *et al.,* 1999). Es ist anzunehmen, dass die Bioverfügbarkeit mit der Sedimenttiefe abnimmt. So war am Standort Neuharlingersieler Nacken der Gehalt an persistenten langkettigen *n*-Alkanolen (C_{22}-C_{28}) in

Sedimentbereichen unterhalb von 280 cm Tiefe zehn Mal höher als die Menge an kurzkettigen *n*-Alkanolen (C_{14}-C_{21}, Freese und Rullkötter, 2003). Das deutet darauf hin, dass mit der Tiefe und damit dem Alter der Sedimente mehr refraktäres organisches Material akkumuliert.

Diese Qualitätsunterschiede spiegelten sich in der Bakteriengemeinschaft durch die Dominanz der *Proteobacteria* in den oberflächennahen Sedimentbereichen wieder (Köpke *et al.,* 2005, Wilms *et al.,* 2006a). Von den meisten *Proteobacteria* ist bekannt, dass sie leicht abbaubares organisches Material verwerten. Das refraktäre organische Material wird vermutlich eher von „Spezialisten" abgebaut. Welche das in den untersuchten Wattsedimenten sind, darüber kann nur spekuliert werden, da solche Substrate in dieser Studie nicht eingesetzt wurden. Allerdings wird von den in den molekularbiologischen Studien nachgewiesenen *Chloroflexi* angenommen, dass sie einige dieser refraktären Substanzen verwerten können (Coolen *et al.*, 2002, Wilms *et al.,* 2006b).

1.2 Vertikale Variabilität mikrobieller Aktivitäten in Wattsedimenten

In dieser Studie wurden mikrobielle Aktivitäten durch die Messung von Sulfatreduktionsraten und Exoenzymaktivitäten quantifiziert. Diese Methoden wurden favorisiert, da zum Einen ein Großteil des organischen Materials in den Sedimenten über die mikrobielle Sulfatreduktion mineralisiert wird (Jørgensen, 1982, Skyring, 1987). Zum Anderen liegen die meisten organischen Verbindungen in den Sedimenten als Polymere vor, die zu groß sind, um von Bakterien inkorporiert zu werden. Deshalb stellt die extrazelluläre Hydrolyse dieser durch die von Bakterien gebildeten Exoenzyme einen entscheidenden Prozess beim Abbau organischer Substanz dar (Chrost, 1991, Meyer-Reil, 1991).

Die höchsten Sulfatreduktionsraten wurden in dieser Studie innerhalb der ersten 5 cm des Sedimentes gemessen, was typisch zu sein scheint für flache, küstennahe Sedimente (Böttcher *et al.,* 2004, Kristensen *et al.,* 2000b). Es lässt sich mit der ständigen Zufuhr leicht abbaubarer organischer Substrate durch Sedimentation erklären. Die mit der Tiefe abnehmenden Sulfatreduktionsraten stimmen mit den Beobachtungen in anderen flachen, küstennahen Sedimenten überein (Kristensen, 1998, Thode-Andersen und Jørgensen, 1989). Diese Abnahme kann u.a. auf die Limitation an leicht abbaubaren organischen Substanzen zurückgeführt werden, da mit der Tiefe und damit dem Alter der Sedimente refraktäres organisches Material akkumuliert (Böttcher *et al.,* 1997) Der Gehalt und die Verfügbarkeit an organischem Material wird vornehmlich durch die

Beschaffenheit der Sedimente beeinflusst. Tonpartikel, wie sie die tieferen Sedimentbereiche des Standortes NSN dominieren, adsorbieren organisches Material (Keil *et al.,* 1994), wodurch dessen Bioverfügbarkeit reduziert wird (Mayer, 1994).

Die erhöhten Sulfatreduktionsraten im Herbst weisen im Vergleich zum Winter in den oberflächennahen Sedimenten auf eine Temperaturabhängigkeit Sulfat reduzierender Bakterien hin (Kristensen *et al.,* 1998, Barnes *et al.,* 1998). Jedoch unterliegen mikrobielle Aktivitäten einer komplexeren Kontrolle durch den simultanen Einfluss anderer saisonaler Effekte die mit der Temperatur korreliert sind (Kristensen *et al.,* 1998), wie z.B. der Produktion von organischem Material durch Phytoplankton.

