MICROBIAL ECOLOGY OF ANAEROBIC CARBON MINERALIZATION IN NAMIBIAN SHELF SEDIMENTS

ELSABÉ M. JULIES

MICROBIAL ECOLOGY OF ANAEROBIC CARBON MINERALIZATION IN NAMIBIAN SHELF SEDIMENTS

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Elsabé Mathilda JULIES

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1. Gutachter: Prof. Dr. Bo Barker Jørgensen

2. Gutachter: Prof. Dr. Kai-Uwe Hinrichs

Weitere Prüfer:

Prof. Dr. Wolfgang Bach
Dr. Volker Brüchert

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For my son Stefan

“Microbial ecology is the study of microbial physiology under the worst possible conditions“(Brock 1966)

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Summary

This PhD thesis is an essential component of the "Namibia Gas" (NAMIBGAS) project, which aims to improve the understanding of the rate and fluxes of hydrogen sulfide and methane to the sediment surface and into the water column on the Namibian shelf, which is one of the most productive upwelling systems on Earth. Organic matter degradation in the sediment drives hydrogen sulfide production, the maintenance of anoxia, and methane formation. Therefore, the primary aims of the thesis are to study carbon transformation processes within the sediment, determine the reactivity of organic carbon in the sediment and, investigate the control of microbial community structure and activity by the amount and accessibility of carbon sources. The emphasis is on linking microbial community structure (identity) to their function (activity) to provide new insights into the microbial ecology that controls carbon turnover in these upwelling sediments. By studying the stepwise degradation of organic carbon a complete process overview of organic carbon mineralization could be obtained.

Firstly, the diagenetic transformation of dissolved organic carbon (DOC), in particular dissolved carbohydrates, was studied by using both biogeochemical methods and molecular techniques. The bulk sediment composition, pore water chemistry, polysaccharide hydrolysis rates, ^{35}S-sulfate reduction rates, and the abundance of active bacteria involved in the initial and terminal processes of organic carbon degradation within the top 15 cm of the sediment from two sampling stations were determined (Chapter 2). Secondly, the diversity of bacteria from the same two sampling stations within the top 12 cm of the sediment, using the 16S rRNA library approach were investigated (Chapter 3). The central question was whether shifts of the community structure of bacteria involved in the major carbon transformation steps of hydrolysis, fermentation, and terminal oxidation are reflected in changes of biogeochemical rates. Finally,

the effect of a sudden high input of DOC into the sediment in the form of high molecular weight substances (the polysaccharide laminarin) and low molecular weight substances (lactate and acetate) on the metabolic activity and community structure of bacteria involved in initial hydrolytic and fermentation steps and terminal oxidation was investigated during two separate experiments (Chapter 4). Substrate addition simulates the high input of organic matter into the sediment after a phytoplankton bloom, following upwelling events.

In the different experiments performed in this thesis, only two structurally specific polysaccharides, laminarin and pullulan were used as proxies of carbohydrates to quantify the initial transformation of high molecular weight DOC in Namibian shelf sediments, because of the complexity of DOC and the limited methodology for its characterization.

A tight coupling between the active heterotrophic and sulfate-reducing bacterial communities existed in the top 10 cm of the sediment, which is reflected in the low volatile fatty acid (VFA) concentrations. The coupling between initial steps and terminal oxidation appears to be controlled by the availability of degradable dissolved organic matter. Gradients in biogeochemical rates and bacterial abundance were not reflected in the depth profiles of particulate organic carbon (POC), DOC, VFA, or total dissolved carbohydrates. This is because an increasing amount of DOC, including the carbohydrate fraction, became unavailable to microbial communities with depth, resulting in an increasing substrate limitation for the active bacteria with depth. The shift in the microbial community composition with depth as revealed by 16S rRNA clone libraries was due to the decrease in organic carbon substrate availability, which results in niche diversification that is related to the physiological abilities of the microorganisms. Experiments in which the sediment was amended with organic carbon also revealed that microorganisms in the top 6 cm of the sediment and in the bottom sediment (14-16 cm depth)

have a differential response to a pulse of DOC into the sediment. Both the addition of low molecular weight -DOC and high molecular weight - DOC to the top sediment (0-2 cm), where higher sulfate reduction rates were measured, and to the bottom sediment (14-16 cm), where lower sulfate reduction rates were measured, revealed the presence of different sulfate reducing bacterial communities in these two depth intervals. Bacteria capable of accelerating hydrolysis and carbon oxidation rates in response to enhanced organic matter input dominate in the top 2 cm of the sediment, while the bacteria at a depth of 14 cm exhibit slower response to substrate input. The ecological niche of sulfate reduction is dominated by fast growing, incomplete oxidizing sulfate reducing bacteria (SRB) in the top sediment, while slower growing completely oxidizing SRB dominate in the deeper sediment. A direct relationship between the decrease in abundance of active Bacteria (Bacteroidetes, Gammaproteobacteria, and SRB) and the decrease in bacterial sulfate reduction rates with depth was revealed by Catalyzed Reporter Deposition Fluorescent In Situ Hybridization (CARD-FISH). **The increasing limitation of electron donor supply (organic carbon substrate availability) results in microbial community shifts and a decrease in the abundance of active bacteria with depth. This is manifested in the steep decrease of metabolic rates with depth.** In addition, the differential response of bacteria to organic carbon addition in the top 6 cm of the sediment compared to the bottom 14-16 cm indicates the **adaptation of bacteria to the occurrence of more reactive DOC in the top sediment, while the bacteria in the deeper sediment are adapted to the presence of less reactive DOC.**

The differences in biogeochemical rates and gradients of two of the sampling stations that were in close proximity and experience similar primary productivity, was attributed to hydrographic controls on particle retention and sediment re-suspension in the bottom boundary

layer. Differences in phylogenetic diversity and the abundance of active bacteria between the two sampling stations were indirectly resulting from different hydrographic factors at these stations, suggesting that **the bottom boundary layer is a critical compartment in the transformation of organic matter.**

This thesis provides the first report on the phylogenetic analysis of bacterial diversity in Namibian shelf sediments. **The organic-rich sediments on the Namibian shelf support a high microbial diversity, especially in the top 6 cm of the sediment where higher biogeochemical rates were measured. The gradient in microbial activity with depth is reflected in the microbial diversity, because of different selective pressures existing throughout the depth of the sediment.** 16S rRNA clone libraries revealed that bacteria with hydrolytic and fermenting abilities include members from the γ-proteobacteria, Bacteroidetes, Actinobacteria, Acidobacteria, Firmicutes, and Thermomicrobia. Bacteroidetes bacteria play an important role in the initial degradation of carbohydrates in these anaerobic marine sediments, as revealed by their dominance in the clone libraries, the high abundance of active members, detected by CARD-FISH, and their rapid response to laminarin addition revealed by Denaturing Gradient Gel Electrophoresis. Sequences affiliated to Actinobacteria dominated the clone libraries from bottom sediments at Station 5. However, CARD-FISH data revealed a low abundance of active members of these bacteria. SRB were diverse and belong to the δ-proteobacteria. Cloned sequences within the δ-proteobacteria affiliates to the Desulfobulbaceae and Desulfobacteriaceae families.

Another important finding of this thesis was that the ecological niches occupied by different sulfate reducing bacteria throughout the sediment depth are a function of the physiological abilities of the bacteria, especially their ability to utilize different substrates. This thesis

emphasizes **the importance of an ecological classification of microbial communities, rather than using phylogenetic classification in isolation to understand the structure and function of microbial communities involved in biogeochemical cycles.**

Zusammenfassung

Die vorliegende Arbeit wurde durchgeführt als ein wesentlicher Bestandteil des „Namibia Gas Projektes“ (NAMIBGAS). Zielsetzung dieses Projektes ist es, die Zusammhänge der Fluxe und Umsatzraten besser zu verstehen, und das Verständnis über diese Prozesse in einem der produktivsten Auftriebsysteme der Erde zu vertiefen. Der mikrobielle Abbau organischer Stoffe im Sediment zu CO_2 fördert die Schwefelwasserstoffproduktion, hält dadurch das Sediment anoxisch und führt zu Methanbildung. Das vorrangige Ziel dieser Arbeit ist es, die einzelnen Schritte der Umwandlungssprozesse des Kohlenstoffs im Sediment zu untersuchen. Der Schwerrpunkt ist dabei, die Reaktivität von organischem Kohlenstoff im Sediment zu bestimmen und zu erforschen, wie die Zusammensetzung der mikrobiellen Gemeinschaft und Aktivität von der Quantität und Verfügbarkeit des Kohlenstoffs kontrolliert wird. Um neue Erkenntnisse über die mikrobielle Ökologie zu erhalten, die den Kohlenstoffumsatz in Sedimenten kontrolliert, liegt der Schwerpunkt dieser Arbeit auf der Verknüpfung der Zusammensetzung der mikrobiellen Gemeinschaft mit ihrer Funktion. Durch die Untersuchung des schrittweisen Abbaus von organischem Kohlenstoff konnte in dieser Arbeit ein vollständiger Überblick über dessen Mineralisierung gewonnen werden.

Die diagenetische Umwandlung von gelöstem organischem Kohlenstoff in gelöste Kohlenhydrate, wurde sowohl mit biogeochemischen Methoden als auch mit molekularbiologischen Techniken untersucht. So konnte die Hauptzusammensetzung des Sediments, die Konzentrationen gelöster Stoffe im Porenwasser, die Polysaccharid-Hydrolyse- und ^{35}S-Sulfatreduktionsraten ebenso bestimmt werden, wie die Abundanz der aktiven Bakterien, die bei den initialen und terminalen Prozessen des organischen Kohlenstoffabbaus, innerhalb der oberen 15 cm des Sediments zweier verschiedener

Probennahme-Stationen, beteiligt waren. (Kapitel 2). Ausserdem wurde die bakterielle Diversität der selben Probennahme-Stationen innerhalb der oberen 12 cm des Sediments anhand von 16S rRNA-Genbanken untersucht (Kapitel 3). Die zentrale Frage war dabei, ob sich Änderungen der Zusammsetzung der mikrobiellen Gemeinschaft, der bei der schrittweisen Kohlenstoff-Umwandlung, Hydrolyse, Fermentation und abschließender Oxidation beteiligten Bakterien in den biogeochemischen Raten wiederspiegeln. Abschließend wurde in zwei getrennten Experimenten der Einfluss von einer hohen Zugabe von gelöstem organischem Kohlenstoff, in Form von hoch molekulargewichtigen Substanzen (Laminarin) und niedrig molekulargewichtigen Substanzen (Lactat und Acetat) zum Sediment untersucht. Dabei galt es den Einfluss dieser Substanzen auf die Zusammensetzung und Stoffwechselaktivität der mikrobiellen Gemeinschaft zu untersuchen, die an den initialen hydrolytischen und Fermentations-Vorgängen sowie der terminalen Oxidation beteiligt sind (Kapitel 4). Die Zugabe von Substrat sollte die starke Zunahme an organischer Materie im Sediment, wie es nach einem Auftriebsgeschehen in Form der Phytoplankton-Blühte auftritt, simulieren.

Aufgrund der Komplexität der gelösten organischen Kohlenwasserstoffe und der begrenzten Methodologie für ihre Charakterisierung, wurden in den verschiedenen Experimenten dieser Arbeit ausschließlich zwei strukturell spezifische Polysaccharide, Laminarin und Pullulan, als Vertreter verwendet. So konnte der erste Schritt der Umwandlung des hoch molekulargewichtigen gelösten Kohlenstoffs im Sediment des Namibischen Shelfs quantifiziert werden.

Es hat sich herausgestellt, dass eine enge Kopplung zwischen den aktiven heterotrophen und sulfatreduzierenden Bakterien in den obersten 10 cm des Sediments bestand, da die Fettsäuren als Zwischenprodukte nur in niedriger Konzentration im Sediment vorlagen. Daher scheint die Kopplung von den ersten Abbauprozessen und terminaler Oxidation von

der Verfügbarkeit gelöster organischer Materie abzuhängen. Die Anzahl der Bakterien und die Verteilung der biogeochemischen Raten zeigen keinen Zusammenhang mit dem Tiefenprofil des partikulären organischen Kohlenstoffs, des gelösten organischen Kohlenstoffs, der intermediären Fettsäuren oder der vollständig gelösten Kohlenhydrate. Das liegt daran, dass ein größerer Anteil von gelöstem organischen Kohlenstoff einschließlich der Kohlenhydrate-Fraktion mit der Tiefe für Mikroorganismen nicht mehr zur Verfügung steht, wodurch die Substratlimitation für aktive Bakterien mit der Tiefe zunimmt. Die strukturelle Veränderung der mikrobiellen Gemeinschaft mit der Tiefe konnte durch die unterschiedlichen Verfügbarkeit von organischen Substraten im Sediment erklärt werden, was zu einer Nischen-Diversifikation in Abhängigkeit der physiologischen Fähigkeiten der Mikroorganismen führt. Die Experimente mit Substratzusatz zeigten, dass Mikroorganismen in den oberen 6 cm des Sediments und in den tieferen Schichten von 14-16 cm Tiefe unterschiedlich auf den Eintrag von gelöstem organischem Kohlenstoff im Sediment reagieren. Die Zugabe von gelöstem organischem Kohlenstoff, mit niedrigem Molekulargewicht und mit hohem Molekulargewicht, zum Oberflächensediment (0-2cm), wo höhere Sulfatreduktionsraten gemessen wurden, und zum Bodensediment (14-16 cm), wo niedrigere Sulfatreduktionsraten gemessen wurde, zeigte die Anwesenheit von verschiedenen sulfatreduzierenden Bakteriengemeinschaften in diesen beiden Tiefenintervallen. Bakterien, die aufgrund eines höheren Eintrags von organischer Materie zu beschleunigten Hydrolyse- und Kohlenstoffoxidationraten fähig sind, dominierten die oberen 2 cm des Sediments, während Bakterien in einer Tiefe von 14 cm eine langsamere Reaktion auf Substratzugabe aufwiesen. Die ökologische Nische für Sulfatreduktion wird von schnell wachsenden, unvollständig oxidierenden, sulfatreduzierenden Bakterien im oberen Sediment dominiert, wohingegen im tieferen Sediment langsam wachsende und vollständig oxidierende sufatreduzierende Bakterein vorherrschen. Der direkte Zusammenhang von tiefenabhängigen

Gradienten bakterieller Sulfatreduktionsraten und der Abundanz von aktiven Bakterien (Bacteriodetes, Gammaproteobacterien, und sulfatreduzierenden Bakterien), wurde durch Catalyzed Reporter Deposition Fluorescence In Situ Hybridisation (CARD-FISH) Daten deutlich. **Die steigende Limitierung von Elektronendonatoren (Verfügbarkeit von organischem Kohlenstoff-Substrat) führte zu einer Veränderung in der Zusammensetzung der mikrobiellen Gemeinschaft und zu einer Abnahme der Abundanz von aktiven Bakterien mit der Tiefe. Dies manifestierte sich in den schnellen Abnahme der metabolischen Umsatzraten mit der Tiefe**. Ausserdem deutet die unterschiedliche Reaktion der Bakterien auf den Substratzusatz zu dem Oberflächensediment (6 cm) verglichen zu den tieferen Sedimentschichten (14-16 cm) an, dass die Bakterien im Oberflächensediment schneller durch Zusatz von reaktivem gelöstem organischem Kohlenstoff stimuliert werden, als die Bakterien im tieferen Sediment. Unterschiede in den biogeochemischen Raten und Gradienten zwischen zwei nah aneinanderliegenden Probennahme-Stationen, mit einer ähnlichen Primarproduktivität wurden auf die hydrografische Steuerung der Partikelretention und Sedimentresuspension in der bodennahen Grenzschicht zurückgeführt. Unterschiede in der phylogenetischen Diversität und der Abundanz aktiver Bakterien zwischen den beiden Probennahme-Stationen resultierten indirekt aus unterschiedlichen hydrografischen Faktoren an diesen Stationen. Das führte zu der Annahme**,** dass **die bodennahe Schicht ein entscheidener Ort für die Umwandlung organischer Stoffe ist.**

Die vorliegende Arbeit liefert einen ersten Einblick in die bakterielle Diversität von Sedimenten des Namibischen Schelfs. **Dieses organisch reiche Sediment des Namibischen Schelfs fördert, besonders in den oberen 6 cm des Sediments wo höhere biogeochemische Raten gemessen wurden, eine hohe mikrobielle Vielfalt. Die Änderung der mikrobiellen Aktivität in Relation zur Tiefe spiegeln sich, aufgrund des**

unterschiedlichen selektiven Druckes, der in der gesamten Sedimentschicht herrscht, in der mikrobiellen Vielfalt wider. 16S rRNA Genbanken zeigten dass Bakterien, die über hydrolytische und fermentative Stoffwechselwege verfügen, zu Angehörigen der γ-Proteobakterien, Bacteroidetes, Actinobacterien, Acidobacterien, Firmicutes und Thermomicrobien gehörten.

Die Gruppe der Bacteroidetes scheinen in diesem anaeroben Meeressediment, bei dem ersten Abbauschritt von Kohlenstoff, eine wichtige Rolle zu spielen. Dies wird unter anderem deutlich durch ihre Dominanz in den 16S rRNA Genbanken, die hohe Abundanz von aktiven Vertretern dieser Bakterien,und ihre schnelle Reaktion auf Laminarin Zufuhr, wie mit Denaturierender Gradienten Gel Elektrophorese (DGGE) gezeigt werden konnte. Sequenzen, die eine phylogenetische Zugehörigkeit zu Actinobacterien zeigten, dominierten die Klondatenbanken der Bodensedimente in Station 5, obwohl die CARD-FISH-Daten nur eine geringe Menge aktiver Bakterien dieser Gruppe aufwiesen.

Die sulfatreduzierenden Bakterien zeigten eine hohe Diversität und gehörten zu den Familien der Desulfobulbaceae und Desulfobacteriaceae aus der Gruppe der δ-Proteobacterien.

Eine wichtige Entdeckung dieser Arbeit war, dass die Besetzung verschiedener ökologischer Nischen durch unterschiedliche sulfatreduzierende Bakterien innerhalb des gesamten Sediments eine Funktion der physiologischen Eigenschaften dieser Bakterien ist, besonders ihre Fähigkeit, unterschiedliche Substrate zu verwerten.

Diese Arbeit hebt besonders die Wichtigkeit der ökologischen Klassifikation mikrobieller Gemeinschaften im Gegensatz zur ausschließlich phylogenetischen Klassifikation hervor, um die strukturelle Zusammensetzung und Funktion der bei biogeochemischen Kreisläufen beteiligter mikrobiellen Gemeinschaften zu verstehen.

Chapter 1

Introduction

The marine carbon cycle

Oceans cover approximately 70% of the Earth's surface (Pidwirny and Duffy 2007) and the biogeochemical cycling of carbon within the marine ecosystem has a significant effect on the global climate by acting as a major sink for anthropogenic CO_2. The marine and terrestrial biospheres absorbed approximately 50% of the 7-8 Pg C y^{-1} CO_2 emitted by anthropogenic activities in the 1990s (Field and Raupach 2004). The marine carbon cycle is influenced by the fixation and release of carbon across two boundaries, the atmosphere-water interface and the sediment-water interface (benthic boundary layer) (Seiter et al. 2005). The difference in partial pressure of CO_2 in the atmosphere and in surface waters is the driving force for the net flux of CO_2 into or out of the ocean, across the atmosphere-water interface. The partial pressure of CO_2 in surface water is controlled by pH, temperature and net carbon production rates (Takahashi 1989). The exchange of CO_2 across the sediment-water interface in the oceans is influenced by the mass movement of carbon in two opposing directions (Fig. 1). First, the balance between remineralization and burial of organic carbon in marine sediments remove carbon from the carbon cycle over geological time periods, and secondly, the dissolution or microbial decomposition of accumulated carbon in the sediment supply remineralized dissolved carbon (Zabel and Hensen 2000). The oceans act as a sink for CO_2 in temperate and high latitudes, but as a source of CO_2 to the atmosphere in the tropics, because of degassing of CO_2 (Field and Raupach 2004). Since the ocean serves both as a source and a sink in the global carbon cycle, it is important to understand the dynamics of the marine carbon cycle and the turnover times of carbon within the ocean.

Carbon fixation occurs mainly through oxygenic photosynthesis within the upper water column by phytoplankton (Hedges 1992). The total annual primary production by photosynthesis in the oceans is approximately 51-64 Gt C (Chester 1999). Primary production rates in coastal regions is 14-18% and in upwelling regions 0.2-0.3% of the global

ocean total production, although they occupy only 9.9% and 0.1% of the total ocean area, respectively (Chester 1999). During consumption of organic carbon in the water column, both particulate organic carbon (POC) and dissolved organic carbon (DOC) are released into the water column (Fig. 1).

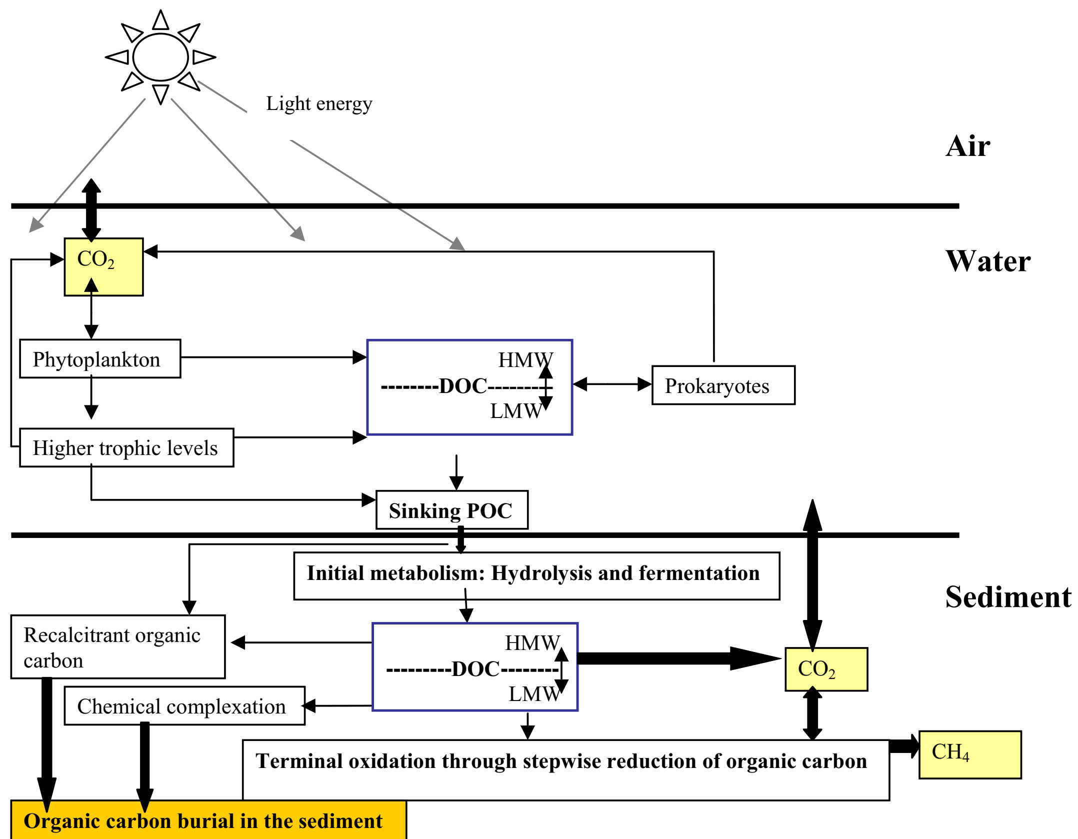

Figure 1: A simplified schematic representation of the organic carbon cycling within the marine ecosystem. The exchange of carbon between the air-water and sediment-water boundaries is shown with thick arrows. DOC within the water column and sediment can be converted back from low molecular weight (LMW) compounds to high molecular weight (HMW) compounds by processes of chemical polymerization.

Dead plant and animal material, particulate organic matter (POM) and dissolved organic matter (DOM) is transported to the sediments as aggregates, referred to as marine snow (Alldredge and Silver 1988; Kiørboe et al. 1995; Kiørboe 2000). In the deep ocean most of

the sinking organic matter is remineralized within the water column, but in shallow coastal regions, 10-50% of the primary production reaches the sediment (Wollast 1998; Wakeham et al 1997; Canfield 1993). The fraction that reaches the sediment is mainly a function of water depth (Zabel and Hensen 2002; Middelburg et al. 1993) and the local productivity. The global ocean POC flux is about 0.5 Gt C y^{-1} (Seiter et al. 2005) to 10 Gt C y^{-1} (Schlitzer 2002) of which 33% occurs in the Southern Ocean south of 30°S, in particular in a zonal belt along the Antarctic Circumpolar Current and in the Peru, Chile, and Namibian coastal upwelling regions (Schlitzer 2002). DOC which is recalcitrant and resists degradation is buried in the sediment, making marine sediments an important potential sink for organic carbon. The estimated amount of carbon which is buried in marine sediments globally, is 0.002-0.12 Gt C yr^{-1} (Seiter et al. 2005), which corresponds to 0.03% of the organic carbon produced in the photic zone (Zabel and Hensen 2002). Continental margins account for 83% of the global sedimentary mineralization (Jørgensen 1983) and accumulate about 87% of the total organic matter buried in sediments (Berner 1982). It is important to determine the amount of organic matter which is mineralized relative to the amount which is preserved in marine sediments, since this affect the levels of CO_2 and O_2 over geological time scales. For this purpose a better understanding of the processes that control organic matter degradation in marine sediments is required. Specific emphasis should be placed on continental margins, with the focus on the flux of organic matter, and the degradation and accumulation of organic carbon in sediments from areas of coastal upwelling with high productivity. In this thesis the microbial ecology within shelf sediments from the Namibian upwelling system, a highly productive marine ecosystem was studied.

The Namibian upwelling system as a highly productive system

Coastal upwelling is a wind-driven process and occurs in all areas where equator-ward winds cause horizontal divergence of surface water, but is particularly intense in eastern boundary currents (Ohde et al. 2006; Summerhayes et al. 1995). The net horizontal offshore transport of surface water is referred to as Ekman transport. In coastal waters Ekman transport can produce intense upwelling of water from deeper layers to replace the surface offshore drift (Berger and Wefer 2002; Summerhayes et al. 1995). The major upwelling regions of the World include the shelves off the western margins of West Africa, Namibia, Peru, the western USA, India, and Southeast Asia (Chester 1999).

The Benguela current is a northward moving current that occupies a zone of about 200 km wide off the west coast of southern Africa from Cape Town north to the latitude of 15°S off the coast of Namibia (Shannon 1985). It is the eastern part of the South Atlantic gyre and the driving force is the south-east trade winds (Ohde et al. 2006), which blow parallel to the coast, giving rise to offshore Ekman transport. The South Atlantic high-pressure system is a permanent feature and varies spatially and temporally in intensity (Ohde et al. 2006). Seasonality in upwelling is more prominent in the southern Benguela system compared to the perennial wind regime of the northern system (Ohde et al. 2006). Spatial variation in upwelling among the different upwelling cells between 16°S and 34°S depends on the strength and direction of the wind field, the vertical structure of the water, instabilities in current flow, variations in the bottom bathymetry along the coast, and remotely forced processes, such as coastally trapped waves and warm water intrusions (references in Ohde et al. 2006; Lass and Mohrholz 2005; Gründlingh 1999). The offshore transport of surface water is replaced by a deep compensation current containing cold, nutrient-rich South Atlantic Central Water (Wefer et al. 1996). Biological productivity is greatly enhanced by the upwelling of nutrient-rich subsurface water into the euphotic zone and the retention of

phytoplankton in well-lit waters by stratification of the water column. The high primary productivity within the Benguela upwelling region (Fig. 2c) supports large populations of fish, marine mammals and birds (Gründlingh 1999; Ryther 1969).

In the Benguela upwelling system primary producers are dominated by diatoms and to a lesser extent by dinoflagellates (Ohde et al. 2006). The high primary productivity on the shelf of 175-240 mmol C m^{-2} d^{-1} (Summerhayes et al. 1995) and the shallow water depth resulted in the formation of a thick diatomaceous mud belt of about 500 km long on the inner shelf between 19° and 26°S around 60-100 m water depth (Struck et al. 2002; Bremner 1978) with organic carbon contents of up to 15% of the dry weight (Inthorn et al. 2006) (Fig. 2d). The maximum age of the diatomaceous mud is equivalent to 3100 years, determined by ^{14}C-dating (Struck et al. 2002). Namibian shelf sediments are mainly biogenic and the input of terriginous organic matter into the sediment is negligible (Pichevin et al. 2004). Aerobic degradation of dead organic matter in the water column in combination with the occasional intrusion of water with low dissolved oxygen below the halocline from north of the Angola-Benguela front into the northern Benguela system result in suboxic to anoxic bottom water (Monteiro et al. 2006) (Fig. 2a). Therefore, up to 90% of the organic carbon degradation within the sediment of the Benguela upwelling region is anaerobic (Ferdelman et al. 1999).