Auch die Aktivität von Exoenzymen wurde als temperaturabhängig beschrieben (Nausch *et al.,* 1998). Die Ergebnisse dieser Studie unterstützen die Ansicht, dass die Synthese und Aktivität der Exoenzyme vor allem über die Verfügbarkeit der Substrate reguliert wird (Boetius, 1995, Chrost, 1991). Da vermutlich die natürlichen Substrate der hier eingesetzten Enzyme, wie Peptide, Cellulose und Chitin, bereits in den der Wassersäule oder den oberflächennahen Sedimentschichten verwertete wurden, wurde durch die Zugabe der Substratanaloga die Konzentration an potentiellen Substraten *in situ* erhöht. Das könnte zur vermehrten Synthese der Exoenzymen geführt haben, was die vergleichbaren Aktivitäten in 10 cm und 100 cm Sedimenttiefe erklären würde.

Mikrobielle Aktivitäten sind allerdings nicht nur auf die oberflächennahen Sedimente beschränkt. Einige Mikroorganismen sind auch unter den limitierenden Bedingungen aktiv, die in tieferen Sedimenten herrschen, wie Sulfatreduktionsraten von bis zu 1 nmol cm^{-3} d^{-1} in 550 cm Sedimenttiefe zeigen. Auch wenn die Aktivitäten in den tieferen Sedimenten um ein vielfacheres geringer sind als an der Sedimentoberfläche, darf ihr Beitrag zur Gesamtaktivität des Systems (Integration über die Tiefe) nicht unterschätzt werden. Schließlich wurden Bakterien mit signifikanten Stoffwechselaktivitäten in über 500 m Sedimenttiefe unter dem Meeresboden nachgewiesen (Parkes *et al.,* 1994, Barnes *et al.,* 1998).

1.3 Ökophysiologie von Isolaten aus Wattsedimenten

Während dieser Studie wurde eine Vielzahl phylogenetisch diverser Bakterien aus den Sedimenten isoliert. Diese konnten sieben verschiedenen phylogenetischen Gruppen zugeordnet werden. Bezugnehmend auf einen Vergleich der Sequenz der 16S rRNA der Isolate mit Sequenzen der Datenbank des NCBI Servers können einige der Isolate als neue Arten oder sogar vermutlich neue Gattungen angesehen werden.

Da die Isolate aus den höchsten positiven Verdünnungsstufen der Kultivierungsansätze gewonnen wurden, scheinen sie *in situ* abundant zu sein. Ferris *et al.* (1996) zeigten dass Isolate, die aus MPN-Reihen gewonnen wurden, sehr wohl zu abundanten 16S rDNA Sequenzen korrespondierten. Die MPN-Technik ist bisher immer noch die Methode der Wahl, um abundante Bakterien zu kultivieren. Dass das aber nur begrenzt möglich zu sein scheint, zeigt die bereits erwähnte Diskrepanz zwischen den Ergebnissen der mikrobiologischen und molekularbiologischen Untersuchungen. Bruns *et al.* (2000) konnten z.B. auch in hohen Verdünnungsstufen von MPN-Reihen keine abundanten Spezies nachweisen. Das verdeutlicht, dass die Beschränkung auf eine Methode für die Analyse von mikrobiellen Gemeinschaften zu Fehlinterpretationen der ökologischen Bedeutung der Bakterien im Habitat führen kann. Daher sollte eine Kombination aus kultivierungsabhängigen und –unabhängigen Methoden favorisiert werden.

Das sollte nicht nur bei den phylogenetischen sondern auch bei den physiologischen Untersuchungen berücksichtigt werden. Mittlerweile stehen Methoden zur Verfügung, welche die Bestimmung von Stoffwechselaktivitäten *in situ* ermöglichen, wie z.B. MICRO-FISH (Cottrell und Kirchman, 2000). Diese ersetzten jedoch nicht die physiologische Charakterisierung von Isolaten im Labor.