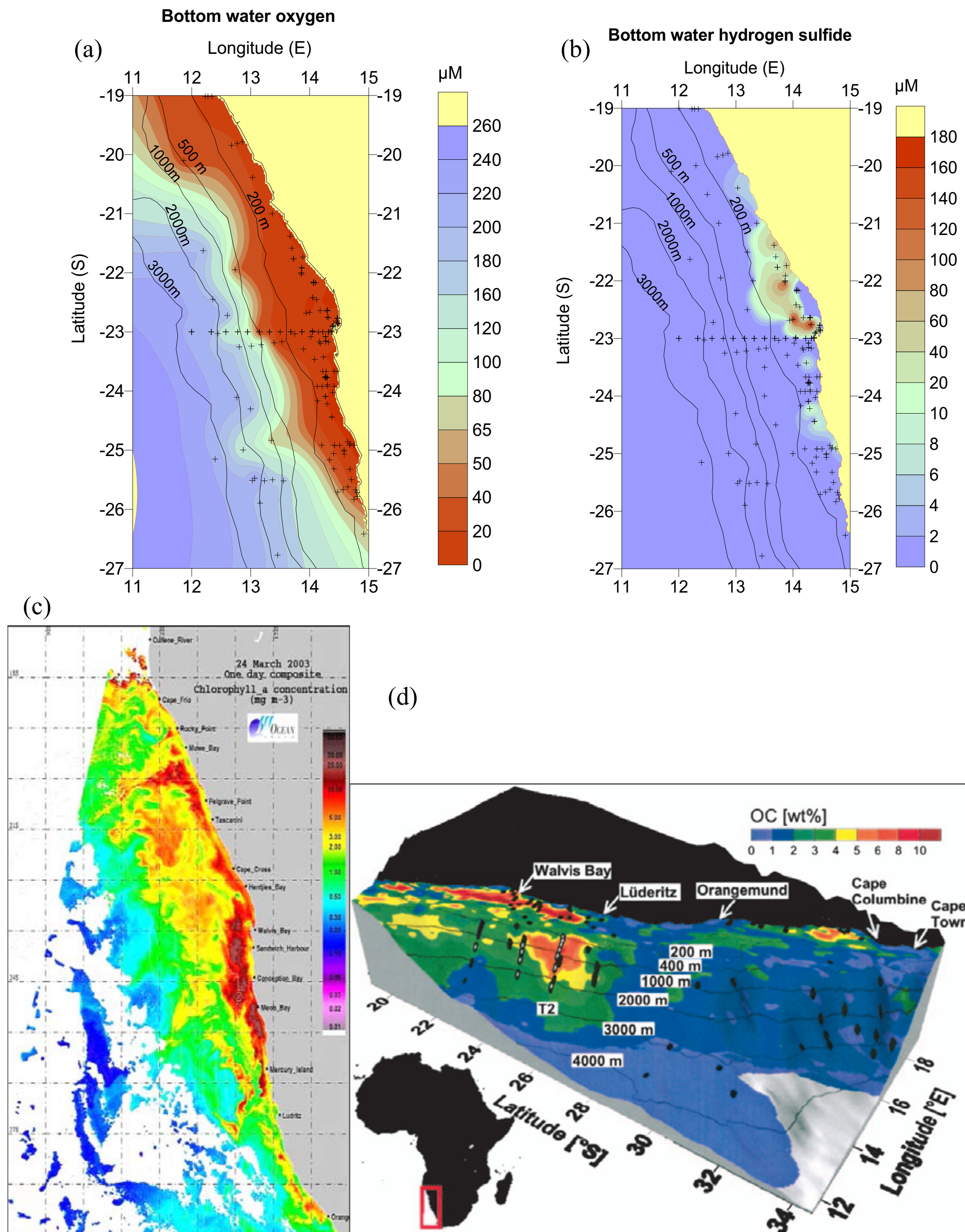

Figure 2: a) Dissolved oxygen and b) hydrogen sulfide concentrations in the bottom water on the Nambian shelf (from Brüchert et al. 2006), c) Chlorophyll a concentrations on the Namibian shelf (SEAWIFS image, NASA), and Organic carbon in the sediment on the Namibian shelf (Inthorn et al. 2006).

Degradation of organic carbon in marine sediments

Organic matter within marine sediments originates from the sedimentation of dead plant and animal material and waste organic matter from the water column. The nature of the deposited material and the chemical, biological, or physical processes that affects this material after its deposition determines the geochemistry of marine sediments (Burdige 2006). These processes are referred to as early diagenesis and are mainly mediated by bacteria, because marine sediments often become anoxic close to the sediment-water interface (Burdige 2006). Suboxic and anoxic bottom water in coastal upwelling regions with high primary productivity, result in the formation of anoxic sediments, since most of the oxygen is consumed within the first few millimeters of the sediment during organic matter mineralization (Canfield 1993). Therefore most of the decomposition of organic matter in organic-rich marine sediments is anaerobic. The rate of organic matter remineralization within the sediment is determined by: a) the amount and reactivity of the available organic substrate, b) supply of oxidants, c) the composition and activity of the prevailing microbial community, d) temperature, and e) sediment fabric (Zabel and Hensen 2002).

The organic carbon which is not degraded within the water column and reaches the sediment consists of a complex mixture of biopolymers including proteins, carbohydrates, lipids, and uncharacterized organic matter (more than 50%) (Hedges 1988). Carbohydrates are acid-hydrolysable sugars and among the most abundant components of organic carbon within marine sediments (Cowie and Hedges 1994). Extracellular carbohydrates are exudates of phytoplankton, and stored carbohydrates serve as an internal energy reserve for phytoplankton (Alderkamp 2006). The most abundant stored carbohydrate in marine phytoplankton is Chrysolaminarin (Alderkamp 2006; Bold and Wynne 1985), which can contribute up to 80% of the organic carbon of diatoms (Alderkamp et al. 2006). Due to the presence of the thick diatomaceous mud belt on the Namibian shelf, laminarin probably

contributes significantly to the carbohydrates present in the sediments. Activity of hydrolytic enzymes capable of hydrolyzing laminarin and pullulan has been demonstrated in a wide range of marine sediments (Arnosti 1995; Hoppe et al. 2002; Arnosti and Jørgensen 2006). For these reasons laminarin and pullulan were used as model substrates in this thesis to study carbohydrate degradation.

Bacteria take up hydrophilic substrates such as carbohydrates through trimeric channel proteins which form water-filled pores with an uptake limit of 600 Dalton throughout the bacterial outer membrane (Weiss et al. 1991; Arnosti and Repeta 1994). The molecular mass of the biopolymers in the dissolved organic carbon (DOC) in sediments exceeds 600 Dalton and since most bacteria are unable to assimilate and transport such large molecules, the first step in organic carbon degradation is the exoenzymatic hydrolysis of macromolecules (Weiss et al. 1991) (Fig. 3). The products of hydrolysis are then further consumed by fermenting and acetogenic bacteria that produce low molecular weight (LMW) DOC compounds including volatile fatty acids (VFA) such as lactate, acetate, formate, propionate, butyrate, valerate and other end products such as alcohols, aromatic compounds, and hydrogen (Middelburg et al. 1993) (Fig. 3). Bacteria involved in anaerobic fermentation of carbohydrates typically assimilate 10% of the substrate carbon (Clark 1989) and excrete the remaining part as LMW organic compounds (Arnosti and Repeta 1994).

Carbohydrates with complex chemical structures that may resist extracellular enzymatic hydrolysis or may present transport or uptake problems persist in dissolved forms (Arnosti and Holmer 1999; Arnosti and Repeta 1994). Such carbohydrates form part of the uncharacterized fraction of DOC in the pore water. The majority of the uncharacterized DOM in marine sediments is very resistant to degradation and consists of LMW compounds (Hedges et al. 2000; Keil et al. 1994; Benner et al. 1992). Due to the immense complexity of marine DOM, its characterization remains a challenge and has been investigated by various

methods including high-temperature combustion of freeze dried samples, the persulfate oxidation method with infrared detection, ^{13}C– and ^{15}N-NMR (nuclear magnetic resonance spectroscopy), pyrolysis gas chromatography-mass spectrometry (references in Hansell and Carlson 2002), and more recently Fourier Transform NMR (Koch and Dittmar 2006; Dittmar et al. 2006).

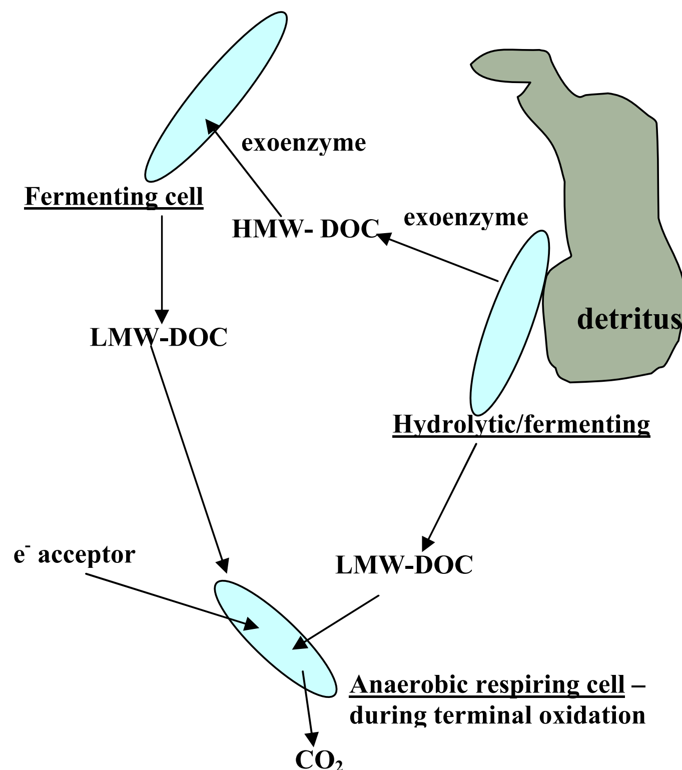

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Figure 3: Schematic presentation of initial degradation of organic matter by hydrolysis and fermentation and terminal oxidation in marine sediments. HMW-DOC = high molecular weight dissolved organic carbon, LMW-DOC = low molecular weight dissolved organic carbon.

The products of hydrolysis and fermentation, mainly VFA and H_2, are decomposed through a series of redox reactions by various prokaryotes that utilize a cascade of oxidants throughout the depth of the sediment to form CO_2 as end product (Fig. 3). The sequence in which the redox reactions occur depends on the availability of oxidants and the energy yield

from the oxidation of organic matter, resulting in the sequential depletion of electron acceptors throughout the sediments depth (Froehlich et al. 1979, Table 1).

Table 1: The sequence of redox reactions occurring throughout the sediment during organic matter oxidation with the various electron acceptors and the standard free energy yield (ΔG°), per mol organic carbon (from Jørgensen 2000).

Reaction	**ΔG° (kJ mol^{-1})**
(a) Oxic respiration $CH_2O + O_2 \rightarrow CO_2 + H_2O$	-479
(b) Nitrate reduction $5CH_2O + 4NO_3^- \rightarrow 2N_2 + 4HCO_3^- + CO_2 + 3H_2O$	-453
(c) Manganese (IV) reduction $CH_2O + 3CO_2 + H_2O + 2MnO_2 \rightarrow 2Mn^{2+} + 4HCO_3^-$	-349
(d) Iron (III) reduction $CH_2O + 7CO_2 + 4Fe(OH)_3 \rightarrow 4Fe^{2+} + 8HCO_3^- + 3H_2O$	-114
(e) Sulfate reduction $2CH_2O + SO_4^{2-} \rightarrow H_2S + 2HCO_3^-$	-77
(f) Methanogenesis $CH_3COO^- + H^+ \rightarrow CH_4 + CO_2$	-28

In anoxic marine sediments sulfate reduction is the major terminal oxidation step (Jørgensen 1982). Rates of sulfate reduction in marine sediments vary over several orders of magnitude from a few picomole cm^{-3} d^{-1} in very deep sediments to thousands of nmol cm^{-3} d^{-1} in near-surface coastal sediments (Canfield and Teske 1996). Sulfate reducing bacteria (SRB) have a syntrophic relationship with hydrolytic and fermenting bacteria, since the end products of hydrolysis and fermentation, mainly acetate, serve as substrate for SRB. When fermentation and sulfate reduction are closely coupled in the sediment, the in-situ concentrations of VFA and H_2 are low (Finke et al. 2007; Hoehler et al. 1998; Wellsbury and Parkes 1995). In contrast, high VFA concentrations in the sediments reflect an imbalance between fermentation processes and terminal oxidation (Arnosti et al. 2005; Hoehler et al.

1998). It is important to establish how close the coupling between initial and terminal steps is and to identify the rate limiting steps during organic carbon mineralization, because this will affect the quantity and composition of the DOC that accumulate in marine sediments.

The amount of hydrolysable DOC decreases with increasing decomposition and diagenetic alteration throughout the sediment depth (Cowie and Hedges 1994). The microbial decomposition of organic matter in marine sediments is expressed by the multi-G model, which defines the decay of organic matter as the sum of exponentially (ie. first-order) declining factors and the non-reactive fraction (Berner 1980). Microbial degradation of organic matter consists of two first-order reactions with different rate constants, which include the decomposition of labile and refractory material (Newell et al. 1981). The organic carbon within the deeper sediments where the rate of sulfide formation exceeds its oxidation rate is more resistant to further mineralization and sustains the low rate of sulfate reduction over great sediment depths (Jørgensen 1982). LMW-DOC that resists degradation accumulates with depth in sediment pore water (Burdige and Gardner 1998; Amon and Benner 1996; Hedges 1988). Hence, pore water DOC concentrations reflect the balance between rates of production and consumption (Alperin et al. 1999). Despite the high productivity on global shelves, only 0.2 Pg C y^{-1} POC of the total production (6.2 Pg y^{-1}) is buried on the shelf and approximately 8 % is exported to the adjacent slopes (Field and Raupach 2004). Factors affecting the preservation of organic carbon in marine sediments include the quantity and quality of the organic carbon source; sedimentation rates (Canfield 1994); bacterial grazing; adsorption of organic matter onto particle surfaces (Alperin et al. 1999; Keil et al. 1994; Heinrichs 1992); chemical complexation reactions of LMW-DOC (Heinrichs 1992) and the inhibition of bacteria by the accumulation of metabolites, such as sulfide (Okabe et al. 1995).

Studying the role of Bacteria in organic carbon degradation in marine sediments

The interactions between bacteria and marine DOM play a major role in the global carbon cycle (e.g. Amon and Fitznar 2001) and received for the first time attention after the formulation of the "microbial loop" hypothesis (Pomechester 1974; Azam et al. 1983). Traditionally, microbial identification, quantification, and physiological studies depended on the isolation and culturing of microorganisms (Amann et al. 1995; Rappé and Giovannoni 2003). The introduction of direct microscopic counting techniques to quantify microorganisms revealed a discrepancy between plate counts and microscopic counts, demonstrating that plate counts underestimate cell numbers (Eilers et al. 2000; Glöckner et al. 1999; Amann et al. 1995) and resulted in the phenomenon referred to as "the great plate count anomaly" (Staley and Konopka 1985). The limitations of culture dependent techniques to study microbial diversity and abundance have been identified in several studies and are discussed extensively in the literature (MacGregor et al. 2002; Rappé and Giovannoni 2003; Hugenholtz et al. 1998; Amann et al. 1995). The introduction of the full cycle 16S rRNA approach, about a decade ago (Amann et al. 1995), opened up new possibilities to study the diversity and abundance of active microorganisms in environmental samples. A snap shot of the microbial diversity in the environment can be obtained by DNA extraction, cloning and sequencing of complete 16S rRNA. While molecular techniques have increased our knowledge of microbial diversity considerably, the common techniques used still have shortcomings like the production of chimeric sequences, polymerase chain reaction (PCR) bias by the preferential amplification of certain templates, and the limitation of the amount of sequences that can be gathered by 16S rRNA clone libraries (Stach and Bull 2005; Eilers et al. 2000; Amann et al. 1995). Genetic fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE) are often used to study temporal and spatial changes in bacterial genetic diversity (Muyzer 1999; Muyzer and Smalla 1998). DGGE provides a rapid way to

obtain qualitative and semi-quantitative information on the composition of the microbial population and it is highly sensitive to detect sequence differences, decreasing the possibility of chimeras (Muyzer et al. 1993). Recent molecular techniques that are commonly used to quantify active members of microorganisms in marine sediments include quantitative dot blot hybridization, which quantifies specific 16S rRNA relative to the total 16S rRNA and, fluorescent in situ hybridization (FISH), which detects individual active cells in the environment with fluorescently monolabeled rRNA-targeted oligonucleotide probes (Amann et al. 1995).

Traditional studies of the involvement of microorganisms in the degradation of organic carbon in marine sediments used to follow a black box approach in which bacteria are studied by only considering the extent to which they are involved in the biogeochemical transformations of organic matter. The taxonomy and phylogenetic composition of these bacteria were ignored. Yet, functional diversity is more important than phylogenetic diversity, since carbon cycling within an ecosystem is affected by the functional characteristics of organisms, rather than by their taxonomic identity (Fierer et al. 2007). Recent microbial ecological studies recognize that bacteria involved in organic carbon degradation in the marine environment belong to different microbial communities with different rates of consumption of various DOC components (Cottrell and Kirchmann 2000) and different growth rates (Cottrell and Kirchman 2003). Only a few studies have explored the relationship between species richness and ecosystem functioning in marine environments (Lin et al. 2006; Mills et al. 2005; Emerson and Huxham 2002). Ecosystem functioning refers to the metabolic activities of microorganisms, the transformation of organic matter and the flow of nutrients, water and atmospheric gases. Exploring microbial community structure in relation to metabolic activity improves the comprehension of carbon flow between the initial and terminal members of the sedimentary microbial food chain. Such studies are complicated

by the lack of a reliable "common species concept" in microbiology (Roselló-Mora and Amann 2001) and methodological constraints to study microbial diversity in sediments (Fierer et al. 2007). Therefore it remains indispensable that geochemical analyses, experiments to determine biogeochemical rates, and molecular techniques are combined to study the microbial community structure and its role during carbon transformation. Microbial ecology should aim to identify the key factors that regulate the abundance and activity of individual microbial populations and ultimately the global carbon cycle.

Aims of the thesis

In the marine sediment of the Namibian continental shelf, between 19°S and 27°S, 90% of the sulfate reduction measured with the ^{35}S radiotracer method of Jørgensen (1978), occurs within the surface 10 cm of the sediment, but sulfate gradients are less steep compared to sulfate reduction rate profiles within the same zone (Brüchert et al. 2006; Brüchert et al. 2003; Schulz et al. 1999). Therefore, the steep sulfate reduction rate profiles within the surface sediment are due to a limitation of electron donor (carbon source) supply, rather than electron acceptor (mainly sulfate) supply in the deeper sediment. The hydrogen sulfide formed during organic matter degradation by sulfate reduction can be oxidized to colloidal elemental sulfur mainly by large sulfur bacteria, including *Thiomagarita* and *Beggiatoa* (Schulz et al. 1999), or it may react with iron to form pyrite. Occasionally, high hydrogen sulfide concentrations in the bottom water are detected (Fig. 2) and concentrations of up to 22 mM in near surface sediments has been measured (Brüchert *et al*. 2003) due to insufficient sulfide oxidation or removal by its reaction with iron. The presence of hydrogen sulfide in the water column has a negative impact on the living marine resources within the Benguela upwelling system (Weeks et al. 2002) and hydrogen sulfide is toxic to some marine invertebrates such as amphipods (Knezovich et al. 1996). In order to understand the

formation of these sulfidic bottom waters and the suspected limitation of electron donor supply in the deeper sediment, it is critical to determine the accumulation rate of reactive organic carbon.

Several studies focus on studying carbon degradation in organic rich sediments (more than 2% dry weight organic carbon) from the perspective of the electron acceptors, especially SO_4^{2-} (Niggemann et al. 2007; Brüchert et al. 2006; Alperin et al. 1994). Fewer studies focus on the more challenging aspect of the stepwise degradation of organic carbon to obtain a complete process overview of organic carbon mineralization. In addition, numerous studies to establish the diversity of bacteria involved in terminal oxidation processes in organic rich sediments, mainly sulfate reduction, exist (Mussmann et al. 2005; Sahm et al. 1999; Llobet-Brossa et al. 1998). However, these bacteria are involved in only one step in the long chain of carbon transformation processes and little information exist about the identity, activity, and ecological role of the bacteria that are involved in the preceding steps. In this thesis the biogeochemical rates of both initial hydrolytic and fermentation steps, and terminal oxidation steps were investigated. In addition the identity and activity of the microorganisms involved in all the different steps of organic carbon mineralization was investigated. The NAMIBGAS project aims to improve the understanding of the rate and fluxes of hydrogen sulfide and methane to the sediment surface and into the water column. This PhD thesis is an essential component of the NAMIBGAS project, because the organic matter degradation processes are the ultimate driver for hydrogen sulfide production, the maintenance of anoxia, and methane formation. The primary aims of the thesis are to obtain a better understanding of carbon transformation processes within the sediment, determine the reactivity of organic carbon in the sediment and, investigate the control of microbial community structure and activity by the availability and accessibility of carbon sources. This will provide an understanding of the bottom-up regulation of microbial communities involved in organic carbon mineralization.

The focus is on carbohydrates as a carbon source, because of its high contribution to the DOC pool in marine sediments and its persistence throughout the sediment depth reported by numerous studies (Arnosti and Holmer 1999; Cowie et al. 1995; Lewis and Rowland 1993). The thesis aims to link microbial community structure (identity) to their function (activity) to provide new insights into the microbial ecology that controls carbon turnover in these upwelling sediments.

The main research questions that were addressed are:

1. How close is the coupling between the initial and terminal processes in carbon mineralization?
2. How does the high amount of organic carbon present in these sediments affect the metabolic activity, distribution and abundance of microorganisms involved in hydrolysis, fermentation and sulfate reduction?
3. What chemical changes of particulate and dissolved organic matter in the topmost 15 cm determine the gradient in the rates of organic matter degradation?
4. Are changes in biogeochemical rates reflected in the abundances and diversity of bacteria involved in the major carbon transformation steps of hydrolysis, fermentation, and terminal oxidation?
5. What is the effect of LMW-DOC (such as VFA) and HMW-DOC (such as carbohydrates) addition, simulating an increase flux of dissolved carbon to the sediment, on the metabolic activity and community structure of microorganisms throughout the sediment depth?

Outline of the thesis

In the first manuscript (**Chapter 2**) lateral and vertical variation in bacterial activity, the chemical changes in POC and DOC, and the coupling of initial hydrolytic and fermentation

steps to the terminal sulfate reduction step within the top 15 cm of the sediment of two closely spaced sampling stations were investigated. This was achieved by using biogeochemical methods to determine bulk sediment composition, pore water chemistry, polysaccharide hydrolysis rates and ^{35}S-sulfate reduction rates as well as molecular techniques to determine the abundance of active bacteria involved in the initial and terminal processes of organic carbon degradation. Due to the complex nature of DOC in marine sediments and the limited methodology for characterization, only two structurally specific polysaccharides, laminarin and pullulan were used as proxies of carbohydrates to quantify the initial transformation of complex organic matter in Namibian shelf sediments.

The second manuscript (**Chapter 3**) investigates the diversity of bacteria from the same two sampling stations within the top 12 cm of the sediment, using the 16S rRNA library approach. Bacteria that play a role in organic carbon transformation and the sulfur cycle were the focus of this study. The aim of this manuscript is to characterize the microbial community structure along a vertical depth profile from two different sampling stations with similar high primary productivity, by linking the identity and activity of microorganisms involved in the transformation of organic carbon. The central question was whether changes in biogeochemical rates are reflected in shifts of the community structure of bacteria involved in the major carbon transformation steps of hydrolysis, fermentation, and terminal oxidation.

Chapter 4 presents the results of two different experiments. In the first experiment the effect of LMW-DOC addition in the form of lactate and acetate, on the terminal oxidation step of sulfate reduction was investigated in intact cores. In the second experiment a flow-through experimental setup was used to investigate the effect of a high input of HMW-DOC on the metabolic activity and community structure of bacteria. This simulates the high input of organic matter into the sediment after a phytoplankton bloom. The rates of substrate consumption, fermentation, and terminal metabolism were monitored. Shifts in the

composition of the microbial community were investigated with Denaturing Gradient Gel Electrophoresis.

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Chapter 2

Diagenetic transformation of organic matter in Namibian shelf sediments: Limitation of microbial activity and bacterial abundance

(Submitted to Limnology and Oceanography)

Julies Elsabé. M. [*1,4], Carol Arnosti [3], Bernhard M. Fuchs[1], and Volker Brüchert[1,2]

[1]Max Planck Institute for Marine Microbiology, Bremen, Germany

[2]Department of Geology and Geochemistry, Stockholm University, Sweden

[3]Department of Marine Sciences, 12-7 Venable Hall, University of North Carolina, Chapel Hill, NC 27599-3300, USA

[4]Department of Biology, University of Namibia, Windhoek, Namibia

[*]Corresponding author: Julies Elsabé Mathilda (ejulies@unam.na)

Abstract

Based on data from two closely spaced stations within the anoxic region of the central Namibian coastal upwelling system, we found a steep downward decrease in bacterial sulfate reduction rates that compared well with gradients in the abundance of active Bacteria (Bacteroidetes, Gammaproteobacteria, and sulfate-reducing bacteria) as detected by Catalyzed Reporter Deposition Fluorescence In Situ Hybridization (CARD-FISH). The tight coupling between the active heterotrophic and sulfate-reducing bacterial communities appeared to be controlled by the availability of degradable dissolved organic matter. Gradients in rates and bacterial abundance were not reflected in the depth profiles of particulate organic carbon, dissolved organic carbon, volatile fatty acids, or total dissolved carbohydrates. An increasing amount of dissolved organic carbon, including the carbohydrate fraction, became unavailable to microbial communities with depth, whereas volatile fatty acids were rapidly turned over and had low concentrations. Depth gradients of polysaccharide hydrolysis and sulfate-reducing activity were steeper than the decrease in bacterial abundance and indicated increasing substrate limitation of the active bacteria with depth. Significant differences were found between the two stations in concentrations and gradients of dissolved organic carbon, total carbohydrates, and in rates of polysaccharide hydrolysis and sulfate reduction. These differences could not be attributed to the carbon degradation effects controlled by variation in oxygen availability. Instead, the contrasting results emphasize the importance of particle retention and sediment re-suspension in the bottom boundary layer.

Introduction

The rates and pathways of organic matter mineralization in marine sediments affect the flux and distribution of carbon, nutrients, and oxygen in the ocean as well as the amount of organic carbon which is ultimately buried (Burdige 2007). Most of the oceanic primary production occurs in continental shelf regions, although they account only for 8% of the total ocean area (Wollast 1991). Due to the shallow depth of continental shelf regions, up to 50% of the organic matter produced during primary production sinks to the bottom (Wollast 1998). About 80-90% of the sinking organic matter is readily remineralized to CO_2 by Bacteria (Jørgensen 1983; Wakeham et al. 1997; Burdige 2007) and therefore, continental margin sediments play a critical role in the global marine carbon cycle (Berner 1982).

The sediments of the Namibian continental shelf between 19°S and 27°S accumulate in one of the most productive coastal upwelling systems on earth (Carr 2002). Estimated primary productivity on the shelf is 175-240 mmol C m^{-2} d^{-1} (Summerhayes et al. 1995). The high productivity and shallow water depth in combination with the near-permanent bottom water anoxia have created a diatomaceous mud belt of organic-rich sediments (Bremner 1978) with an organic carbon content as high as 15 percent in surface sediments (Inthorn et al. 2006). Particulate organic carbon (POC) in the sediment consists of complex biopolymers, which need to be hydrolyzed by extracellular enzymes into dissolved molecules with a molecular mass smaller than 600 Dalton in order to allow assimilation by bacteria (Weiss et al. 1991). Some of the dissolved organic carbon (DOC) is rapidly remineralized, however, a part of the DOC is recalcitrant and resists rapid microbial degradation (Williams and Druffel 1987; Arnosti 2000). Several studies have been performed to quantify organic carbon mineralization through sulfate reduction on the Namibian shelf (Brüchert et al. 2006; Brüchert et al. 2003). However, little is known about initial transformation of the organic macromolecules in the sediment and potential bottlenecks in organic carbon mineralization

as well as the diversity and abundance of the microorganisms involved. Namibian shelf sediments can be considered as a case example of organic-rich continental margin sediment in an upwelling region that typically offers abundant substrate to bacteria.

The first aim of this study was to investigate the coupling between the initial and terminal processes in carbon mineralization in order to evaluate the dynamics of the DOC pool in the sediment and the availability of the high molecular weight (HMW) fraction for remineralization. We focused on the hydrolysis of selected structurally and compositionally defined carbohydrates, laminarin and pullulan, to quantify the initial anaerobic transformation of complex organic matter in Namibian shelf sediments. Presently only a few studies (Arnosti and Holmer 1999; 2003) have assessed the contribution of carbohydrates to the total organic carbon budget in marine sediments by combining biogeochemical rate measurements during initial and terminal steps in anaerobic organic carbon mineralization with measurements of total dissolved carbohydrates, DOC and POC.

The second aim of this study was to investigate how the metabolic activity, distribution, and abundance of microorganisms involved in hydrolysis, fermentation, and sulfate reduction along sediment depth profiles are affected by the high amounts of substrate in these organic-rich sediments. The factors controlling the activity of marine microbial enzymes are poorly understood (Hoppe et al. 2002) and total microbial numbers or microbial production is often only weakly correlated with enzyme activity (Mayer 1989). Using bulk sediment composition, pore water chemistry, experimental rate measurements of polysaccharide hydrolysis, and direct determination of ^{35}S-sulfate reduction rates we first investigated the lateral variation in bacterial activity within an oceanographic upwelling system with perennial anoxia. Then we investigated what chemical changes of particulate and dissolved organic matter in the topmost 15 cm determine the gradient in the rates of organic matter degradation. Finally, we posed the question whether changes in

biogeochemical rates were proportionately reflected in the abundances of Bacteria involved in the major carbon transformation steps hydrolysis, fermentation, and terminal oxidation.

Answering these questions will help to understand the response of the microbial community to high inputs of organic carbon in organic-rich sediments. Comparison of the activity of microorganisms involved in the initial hydrolytic and final CO_2-producing steps of carbon mineralization to their distribution within the sediment would allow us to obtain a complete process overview during anaerobic organic carbon mineralization and help to illuminate the process of carbon flow between microorganisms involved in the sedimentary microbial food chain.

Materials and methods

Study sites and sampling

Sediment samples were collected from two stations, Station 3 and 5 during a cruise on the *RV Alexander von Humboldt* in March 2004. Both sampling stations are characterized by a highly productive water column with an average primary production of 150 mmol C m^{-2} d^{-1} (Mitchel-Innes et al. 2002) and chlorophyll *a* concentrations in the water column as high as 30 mg m^{-3} (Barlow 1982). The two stations were about 130 km apart and located in 70 m and 112 m water depth (Table 1). At both stations the bottom waters had very low oxygen concentrations (< 4.5 µmol L^{-1}) (Table 1), the sediments were very sulfidic (Brüchert et al. 2006), and bioturbation was negligible. Both sampling sites are located within the inner shelf mud belt, defined by Monteiro et al. (2005) as the mid-shelf area (~20-24°S) at water depths between 50 and 140 m. Station 3 is positioned at the coastal edge of this mud belt, whereas Station 5 is centered within the mud belt. The sediments of the inner shelf mud belt have the highest particulate organic matter (POM) concentrations on the Namibian shelf, because of the combination of high organic carbon fluxes and persistent low bed shear-stress due to the

absence of gravity-wave stresses, Ekman flow and barotropic-baroclinic tidal coupling, which are responsible for resuspension of sediment (Monteiro et al. 2005; Inthorn et al. 2006).

Table 1: Locations and characteristics of sampling stations ((Station 3: 4 March 2004, 14h00 and Station 5: 6 March 2004, 9h00).

	Station 3	Station 5
Coordinates	22°38.3S, 14°18.27E	23°45.08S, 14°18.27E
Depth (m)	70	112
Bottom water temperature (°C)	13.3	11.8
Bottom water oxygen (µmol L^{-1})	< 4.5	< 4.5
Bottom water turbidity (Fluorescence intensity)	0.03	0.02
Sediment description	Dark, olive green mud; first 5cm is soft and fluffy; Below 5 cm depth - mica and fine sand. No bacterial mat visible. Low abundance of Thiomagarita species.	Top 15 cm of sediment extremely soft fluff; Olive green mud; light-colored precipitates at about 15-20 cm depth. Sediment covered by thick layer of Beggiatoa species.