Die Bakterien in diesen dynamischen, stark durchmischten Sedimenten sind vor allem in den oberflächennahen Bereichen sich verändernden Einflüssen ausgesetzt, wie dem Sauerstoffgehalt, der Temperatur, der Salinität. Die intensive Bioturbation und Sedimentumlagerungen durch hydrodynamische Kräfte bewirken, dass die Wattsedimente ein Mosaik aus einzelnen Mikronischen darstellen. Obwohl aufgrund hoher mikrobieller Aktivitäten in oberflächennahen Sedimenten der Sauerstoff meist innerhalb der ersten Sedimentmillimeter aufgebraucht ist, sind durch Aktivität einiger Makrozoobenthos-Arten oxische Mikronischen auch noch in 20 cm Sedimenttiefe zu finden (Kristensen, 2000a). Diesem heterogenen System mit seinen kurzzeitigen Änderungen haben sich die Bakterien durch eine große physiologische Vielseitigkeit angepasst. Von den in dieser Studie isolierten 112 Stämmen sind ca. 34 % fähig, sowohl unter oxischen als auch anoxischen Bedingungen zu wachsen, was bei (Sediment-)Umlagerungsprozessen von Vorteil ist.

Vor allen Dingen die oberflächennahen Sedimente unterliegen großen saisonalen Temperaturschwankungen von ca. –5°C bis 25°C, wobei sich die Sedimentoberfläche kurzzeitig auch noch stärker erwärmen kann. An diese Temperaturen haben sich z.B. die in dieser Studie isolierten Stämme der *Roseobacter*-Gruppe adaptiert. Mit einem Wachstum

im Bereich von 4 bis 35°C und einem Temperaturoptimum der Wachstumsrate von 20 bis 25°C wurden sie als psychrotroph eingestuft (Morita, 1975).

Auf die unterschiedliche Qualität des organischen Materials reagieren Bakterien auf verschiedene Weise. Zum Einen können sie sich, wie die *Roseobacter* verwandten Isolate, durch ein breites Substratspektrum hinsichtlich der verwertbaren Kohlenstoffquellen auszeichnen. Damit sind diese Bakterien gegenüber denen im Vorteil, die nur einige wenige Kohlenstoffquellen verwerten können. Das refraktäre organische Material in den tiefern Sedimenten wird wahrscheinlich von „Spezialisten" verwertet, zu denen vermutlich auch Vertreter der Gruppe der *Chloroflexi* zählen (Coolen *et al.,* 2000, Wilms *et al.,* 2000b). Als eine Folge der unvorteilhaften Bedingungen in tieferen Sedimentschichten kann die hohe Zahl der Sporenbildnern angesehen werden. Durch die Sporenbildung sind Bakterien in der Lage, Mangelphasen zu überstehen, in denen ein wichtiger Nährstoff limitiert ist (Madigan *et al.,* 2000).

1.4 Allgemeine Betrachtung

Die hier vorliegende Studie beschreibt die Wattsedimente als ein biogeochemisch sehr vielfältiges System. Im Hinblick auf die Zusammensetzung der Bakteriengemeinschaften stellen sie ein typisches marines Küstenhabitat dar (Gonzalez und Moran, 1997, Stevens *et al.,* 2004, 2005, Llobet-Brossa *et al.,* 1998), das die höchsten mikrobiellen Aktivitäten nahe der Sedimentoberfläche aufweist. Die große Zahl der Isolate sowie deren hohe Diversität im Vergleich zu anderen Sedimenten zeigt außerdem, dass das Potential der Wattsedimente für die Isolierung neuer Bakterien noch nicht ausgeschöpft ist.

Die oberflächennahen Sedimentbereiche grenzen sich deutlich durch die unterschiedlichen Bakteriengemeinschaften von den tieferen Sedimenten ab. Die hohe Zahl an *Chloroflexus* verwandten Phylotypen in diesen Sedimentbereichen (Wilms *et al.,* 2006b) deutet auf eine Verbindung dieser zur „tiefen Biosphäre" hin, die sich über mehrere hundert Meter in die Tiefe erstrecken kann (Parkes *et al.,* 2005).