Sediments were collected using a multiple sediment corer (MUC) and sub-cores were taken from the MUC as described by Brüchert et al. (2003). As far as possible sub-cores for the hydrolysis experiment were collected from the same cast. Samples for molecular analysis were also collected from one cast.

Density, porosity, total particulate organic carbon (POC), and total particulate organic nitrogen (PON)

The density of the sediment was determined from the weight and volume of the sediment. Porosity of the sediment was determined from its density and water content after drying the samples at 80°C. Total particulate organic carbon (POC) and total particulate organic nitrogen (PON) was measured with a Carlo Erba CNS 1500 Elemental Analyzer with

auto sampler after freeze drying, grounding, and acidification of the sediment with 1 N HCl to remove the inorganic carbon.

^{35}S-Sulfate reduction rates

Sulfate reduction rates were measured with the whole core $^{35}SO_4^{2-}$ radiotracer incubation method (Jørgensen 1978). Duplicate sub-cores were taken from a multiple corer at each station. Each sub core was injected with 2 μL $^{35}SO_4^{2-}$ radiotracer (80 kBq) through silicon sealed ports at 1 cm intervals. Injection of the sub-cores with tracer took place less then 2 hours after sampling. The cores were incubated for 8 hours at 12°C in the dark. After incubation the cores were sectioned and the sediment preserved as described by Brüchert et al. (2003). Sulfate reduction rates were measured by the one-step acidic chromium reduction method of Kallmeyer et al. (2004). Total Reduced Inorganic Sulfur (TRIS) and $^{35}SO_4^{2-}$ were counted on a Canberra-Packard 2400 TR liquid scintillation counter (Packard Ultima Gold XR scintillation fluid).

Potential hydrolysis rates and total dissolved carbohydrates

Two polysaccharides, laminarin, a β(1,3)-D-glucose polymer and pullulan, an α(1,6)-linked polymer of maltotriose, were selected as indicators of potential hydrolysis rates of carbohydrates in Namibian shelf sediments. These two polysaccharides were selected because laminarin is one of the main forms of stored glucan in marine phytoplankton (Bold and Wynne 1985), and can contribute up to 80% of the organic carbon of diatoms (Alderkamp et al. 2006). The sampling area on the shelf between 22° and 24°S is characterized by a thick diatomaceous mud belt (Bremner 1978) and therefore it is expected that Namibian shelf sediment is rich in laminarin. Both laminarinase and pullulanase have been detected in marine bacteria (Antranikian 1992; Arnosti and Repeta 1994; Alderkamp et

al. 2007) as well as in a wide range of marine sediments (Arnosti 1995; Hoppe et al. 2002; Arnosti and Jørgensen 2006).

Measurement of potential hydrolysis rates is based on the quantification of fluorescent molecules that are derived from the hydrolysis of fluorescently labeled laminarin and pullulan (Arnosti 1995; 1996). Injection of the sub-cores with the fluorescently labeled substrate took place less then 2 hours after sampling. The substrates were injected at three depths, 1 cm, 5 cm, and 11 cm, into duplicate sub-cores. The $^{35}SO_4^{2-}$ tracer was not injected into the same cores into which fluorescently labeled polysaccharide were injected in order to prevent contamination of the fluorescence detector with radioactive tracer. The amount of laminarin injected into each of the three different depths of a core was 100 µL, containing 49.7 nmol laminarin, corresponding to 1.8 µmol monomer equivalents. The amount of pullulan injected was 150 µL comprising 4.8 nmol pullulan, corresponding to 2.1 µmol monomer equivalents. The cores were subsequently incubated at 12°C, in the dark for 48 hours. Two of the cores were sectioned immediately to serve as zero time point measurements. After incubation the cores were sectioned in 2 cm intervals. Collection and storage of the pore water was done after Arnosti (2000). One core per station was not injected with fluorescently labeled substrate in order to determine the background fluorescence and the concentration of total dissolved carbohydrates in sediment pore water. Hydrolysis products were separated by a low pressure gel permeation chromatography system and hydrolysis was quantified by a Hitachi L-7480 fluorescence detector, as described in Arnosti (2003). The amount of leakage of substrate into neighboring sections that were not injected with substrate was less than 30% of the substrate injected into a section. The standard deviation of total fluorescence for duplicate injections was 0.05% - 13%.

Volatile fatty acids (VFA), dissolved carbohydrates, dissolved organic carbon (DOC), and sulfate in the pore water

Data presented for VFA analysis were from duplicate cores at each station. All other pore water measurements were determined from a single core. Pore water for VFA measurements was obtained after sediment was centrifuged through Spinex (Phenomenex) centrifugal filter units (0.2 µm) at 2000 rpm for 10 minutes. Low-molecular weight volatile fatty acids, including glycolate, lactate, acetate, formate, propionate, isobutyrate, and butyrate were measured by HPLC according to the method described by Albert and Martens (1997) and modified as described in Arnosti et al. (2005). VFA concentrations in the pore water were determined after calibration with standard mixtures containing glycolate, lactate, acetate, formate, propionate, isobutyrate, and butyrate. Detection limits of the method were 0.2 µmol L^{-1} for lactate, 1 µmol L^{-1} for acetate and formate, 0.5 µmol L^{-1} for propionate and isobutyrate and 2 µmol L^{-1} for butyrate, valerate, and isovalerate.

Pore water for DOC and total dissolved carbohydrate measurements was obtained from centrifuging the sediment in pre-combusted glass centrifuge tubes at 3000 rpm for 5 minutes. The pore water was then filtered through sterile 0.2 µm filters. The first milliliter was discarded and the rest was stored in pre-combusted glass vials at -20°C until further analysis. DOC concentrations were measured by high temperature catalytic oxidation with a total organic carbon analyzer (TOC-5050A – Shimadzu) (Benner and Strom 1993). Pore water samples were acidified before analysis to remove inorganic carbon. Total dissolved carbohydrates were measured with the phenol-sulfuric acid method of Chaplin and Kennedy (1986). Pullulan was used as a standard to construct the calibration curve.

Pore water for sulfate measurements was obtained with a Reeburgh-type pneumatic pore water squeezer, using nitrogen gas (Reeburgh 1967). For sulfate measurements, 1.5 mL pore water was squeezed directly into cryovials containing 500 µL of 5% (w:v) zinc acetate

to precipitate hydrogen sulfide. Sulfate concentration in the pore water was measured by non-suppressed ion chromatography (Brüchert et al. 2003).

Catalyzed Reporter Deposition Fluorescence in situ hybridization and microscopy

Catalyzed Reporter Deposition Fluorescence In Situ Hybridization (CARD-FISH) was used to determine the abundance of microorganisms involved in initial and terminal metabolism during organic carbon mineralization. We chose probes specific for several subgroups of sulfate-reducing Deltaproteobacteria, the Bacteroidetes and the Gammaproteobacteria. Information on the specific probes used and the hybridization conditions are outlined in Table 2.

Table 2: Oligonucleotide probes used during fluorescent in situ hybridization

Probe	Target organism	Position	Sequence (5'-3') of probe	(%) FA[a]	Reference
EUB I-III	Bacteria	338-355	GCTGCCTCCCGTAGGAGT	35	Amann et al. (1990)
NON338	Negative control	338-355	ACTCCTACGGGAGGCAGC	35	Wallner et al. (1993)
ARCH915	Archaea	915-934	GTGCTCCCCCGCCAATTCCT	35	Stahl and Amann (1991)
CF319a	Cytophaga-Flavobacterium-Bacteroidetes	319-336	TGGTCCGTGTCTCAGTAC	35	Manz et al. (1996)
Gam42a	Gammaproteobacteria	1027-1043	GCCTTCCCACATCGTTT	35	Manz et al. (1992)
DSB985	Desulfobacter, Desulfobacula	985-1003	CACAGGATGTCAAACCCAG	20	Manz et al. (1998)
DSS658	Desulfosarcina, Desulfobaba, Desulfococcus, Desulfofrigus	658-675	TCCACTTCCCTCTCCCAT	60	Manz et al. (1998)
DSR651	Desulforhopalus	651-668	CCCCCTCCAGTACTCAAG	35	Manz et al. (1998)

[a] Formamide (FA) concentration in the hybridization buffer

Duplicate sub-cores were sectioned in two centimeter intervals. 0.5 cm^3 of sediment was fixed for 24 hours in 1.5 mL formaldehyde (4% v:v). The sediment was then washed twice with 1 x phosphate-buffered saline (PBS; 4 mmol L^{-1} sodium phosphate (pH 7.2), 130 mmol L^{-1} NaCl) and finally stored in a mixture of 1 x PBS and ethanol (2:3 v:v) at -20°C. Fixed samples were diluted 5-fold with a mixture of 1 x PBS and ethanol (2:3 v:v) and then

sonicated with an MS73 probe (Sonoplus HD70; Bandelin, Berlin, Germany) at a setting of 20 s, with an amplitude of 42 µm, and <10 W for 80 s. The sample was then filtered on 0.2 µm-pore-size GTTP carbonate filters. The filters were allowed to dry and then stored at -20°C until hybridization or staining with 4,6-diamidino-2-phenylindole (DAPI) to determine the abundance of specific groups of Bacteria and total Bacteria in the sediment.

Hybridization and microscopic counting of hybridized and DAPI-stained cells were performed as described by Pernthaler et al. (2002). Cells revealing probe-conferred fluorescence and DAPI-stained cells were counted in 20 randomly chosen counting grids per filter, corresponding to about 1,000 DAPI- stained cells. Cell counts were corrected by subtracting signals observed with probe NON338. Formamide concentrations used during hybridization and specificity of the probes used are shown in Table 3. Nucleotide probes used in this study were purchased from Biomers.net (Ulm, Germany) with Cy3 fluorochrome at the 5' end.

Results

Sediment characteristics

At Station 3 the top 5 cm of the sediment consisted of a dark, olive green, fluffy mud. Below 5 cm depth the sediment contained abundant mica and fine sand. The porosity decreased from 95% at the surface to 90% at 5 cm depth. Below 5 cm depth the porosity was about 80% for the remainder of the 20 cm long core. At Station 5 the top 15 cm of the sediment was extremely soft and fluffy in appearance and consisted of an olive green mud with a porosity of 93%. Below 15 cm depth the sediment was less porous, with a porosity of about 87% and light-colored precipitates was observed at 15-20 cm.

At both stations re-suspension of sediment was detected as a higher turbidity in the bottom water compared to the rest of the water column. The highest bottom water

fluorescence at Station 5 was only about half that at Station 3 (Table 1). In addition, the bottom turbidity layer extended over 20 m at Station 3, and only over 10 m at Station 5 (data not shown).

Total particulate organic carbon (POC), dissolved organic carbon (DOC), dissolved carbohydrates, and total particulate organic nitrogen (PON)

Particulate organic carbon (POC) constituted most of the total carbon in these sediments, since analyses of acidified and non-acidified samples revealed that carbonate carbon accounted for only 0.1-0.4% dry weight at Station 3. Carbonate carbon was only 0.1% by weight in the top 5 cm of the sediment and slowly increased below 5 cm depth to ~0.4% at a depth of 30 cm.

At Station 3 POC concentrations in the topmost 3 cm of the sediment were 7% by dry weight and decreased to 4% at 10 cm depth (Fig. 1a). PON decreased gradually from 1% dry weight at the surface to 0.5% at 10 cm depth (Fig. 1a). Below a depth of 10 cm, POC and PON remained relatively constant. The C:N ratio in the sediment at Station 3 increased from 7 at the surface to 10 at 14 cm depth (Fig. 1b). Below a depth of 14 cm, C:N ratios remained constant. At Station 5, POC increased from 4% dry weight in the surface sediment to 8% at a depth of 14 cm (Fig 1a). While POC increased with depth, PON remained constant. Below 14 cm depth POC decreased again gradually from 8% to 6% dry weight at 20 cm depth. The C:N ratio at Station 5 increased slightly with depth from 6.5 at the surface to 7 in the bottom of the core (Fig. 1b).

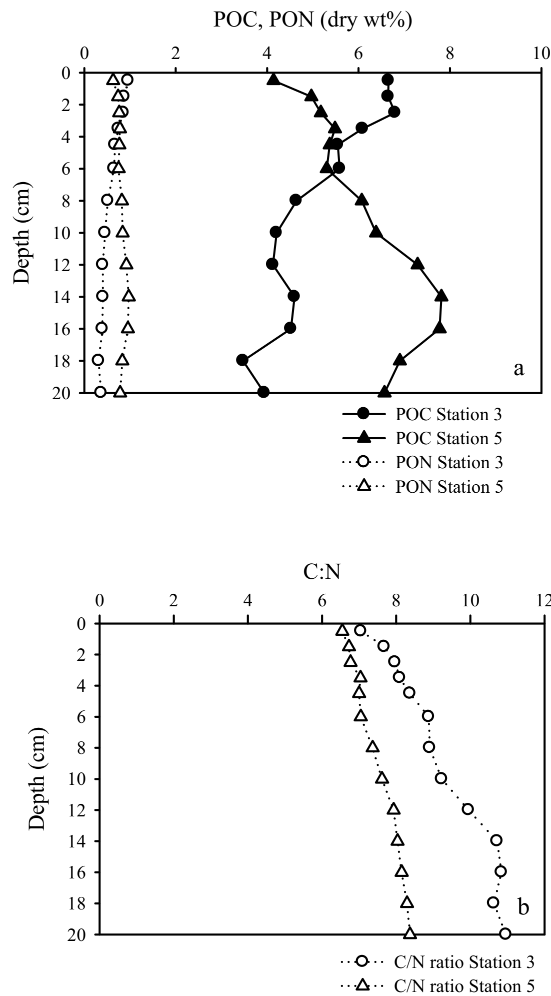

Figure 1: (a) Concentrations of total particulate organic carbon (POC) and particulate organic nitrogen (PON) and (b) C:N ratios in the sediment at Station 3 and Station 5.

DOC concentrations in the topmost 4 cm of the sediment at Station 3 were between 1.5 mmol L^{-1} and 1.6 mmol.L^{-1} and increased slightly with depth to reach a concentration of 1.8 mmol L^{-1} below 6 cm (Fig. 2). Total dissolved carbohydrate concentrations decreased slightly with depth from 1.5 mmol L^{-1} in the topmost 2 cm to 1 mmol L^{-1} at 13 cm depth (Fig. 2). The contribution of dissolved carbohydrates to the total DOC pool decreased with depth from ~96% in the topmost 2 cm to ~50%-65% below 8 cm depth. At Station 5 DOC concentrations in the surface 5 cm varied between 1 mmol L^{-1} and 1.5 mmol L^{-1} and gradually increased

with depth to a concentration of 3 mmol L^{-1} at a depth of 16 cm (Fig. 2). Total dissolved carbohydrate concentrations increased only slightly with depth at Station 5 from 0.2 mmol L^{-1} in the topmost 2 cm to 0.6 mmol L^{-1} at 13 cm depth. The contribution of dissolved carbohydrates to the DOC pool was markedly lower at Station 5 compared to Station 3, and varied between 11% and 30%.

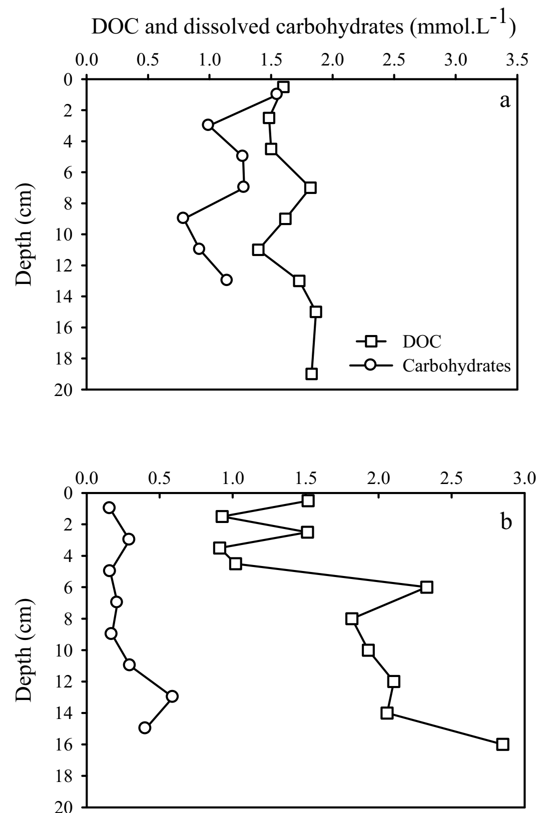

Figure 2: Concentrations of dissolved organic carbon (DOC) and total dissolved carbohydrates at (a) Station 3 and (b) Station 5.

Intermediates

At both stations, volatile fatty acid (VFA) concentrations were in the low µmol L^{-1} range and did not show significant depth trends (p = 0.06–0.38, df = 3). Only the concentrations of

lactate, acetate, formate, and propionate were above detection limit. The average variation between duplicate cores was 0.5-3 µmol L^{-1}.

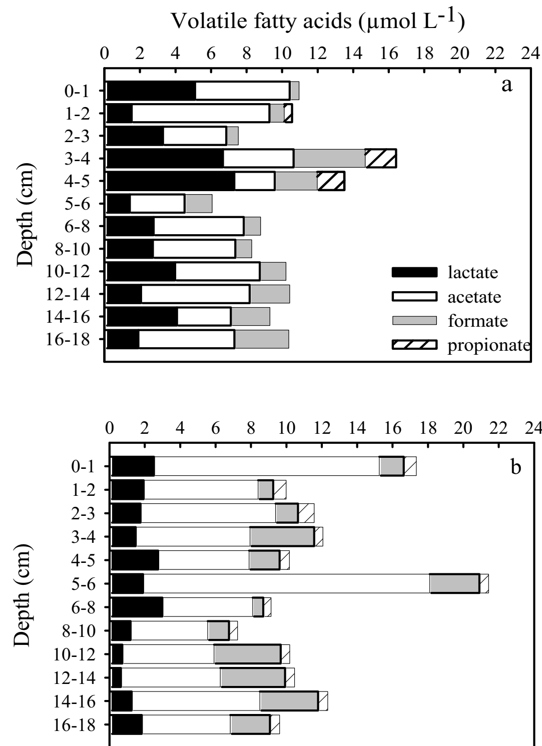

Figure 3: Volatile fatty acid concentrations from (a) Station 3 and (b) Station 5. Concentrations represent the average from two cores.

At both stations the most abundant VFA was acetate, which varied between 1 µmol L^{-1} and 8 µmol L^{-1} at Station 3 and was slightly higher in the top 4 cm of the sediment at Station 5 (8 µmol L^{-1} to 14 µmol L^{-1}) compared to the sediment below 4 cm depth (2 µmol L^{-1} to 8 µmol L^{-1}) (Fig. 3). Lactate concentrations were slightly higher at Station 3 (0.5 µmol L^{-1} to 14 µmol L^{-1}) than at Station 5 (0.1 µmol L^{-1} to 4 µmol L^{-1}). Formate concentrations were similar at both stations and varied from below detection limit to a maximum of 6 µmol L^{-1}.

Initial metabolism - Potential hydrolysis rates

Both core sets showed significant substrate hydrolysis after 48 hours incubation. Potential hydrolysis rates of laminarin were significantly higher ($p < 0.05$, df = 3) than for pullulan in the surface sediment (0-2 cm and 4-6 cm depth) at both stations (Fig. 4a and b). However, potential hydrolysis rates of the two polysaccharides did not differ significantly in the 10-12 cm depth interval ($p = 0.3$, df = 3 at Station 3 and $p = 0.1$, df = 3 at Station 5).

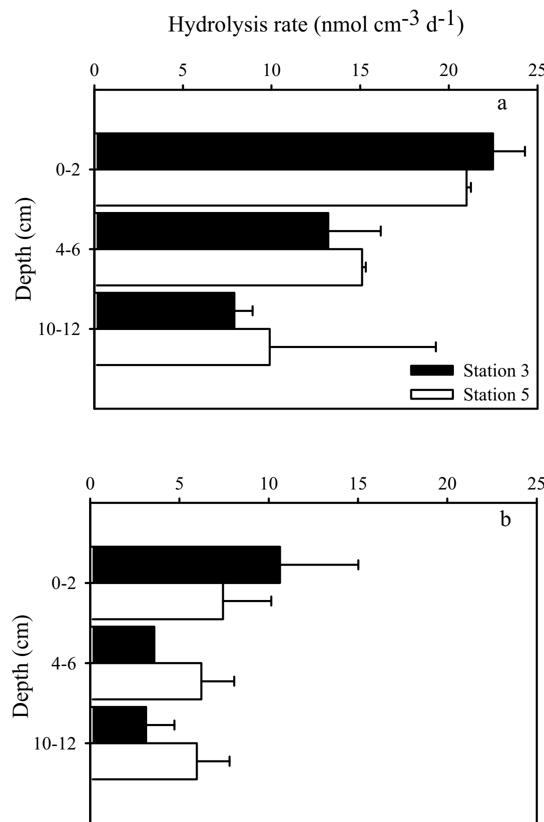

Figure 4: Potential hydrolysis rates of (a) laminarin and (b) pullulan at Station 3 and Station 5. Rate measurements were done in two parallel cores after incubation with substrate for 48 hours.

The highest rates were measured for laminarin in the top 2 cm. At both stations hydrolysis rates of laminarin decreased with depth and were 50% lower in the deeper sediment (10-12 cm) compared to the surface sediment (0-2 cm). Statistical analysis showed

that the potential hydrolysis rate of laminarin at a depth of 10–12 cm is significantly slower than in the top 2 cm of the sediment ($p < 0.05$ df = 3). For pullulan, the decrease of the hydrolysis rate with depth was less steep and no significant difference was found between rates at a depth of 10-12 cm and in the top 2 cm of the sediment ($p = 0.09$, df = 3 and $p = 0.1$, df = 3 at Stations 3 and 5, respectively).

Terminal metabolism - Sulfate reduction rates

At Station 3, sulfate reduction rates were the highest in the surface section (0-2 cm) of the sediment and decreased abruptly by a factor of 10 at 4 cm depth (Fig. 5a). At Station 5 the highest sulfate reduction rates were less than a third of those at Station 3, and the downward decrease was much more gradual (Fig. 5b). Sulfate concentration decreased to 13 mmol L^{-1} at a depth of 14 cm at Station 5 and to 14 mmol L^{-1} at a depth of 8 cm at Station 3. The decrease in sulfate concentration was steeper in the top 8 cm of the sediment at Station 3 than at Station 5 (Fig. 5a,b). Depth integration of the measured rates over the analyzed depth interval at the two stations showed that the amount of carbon mineralization in the sediment was similar, 4.3 ± 1.8 mmol m^{-2} d^{-1} at Station 3 and 5.2 ± 0.4 mmol m^{-2} d^{-1} at Station 5.

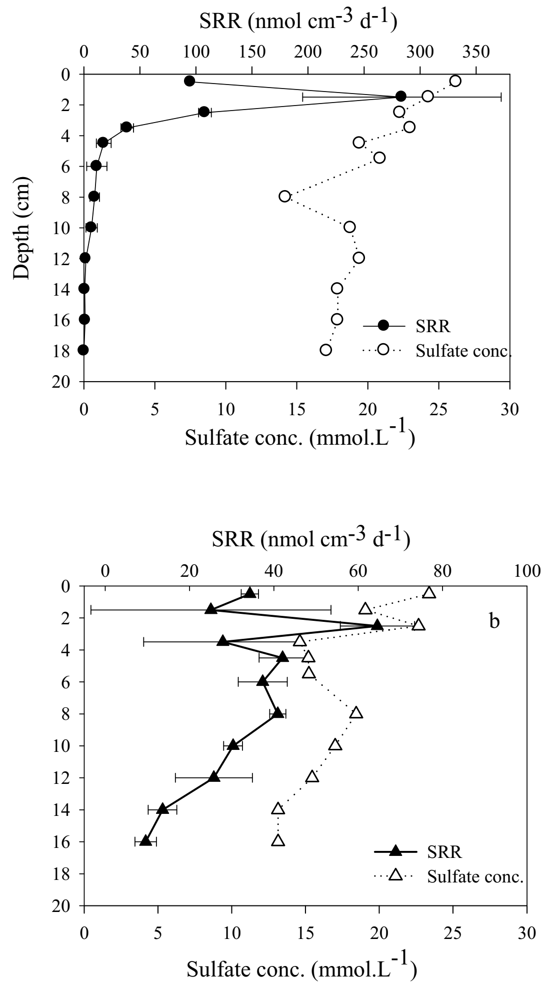

Figure 5: Sulfate reduction rates and sulfate concentrations measured at (a) Station 3 and (b) Station 5. Sulfate reduction rate measurements are from duplicate measurements from two parallel cores, while sulfate concentration measurements are from a single core at each station.

Distribution, abundance and community structure

Total DAPI, Bacteria, and Archaea cell counts: At the surface of both stations, more than 80% of the total DAPI-counted cells hybridized to the general bacterial probe EUB I-III (Fig. 6 a and b), indicating that most of the active prokaryotes were Bacteria. Archaea accounted only for 2% of the total DAPI stained cells in the surface sediment. These detection levels dropped with depth and amounted to only 30% of the total DAPI cell counts between 10 and

12 cm depth for Bacteria and Archaea combined at Station 3 and 46% of the total DAPI stained cell counts between 16 an 18 cm depth at Station 5.

At Station 3, total DAPI cell numbers were a maximum at 2-4 cm depth reaching 21.5 x 10^8 ± 1.3 x 10^8 cells mL^{-1} wet sediment (Fig. 6a). The amount of active Bacteria detected by the general bacterial probe EUB I-III were 12.8 x 10^8 ± 1.3 x 10^8 cells mL^{-1} wet sediment, while active Archaea cell numbers detected by the general probe ARCH915 were only 0.8 x 10^8 ± 0.05 x 10^8 cells mL^{-1} wet sediment. Total DAPI stained cells, active Bacteria, and Archaea cell numbers decreased steeply with depth below 4 cm depth. This sharp decrease in bacterial abundance with depth at Station 3 corresponds well to the transition to more mica- and quartz-rich sand. At Station 5, total DAPI stained cell numbers remained high within the top 10 cm of the sediment, varying between 9 to12 x 10^8 cells mL^{-1} wet sediment (Fig 6b). Active bacterial cell numbers within the top 10 cm were between 6 and 11.6 x 10^8 cells mL^{-1} wet sediment, while active Archaea cell numbers were between 0.1 and 0.3 x 10^8 cells mL^{-1} wet sediment. Below 10 cm depth total DAPI stained cells, active Bacteria, and Archaea cell numbers decreased sharply, reaching 1.3 x 10^8, 0.5 x 10^8, and 0.1 x 10^8 cells mL^{-1} wet sediment, respectively (Fig 6b).

Bacteroidetes and Gammaproteobacteria: A strong fluorescent signal was observed with the probes CF319a and Gam42a, which target the phyla Gammaproteobacteria and Bacteroidetes, indicating highly active members of these phyla. Bacteroidetes and Gammaproteobacteria contributed about 20-40% to the total DAPI-stained cells in the top 4 cm, but less than 10% below a depth of 8 cm (Fig. 6c,d). This depth trend was similar at Station 5, where these bacteria represented up to 50% of the total DAPI cell counts and decreased to less than 10% of the total DAPI cell counts below 12 cm depth. At both stations, Bacteroidetes bacteria were 3-4 times more abundant than Gammaproteobacteria.

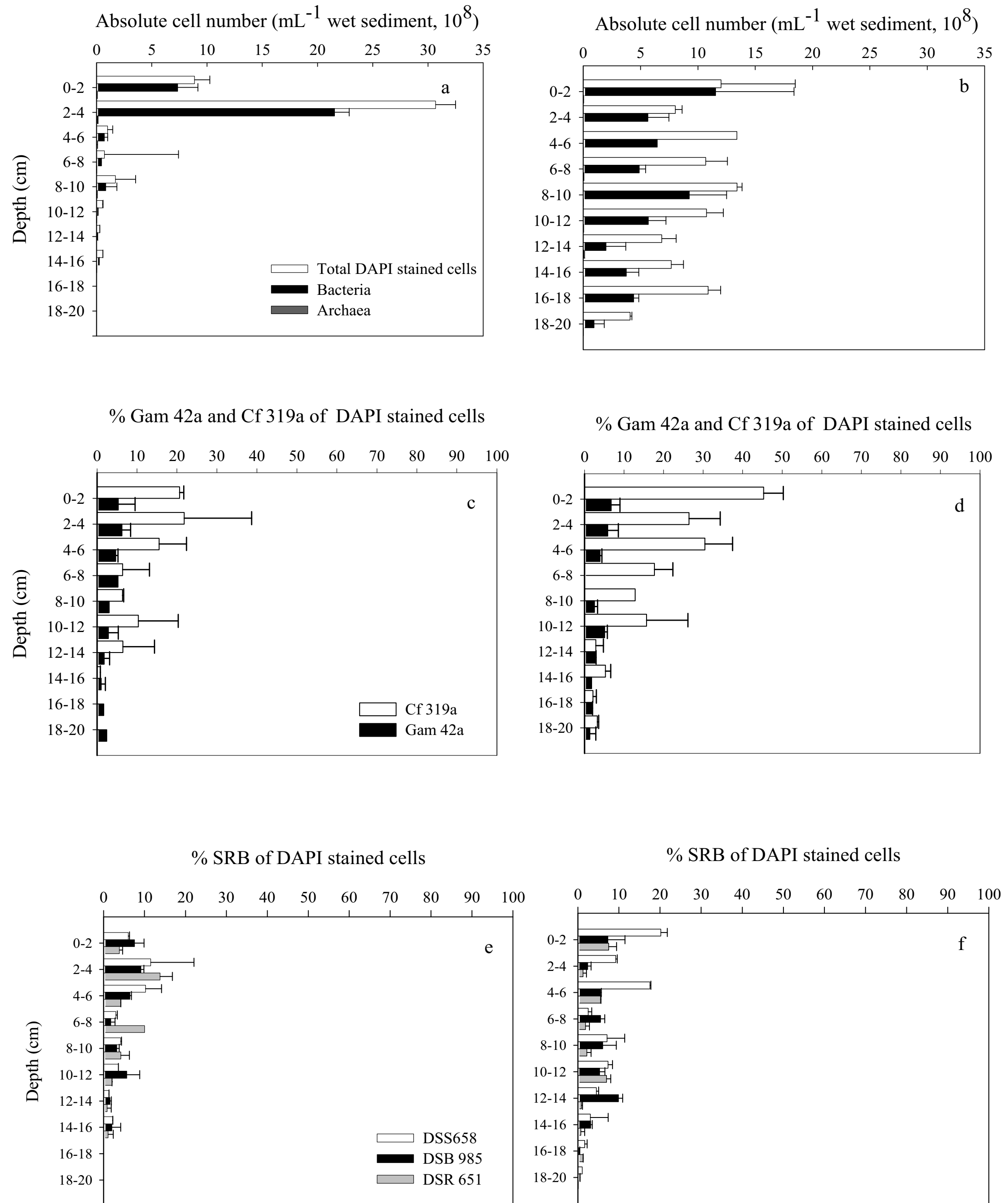

Figure 6: (a and b) DAPI (total cells), EUB I-III (Bacteria), and ARCH915 (Archaea) cell counts at (a) Station 3 and (b) Station 5; (c and d) cell counts of Bacteroidetes (Cf 319a) and Gammaproteobacteria (Gam 42a) as a percentage of DAPI- stained cells at (c) Station 3 and (d) Station 5; (e and f) sulfate reducing bacteria (SRB) as detected by probes DSS658, DSB985, and DSR651 as a percentage of DAPI-stained cells at (e) Station 3 and (f) Station 5.