Wattsedimente stellen aber auch durch ihre physiko-chemischen Eigenschaften eine Besonderheit dar. Da ist zum Einen die vertikale Sedimentabfolge des Standortes NSN zu nennen. Es ließen sich drei große Sedimentbereiche unterscheiden: ein oberer sandiger, ein mittlerer muschelhaltiger und ein unterer toniger. Die unerwartet hohen Sulfatwerte in 240 cm Tiefe sind vermutlich auf ständige Sulfateinträge in die tieferen Wattsedimente durch die als Aquifer dienende Muschelschicht zurückzuführen. In die tieferen

Sedimentbereiche des Standortes Gröninger Plate scheint, wie die Tiefenprofile der Chloridkonzentrationen vermuten lassen, Süßwasser eingetragen zu werden.

Diese physiko-chemischen Sedimenteigenschaften beeinflussten scheinbar auch die Zusammensetzung der mikrobiellen Gemeinschaften wie deren Aktivitäten. Es kam zu Veränderungen, wenn sich sowohl die Verfügbarkeit organischen Materials wie auch die Lithologie änderte.

2. Ausblick

Die Ergebnisse dieser Arbeit weisen darauf hin, dass auch in den tieferen Sedimentschichten erhöhte mikrobielle Aktivitäten zu finden sein können und das diese u.a. von der Lithologie beeinflusst zu sein scheinen. Um diese Aussagen zu verifizieren, sollten die mikrobiellen Aktivitäten hochauflösender über die Tiefe gemessen werden.

Die Modellierung der Netto-Sulfatreduktionsraten ließ ein Maximum dieser im Bereich der Sulfat-Methan-Übergangszone erkennen. Im Hinblick auf die Diskussion der anaeroben Methanoxidation sollte daher parallel zu den bisher berücksichtigten chemischen Parametern die Konzentration von Methan im Sediment bestimmt werden. Weiterhin sollten Methanbildungsraten gemessen werden, um auf mögliche Stoffflüsse Rückschlüsse ziehen zu können.

Aktivitätsparameter stellen ein unverzichtbares Werkzeug in der Ökosystemanalyse dar, weshalb die Aufnahme weiterer mikrobieller Aktivitäten, wie z.B. die Bestimmung des „heterotrophen Potentials“ mittels radioaktiv markierter Substrate, berücksichtigt werden sollte. In Verbindung damit können zusätzliche Informationen durch die Bestimmung von Stoffwechselzwischenprodukten und –endprodukten und damit auch potentieller Substrate, wie z.B. Aminosäuren, kurzkettige Fettsäuren und Alkohole, gewonnen werden.

Durch die Anwendung weiterer molekularbiologischer Methoden wie CARD-FISH und *real-time* PCR ließen sich die Abundanzen aber auch die Diversität ausgewählter Gruppen, wie z.B. von Vertretern der *Roseobacter*-Gruppe, Sulfatreduzierern und Methanogenen im Sediment *in situ* untersuchen.

In dieser Studie wurde eine große Stammsammlung erstellt, wobei einige der Isolate neue Arten aber auch neue Gattungen darzustellen scheinen. Vor allem diese gilt es phylogenetisch einzuordnen sowie physiologisch zu charakterisieren. Damit sind Einblicke in die Anpassung der Isolate an ihr Habitat und Schlussfolgerungen auf deren Funktion *in situ* möglich.

Im Hinblick auf die noch offenen Fragen zur Sedimentstruktur sollte eine Transektbeprobung fokussiert werden, um zu klären, inwieweit unterirdischen (Grund)Wasserströmen auftreten und welchen Einfluss sie auf die chemischen Sediment- und Porenwasserdaten haben.

Kapitel VI

Literatur

1. Literaturverzeichnis der Einleitung und Diskussion

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Erklärung

Hiermit bestätige ich, dass ich die vorliegende Dissertation selbständig verfasst und nur die angegebenen Quellen und Hilfsmittel verwendet habe.

Weiterhin erkläre ich, dass diese Dissertation weder in ihrer Gesamtheit noch in Teilen einer anderen wissenschaftlichen Hochschule zur Begutachtung in einem Promotionsverfahren vorliegt oder vorgelegen hat.

Dresden, den 28.09.06

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