Sulfate reducing bacteria (SRB): At Station 3 the relative abundance of the total SRB, detected by the probes DSS658, DSB985, and DSR651, decreased steeply from ~20-40% of the total DAPI cell counts in the top 6cm to ~15% and less of the total DAPI cell counts below 6cm depth, while the decrease in the relative abundance of SRB with depth at Station 5 was more gradual (Fig. 6e,f). Bacteria targeted by the probe DSS658 made up the majority of the SRB at both stations (Station 3: 6-19% of the total DAPI cell counts and Station 5: 9-19% of the total DAPI cell counts) (Fig. 6e,f). SRB targeted by the probes DSR 651 and DSB985 had a much lower abundance (~ 3 times lower) in the top 6cm of the sediment compared to the Bacteria targeted by probe DSS 658. SRB abundance in the bottom of the cores (16 cm depth) was less then 2% of the total DAPI cell counts at both stations.

Discussion

Sedimentary control on bulk sediment composition

According to Monteiro et al. (2005) and Rogers and Bremner (1991), Namibian shelf sediments are resuspended in the bottom water between the coast and depths of about 50 meters, due to increased bed stresses caused by the long-period gravity waves during winter. This interpretation is supported by the higher bottom water fluorescence at Station 3 compared to Station 5 (Table 1). The increased turbidity reflects the enhanced retention of sinking particles in the bottom boundary layer due to suspension and the higher turbulence above the sediment-water interface (Dade et al. 2001; Hill and McCave 2001). Station 3 is at the edge of this inner shelf mud belt and is likely to experience varying bed stress on an annual and inter-annual basis (Lass and Mohrholz 2005).

In agreement with this interpretation are the depth profiles of POC, PON and C:N ratios (Figs. 1a,b). At Station 3, POC decreased from 7 weight% (wt.%) in the dark, olive green, fluffy diatom mud at the surface to 3.5 wt.% at 18 cm depth, where the diatom mud

contained more mica and fine sand grains. The higher terrigeneous content in the deeper layers of the core, suggests higher bed shear stresses than today or a higher contribution of wind-blown detritus from the Namibian desert. Since Station 5 is located in greater water depth and further offshore, the sediment was more homogeneous and sand and mica grains were a minor component of the sediment. The biogenic carbonate contribution was minor, and particulate inorganic carbon varied between 0.1-0.4 wt.%, with a gradual downward increase (data not shown). We attribute the initial increase in POC concentration with depth at Station 5 (Fig. 1a) to the selective dissolution of opal during the decomposition of diatoms in the top 10 cm of sediment (Sayles et al. 1996). At both stations, C:N ratios are close to the Redfield ratio of 6.7 at the top of the core and increase gradually with depth, which reflects the selective degradation of labile nitrogen-containing organic molecules. This is supported by direct measurements of total hydrolysable amino acid concentration profiles in these sediments (Ahke 2006). The downward increase in C:N ratios is substantially greater at Station 3 compared to Station 5, and is consistent with enhanced sediment reworking during deposition of the older, buried layers of this sediment.

Relationship between biogeochemical rates and bacterial abundance

The high detection level of hybridized cells in the surface sediment indicates high cellular contents of 16S rRNA and suggests high metabolic activity of microorganisms (Amann et al. 1995). In contrast, the lower detection yield with probes EUB I-III and ARCH915 below 14 cm depth may be attributed to increasingly lower rRNA contents and increasing starvation of the active bacterial population. Direct measurements of hydrolysis and sulfate reduction rates support this interpretation. Depth gradients of polysaccharide hydrolysis and sulfate-reducing activity were steeper than the decrease in bacterial abundance.

Bacteroidetes and Gammaproteobacteria: Members of the Bacteroidetes and Gammaproteobacteria possess enzymes for polysaccharide hydrolysis and fermentation (Alderkamp et al. 2007; Kirchman 2002). The majority of the Bacteroidetes phylum is gram-negative bacteria and includes aerobic, microaerophilic (Manz et al. 1996), and anaerobic fermentative species (Ravenschlag et al. 2001; Llobet-Brossa et al. 1998) that have the ability to degrade complex organic macromolecules (Weller et al. 2000; Manz et al. 1996; Holmes 1991). Rosselo-Mora et al. (1999) and Battin et al. (2001) have illustrated that Bacteroidetes populations respond to substrate addition in marine sediments. We recognize that the Bacteroidetes and Gammaproteobacteria include bacteria with various metabolisms and not all the members of these groups have hydrolytic and fermenting capabilities. However, the distribution of these bacteria throughout the depth of the sediment can give an initial indication of a possible link between hydrolysis rates and the abundance of potential hydrolytic and fermenting bacteria. The establishment of such a link should be verified with more specific bacterial probes, functional gene markers, or Micro autoradiography combined with FISH (MAR-FISH). The high abundances of the Bacteroidetes bacteria in the top 6 cm of the sediment are in agreement with results found in a study on the microbial diversity of intertidal flat sediments from the North Sea (Llobet-Brossa et al. 1998). The higher metabolic rates in the surface 6-8 cm of the sediment is matched by a higher abundance of Bacteroidetes, Gammaproteobacteria, and sulfate reducing bacteria, compared to the deeper sediment. This is not surprising, since most of the carbon input into the sediment is due to vertical flux of settling organic matter, providing fresh organic matter and stimulating microbial activity in the surface sediment. The proportionate decrease in cellular abundance and detection level relative to DAPI counts for both the potential hydrolytic and sulfate-reducing bacteria suggests that they become increasingly carbon-limited at greater sediment depth, which is consistent with the gradual increase in the C:N ratio of the organic matter.

Sulfate reducing bacteria: The probes DSS658, DSB985, and DSR651 were selected for the purpose of quantifying the SRB. Evidently, not all SRB in the sediment were targeted by the probes used in this study. We chose these probes because genera targeted by the probes and their close relatives appeared in clone libraries constructed from extracted DNA at the same sampling stations (Julies et al. in prep.). In addition sulfate reducing Archaea are not detected by these probes however, the relative abundance of Archaea in the sediment depth investigated by us was lower than 25% of the total DAPI stained cells (Fig 6a,b). We therefore assume that a significant fraction of the SRB in the sediment was detected by the probes used and that the probes had a high specificity, targeting only SRB.

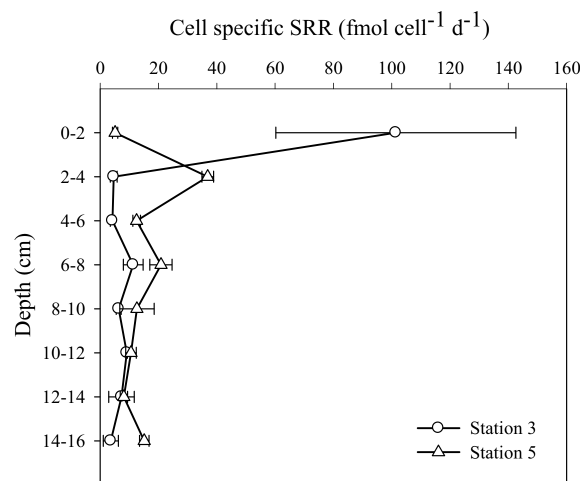

Figure 7: Cell-specific sulfate reduction rates at Station 3 and Station 5.

By assuming that the relative detection rate of active SRB was equal at the two stations and did not change with depth, cell-specific sulfate reduction rates can be calculated by combining FISH cell counts of SRB and sulfate reduction rates at the two stations. These calculations showed that the cell-specific rates differed significantly between the two stations in the top 4 cm of the sediment ($p < 0.05$, df = 1), but converged below this depth to rates between 3 and 12 fmol cell^{-1} d^{-1} (Fig. 7). Below a depth of 8 cm cell-specific rates were

slightly higher at Station 5 compared to Station 3, but this difference was statistically insignificant (p = 0.08, df = 1). These rates are within the same range as reported from other studies (e.g., Parkes et al. 2007; Sahm et al. 1999). A very high rate of ~101 fmol $cell^{-1}$ d^{-1} was calculated for the topmost centimeter of sediment at Station 3, which decreased rapidly to 7 fmol $cell^{-1}$ d^{-1} in the 2-4 cm depth interval. At Station 5 cell-specific sulfate reduction rate in the top 2cm of the sediment was only ~5 fmol $cell^{-1}$ d^{-1}, but a subsurface maximum was found at a depth of 4-6cm (~37 fmol $cell^{-1}$ d^{-1}). These differences are largely due to differences in the measured rates rather than bacterial cell numbers, since the relative abundances of SRB in the top 6 cm of the sediment were relatively similar at the two stations. SRB in the topmost centimeter at Station 3 appear to maximize the cellular turnover rate of the available organic matter, whereas the strategy of SRB at Station 5 suggests the existence of a sulfate-reducing bacterial community with lower energy requirements and thus turnover rates. This contrasting adaptation may possibly be dictated by the transient availability of labile organic matter at Station 3.

Coupling between initial and terminal processes during carbon mineralization

In order to evaluate the dynamics of the DOC pool in the sediment and the availability of the high molecular weight (HMW) fraction for remineralization, it is important to study the turnover of DOC by both initial and terminal steps during carbon mineralization. The balance and coupling between initial hydrolytic and fermenting steps to terminal oxidation steps can be assessed from the pore water DOC concentration or the concentrations of intermediates. DOC produced by hydrolytic enzymes can be taken up by sediment bacteria, or adsorbed onto particle surfaces (Alperin et al. 1999; Hedges and Keil 1995), or chemically transformed via abiotic reactions (Burdige and Gardner 1998; Henrichs 1992). DOC concentrations, therefore, reflect the balance between rates of production and consumption (Alperin et al.

1999). VFA and hydrogen produced during initial hydrolytic and fermenting steps serve as electron donors for bacteria involved in terminal oxidation processes, accounting for 65-90% of the sulfate reduction (Sørensen et al. 1981, Parkes et al. 1989). Therefore, VFA concentrations reflect the balance and coupling between initial metabolism and terminal oxidation.

The low VFA and almost constant DOC concentrations measured within the top 5 cm of the sediment suggest rapid turnover of fermentation products within the surface sediment and is indicative of a tight coupling between initial and terminal processes. A similar tight coupling between initial and terminal steps during carbon mineralization has been illustrated in Cape Lookout Bight sediments (Arnosti and Holmer, 1999; Alperin et al., 1994). The net accumulation of DOC and persistence of low amounts of carbohydrates below 5 cm depth suggest a de-coupling of initial and terminal steps with increasing depth. However, this is not reflected in the VFA concentrations, which remain low throughout the entire sediment core. Anaerobic bacteria that ferment carbohydrates assimilate only about 10% of the substrate carbon (Clark, 1989) and excrete the remainder as LMW organic compounds such as short chain acids, alcohols and CO_2. The acids and alcohols are taken up by other bacteria in the community that oxidize much of the carbon to CO_2. In the deeper sediment where sulfate reduction rates decrease, low VFA concentrations will be maintained by methanogenic bacteria in the sulfate-methane transition (SMT) zone.

Carbohydrates persisted with depth, since carbohydrate concentrations either remained relatively constant or changed slightly with depth. The contribution of carbohydrates to the DOC pool was substantial at both stations and increased at Station 5 from 11% at the surface to 28 % at the bottom of the core (12-14 cm depth). At Station 3 the contribution of carbohydrates to the DOC pool in the surface sediment was high (97%) and decreased gradually with depth to 64% at a depth of 14-16 cm. This is the first report on the

contribution of dissolved carbohydrates to the total dissolved organic carbon pool in Namibian shelf sediments. Arnosti and Holmer (1999) found a similarly high contribution of dissolved carbohydrates to the DOC pool of 85% in the surface and 38% at a depth of 10-16 cm in Cape Lookout Bight sediments. In comparison, sediments from the Peruvian upwelling system, which also consist of laminated and bioturbated diatomaceous mud contained approximately 10% carbohydrates and the contribution of carbohydrates to the total DOC pool decreased with depth (Lewis and Roland 1993). The terminal metabolic rate (sulfate reduction rate) was not limited by the availability of the electron acceptor (dissolved sulfate) at both stations, because the lowest dissolved sulfate concentrations were far above the suggested K_m values of 10–300 µmol L^{-1} for sulfate-reducing communities (Pallud and van Cappellen. 2006). Therefore, the pore water DOC accumulation and carbohydrate persistence can be explained by limited production of an electron donor that can be oxidized by SRB and indicate the resistance of certain carbohydrates to microbial degradation.

Rate of initial metabolism – Hydrolysis rates: Fluorescently labeled polysaccharides can be used to measure endohydrolase activity, which is the major cleavage activity during hydrolysis and is carried out by hydrolytic enzymes bound to the cell wall or released into solution (Hoppe *et al*., 2002). Presently the amount of fluorescently labeled polysaccharides that can be used to study hydrolytic enzyme activity is limited and a method to measure the structure and concentration of different HMW polysaccharides in marine pore water is currently not available. The experimental hydrolysis rates measured in our study does not represent in situ rates, because the two fluorescently labeled polysaccharides used probably represent only a small fraction of the total dissolved carbohydrate pool in the sediment. The amount of polysaccharides added to the sediment was about 100-400% of the naturally occurring total dissolved carbohydrate concentration and the enzymes were probably saturated by these high substrate concentrations. Therefore, hydrolytic activity was likely

zero order, so that the hydrolysis rates measured were independent of substrate concentration. Although we did not measure in situ rates with our experiment, the hydrolysis rates is likely to reflect the activity of enzymes produced by the microbial community in response to a sudden high input of substrate, which is typical after an upwelling event. For this reason we can refer to our rates as potential rates.

The addition of both polysaccharides represented a similar carbon input into the sediment. Laminarin addition represented an addition of 10.8 µmol C, while pullulan addition represented 12.6 µmol C. Potential hydrolysis rates of pullulan at Station 5 do not decrease with depth, as in the case of laminarin, indicating that these two polysaccharides are degraded at different rates within the sediment. Alderkamp et al. (2007) found an absence of pullulanase activity in strains grown on laminarin as sole carbon source. Hence, a possible explanation for the difference in potential hydrolysis rates of the two investigated polysaccharides is that laminarin is more abundant than pullulan in the sediment at Station 5 and as a consequence, there is more laminarinase activity. This needs further investigation and will require a structural and quantitative characterization of high-molecular weight polysaccharides in the pore water to understand the complex interaction of bacterial enzyme systems in marine sediments.

Rate of terminal metabolism – sulfate reduction rates: The rate of terminal metabolism can be measured either as the rate at which reactant (sulfate) is consumed or product (CO_2) is formed. Using a 2:1 stoichiometry for the oxidation of organic matter by bacterial sulfate reduction and integrating the measured rates over the analyzed depth interval at the two stations yielded a CO_2 production rate of 8.6 ± 3.6 mmol m^{-2} d^{-1} at Station 3 and of 10.4 ± 0.8 mmol m^{-2} d^{-1} at Station 5. These rates are similar to those measured in arctic sediments (Arnosti and Jørgensen 2006) and shelf sediments off the coast of Chile (Ferdelman et al. 1997).

The overall low concentrations of VFA indicate efficient uptake by microorganisms. Sulfate reduction rates are therefore limited by the availability of the low-molecular weight fatty acids, and thus the transformation of the degradable fraction of DOC, including the carbohydrates. Additionally, the tight coupling in the abundance of SRB, Gammaproteobacteria, and Bacteroidetes suggests that bacteria belonging to these groups control the turnover of the readily degradable organic matter fraction in the Namibian shelf sediments. There are likely depth-dependent changes in the composition of the active Bacteria within these groups that have not been revealed with the relatively broad coverage achieved by the oligonucleotide probes used here. A closer affiliation of targeted bacteria to the degradation of a specific DOC compound is not yet technically possible.

Formation of refractory DOC and imbalanced carbon turnover

We used the approach presented by Arnosti and Holmer (1999) to estimate the coupling between hydrolysis and terminal oxidation by calculating the turnover times of the dissolved carbohydrates from the measured potential enzymatic hydrolysis rates of laminarin and pullulan and by comparing these numbers to the turnover time of carbohydrates to CO_2 by SRB (Table 3). At Station 3, the estimated turnover times of the total dissolved carbohydrates based on potential hydrolyses rates of laminarin and pullulan were 10.2 days and 21.6 days in the top 2 cm, respectively. The combined turnover time using both hydrolytic enzymes was 6.9 days. The turnover of the carbohydrate pool based on the sulfate reduction rates in the top 2 cm was 3.9 days and thus shorter than the combined hydrolysis rates. Provided these hydrolysis rates reasonably represent carbohydrate turnover, the carbohydrate pool that is accessible to the SRB is insufficient to satisfy the metabolic demand of the SRB. These bacteria therefore satisfy their carbon demand from compound classes other than carbohydrates, likely proteins.

Table 3: Calculated turnover rates of carbon based on measured potential hydrolysis rates and sulfate reduction rates.

Depth (cm)	Station 3	Station 5
Turnover time of carbohydrates based on potential hydrolysis rates of laminarin (days)		
0-2	10.2	1.1
4-6	11.7	1.4
10-12	27.9	4.1
Turnover time of carbohydrates based on potential hydrolysis rates of pullulan (days)		
0-2	21.6	3.2
4-6	43.0	3.3
10-12	70.4	6.8
Turnover of carbohydrates based on combined potential hydrolysis rates of both polysaccharides (days)		
0-2	6.9	0.8
4-6	9.2	1.0
10-12	20.0	2.5
Carbohydrate turnover time based on sulfate reduction rates (days)		
0-2	3.9	1.9
4-6	28.2	1.6
10-12	487.5	5.6

In the top 2 cm at Station 5, the turnover time of the carbohydrates by the two exoenzymes is significantly shorter (1.1 and 3.2 days, for laminarin, and pullulan, respectively) and the combined hydrolysis rate resulted in a turnover time of the carbohydrate pool of only 0.8 days (Table 3). The turnover of the carbohydrate pool based on the sulfate reduction rates in the top 2 cm was 1.9 days. These numbers are reasonably close to each other to argue for a tight coupling between carbohydrate turnover by these exoenzymes and sulfate reduction rates, i.e., a limitation of sulfate reduction rates by the hydrolysis of available organic matter.

With depth a growing imbalance between turnover by hydrolysis and by terminal oxidation is observed at Station 3 with shorter turnover times of the carbohydrates by the combined hydrolysis compared to the terminal oxidation by sulfate reduction. This is particularly true for the laminarin turnover. The imbalance is the strongest in the 10–12 cm section, where the calculated turnover of the carbohydrate pool via sulfate reduction is 488 days compared to 27.9 and 70.4 days for hydrolysis via laminarinase and pullulanase,

respectively. This discrepancy is not apparent at Station 5, where the turnover times of carbohydrates by hydrolysis and terminal oxidation in the three depth sections remain relatively similar with a slightly faster turnover by hydrolysis relative to terminal oxidation. Evidently, the available carbohydrates are efficiently turned over at Station 5. The DOC pool is still dynamic at a depth of 10–12 cm, because carbohydrates make up 28% of the DOC pool.

Faster initial potential hydrolysis rates compared to rates of fermentation and sulfate reduction as observed at Station 3 have also been measured by Brüchert and Arnosti (2003). They may be explained by different modes of response of hydrolytic enzyme-producing bacteria and SRB: the enzyme-producing bacteria may only need to induce a specific enzyme or systems of enzymes to respond to substrate input, while the response of SRB may be slower, most probably through cell division (Brüchert and Arnosti 2003; Arnosti et al. 1994). A second possible explanation for the imbalance observed between hydrolysis rates and sulfate reduction rates may be that some of the hydrolytic products are transformed into structures inaccessible to further enzymatic degradation (Hoppe et al. 2002). The measured divergence in our direct rate measurements provides evidence for the selectiveness of naturally occurring exoenzymes for available organic matter. However, the limited diversity of substrates and substrate analogs used to determine hydrolysis rates insufficiently represents the diversity of chemical structures of organic matter in the sediment. In addition, enzymatic kinetics from model substrates used in studies and actual polymers in marine sediment differ significantly (Arietta and Herndl 2002).

The accumulation of DOC with depth suggests that DOC produced at depth is accessible to hydrolytic degradation, but cannot be completely oxidized. The slow increase in DOC concentration below 8 cm depth is either due to a decreased production from particulate organic matter or more likely, a significant decrease in DOC consumption. The latter is

supported by the substantial decrease in sulfate reduction rates (Fig. 6) and the decrease in the abundance of active Gammaproteobacteria, Bacteroidetes and SRB with depth (Fig. 4). If total dissolved carbohydrates and volatile fatty acids are subtracted from the total DOC pool, the rest consists of dissolved free amino acids (DFAA) and uncharacterized components. DFAA had short turnover times from about 1 day to several weeks in Peruvian (Henrichs et al. 1984) and Chilean (Pantoja and Lee 2003) shelf sediments. Concentrations of total hydrolysable amino acids (THAA) in Namibian shelf sediments are 10 - 20% of POC (Ahke 2006) and are similar to THAA concentrations in Cape Lookout Bight sediments (10-15% of POC – Burdige and Martens 1988). The overall contribution of DFAA to the non-acid-volatile components of the DOC was low in Cape Lookout Bight sediments (~10 μμ at 5-10 cm depth (Burdige and Martens 1990; Arnosti and Holmer 1999). If we assume that the contribution of DFAA to the total DOC pool is similarly low in Namibian shelf sediments, then most of the DOC that is not dissolved carbohydrates or VFA consist of uncharacterized components. Klok et al. (1984) found that POC in diatomaceous ooze on the Namibian shelf contained only 4% free lipid, 11% protein and 4% carbohydrate and 78% of the OM was uncharacterized. Only recently, instruments have become available to explore the compositional variety of DOM (Koch and Dittmar 2006; Dittmar et al. 2006). A decrease in DOC production may be explained by the formation of abundant secondary organic sulfur compounds in the Namibian shelf sediments (Pichevin et al. 2004; Dübecke and Brüchert 2004). In particular, the formation of sulfurized carbohydrates has been invoked as an important mechanism to slow down bacterial degradation (Kok et al. 2000; van Dongen et al. 2003). Organic-sulfur compounds have been observed in Namibian shelf sediments, but their quantitative contribution towards retarding bacterial degradation is not sufficiently established.

This study revealed differences in biogeochemical rates and bacterial abundance between the two sampling stations examined due to enhanced sediment reworking at the shallower station. We base our conclusion on the higher and more extensive bottom water turbidity at this station. Similarity in bottom water oxygen concentrations at the two stations suggests that oxygen does not have a significant influence on the contrasting degradation processes in this system. A very tight coupling of the actively cycling carbon at the more distal station (Station 5) contrasts with the accumulation of increasingly refractory DOC and carbohydrates with depth at the more proximal station (Station 3). This is consistent with the greater degree of sediment suspension and reworking at Station 3. Although the dynamics of carbon transformation in the bottom boundary have not been studied in any detail, the results presented in this study suggest that the bottom boundary layer is a critical compartment in the transformation of organic matter.

The increase in DOC and the persistence of carbohydrates with depth provide evidence for the rapid diagenetic transformation of dissolved organic matter. Only a fraction of the hydrolytic products are completely oxidized to CO_2 at the same rate as the initial hydrolysis. We hypothesize that possible, secondary reactions, such as reactions with sulfides and polysulfides and the formation of organic sulfur compounds may counteract the complete oxidation. The nature of complex organic molecules in the sediment needs further investigation. Despite the high organic carbon concentrations in sediments of the Namibian upwelling system, the biological accessibility of the organic carbon was found surprisingly limited. This is clearly evident from the numbers of active bacteria involved in fermentation and terminal oxidation and the cell-specific sulfate reduction rates, suggesting increasing starvation of bacteria with depth within the first 16 cm of sediment. This study reveals an integrated perspective of the strong physical forcing imposed by water column processes on

biogeochemical carbon dynamics and provides a preliminary understanding of the dynamics of carbon turnover in these sediments.

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Chapter 3

Characterization of microbial community structure in biogeochemical contrasting shelf sediments from the Namibian upwelling system

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Julies Elsabé. M.[*1,3], Volker Brüchert[1,2], and Bernhard M. Fuchs[1]

[1]Max Planck Institute for Marine Microbiology, Bremen, Germany

[2]Department of Geology and Geochemistry, Stockholm University, Sweden

[3]Department of Biology, University of Namibia, Windhoek, Namibia

*Corresponding author. Mailing address: Department of Biology, University of Namibia Private Bag 13301, 340 Mandume Ndemufayo Ave., Pioneerspark, Windhoek, Namibia. Phone: (00-264-(0)61-206 3561. Fax: (00-264-(0)61 206 3791. E-mail: ejulies@unam.na.

Abstract

This study investigates the diversity and abundance of bacteria in organic-rich Namibian shelf sediments from two biogeochemical contrasting sampling stations, using the 16S rRNA library approach and Catalyzed Reporter Deposition Fluorescent In Situ Hybridization (CARD-FISH). We focused on bacteria that play a role in organic carbon transformation and the sulfur cycle. Differences in phylogenetic diversity between the two sampling stations were indirectly resulting from different hydrographic factors at these stations. The shift in microbial community composition with depth is due to gradients in organic substrate availability within the sediment, which results in niche diversification related to physiological abilities of the microorganisms. Sequences that were related to bacteria with hydrolytic and fermenting abilities, included members from the *γ-proteobacteria, Bacteroidetes, Actinobacteria,* and *Acidobacteria,* at both stations, while *Firmicutes*, *Thermomicrobia* and the OP8, WS3, and TM6 divisions were restricted to Station 3. Sequences affiliated to *Actinobacteria* dominated the clone libraries from bottom sediments at Station 5, although CARD-FISH data revealed a low abundance of active members of these bacteria. Sulfate reducing bacteria (SRB) were diverse and belong to the *δ-proteobacteria*. Cloned sequences within the *δ-proteobacteria* affiliates to the *Desulfobulbaceae* and *Desulfobacteriaceae* families at both stations and were detected throughout the depth of the sediment with no significant shift in the community composition with depth, while there was a direct relationship between the abundance of SRB and sulfate reduction rates with depth. This study is the first phylogenetic analysis of the microbial community diversity within shelf sediments from the Namibian upwelling system.

Introduction

Knowledge of the phylogeny and occurrence of various microorganisms within marine sediments is essential for understanding the biogeochemical function and ecology of the uncultured microbial majority and the extent of genetic diversity within sediment bacteria globally (31, 19). Previous studies performed in sediments from the highly productive continental shelf off Namibia in the Benguela upwelling region at two closely spaced sampling stations with contrasting biogeochemical rates, revealed a steep downward decrease in biogeochemical rates that compared well with gradients in the abundance of active bacteria (26). The differences in biogeochemical rates between the two sampling stations are due to enhanced sediment reworking at the shallower station. The distribution of the metabolically active members within a fraction of the microbial community was determined in a previous study, using Catalyzed Reporter Deposition Fluorescent In Situ Hybridization (CARD-FISH) (26). However, this technique fails to provide a detailed characterization of the overall microbial diversity (39). The diversity of bacteria involved in terminal oxidation processes, mainly sulfate reduction has been investigated by several studies in various marine sediments (41, 54, 32). However, sulfate reducing bacteria (SRB) are involved in only one step in the long chain of carbon transformation processes and there is a lack of information about the identity and activity of the bacteria that are involved in the preceding steps, which are hydrolysis and fermentation. Therefore, the aim of our study is to characterize the microbial community involved in the different steps of organic carbon mineralization along a vertical depth profile within sediments from the highly productive continental shelf off Namibia in the Benguela upwelling area.

This study provides new insights into the microbial ecology in these upwelling sediments by linking identity and activity of microorganisms involved in the transformation of organic carbon. We hypothesize that the organic-rich sediments on the Namibian shelf (25) support a

high microbial diversity, especially in the top 6 cm of the sediment where higher biogeochemical rates were measured, and that the gradients in microbial activity with depth is reflected in the microbial diversity, because of different selective pressures existing throughout the depth of the sediment. Therefore, we expect a change in the composition of the microbial community with depth. Additionally, we expect the differences in biogeochemical rates between the two sampling stations to be reflected in the composition of the microbial communities within the sediment at these two stations.

The inability of culture-dependant techniques to capture the full diversity and microbial community structures of marine sediments have been demonstrated in several studies (35, 22, 1). Our current knowledge about marine microbial diversity and distribution depends on the development and use of culture-independent molecular methods. In the absence of cultivation, the only way to understand the role of different microorganisms within their environment is through culture –independent characterization linked to determination of in situ metabolic activity (19). We studied microbial diversity in Namibian shelf sediments by polymerase chain reaction (PCR) based 16S rRNA cloning and sequence analysis. The biases in this approach during DNA retrieval and amplification are discussed in several reviews and studies (57, 35, 12). Clone libraries are useful for obtaining a snap-shot of microbial diversity within the sediment. This study provides an insight into the microbial diversity in the sediment and understanding of the community structure illuminates the key players in organic carbon transformation within the Namibian upwelling system. It provides information that can be useful for the exploration of other productive marine ecosystems.

Materials and methods

Study site and sampling

Sediment samples were collected during March 2004 on an expedition to the Namibian shelf with the *RV Alexander von Humboldt* from two stations that were in close proximity to each other. Station 3 is located at 22°38.3S, 14°18.27E and Station 5 at 23°45.08S, 14°18.27E. The water depth is 70 m at Station 3 and 112 m at Station 5. The physical and biogeochemical characteristics of the sediment at the two sampling stations are summarized in Table 1. At the time of sampling, the bottom water temperature was 13.3 °C at Station 3 and 11.8 °C at Station 5. Concentrations of dissolved oxygen in the bottom water measured about 2 m above the sediment surface were less than 4.5 µmol L^{-1} at both stations. Sediments at these two stations are rich in organic carbon and have C:N ratios of 7 at Station 5 in the top 6 cm of the sediment, gradually increasing to 8 at a depth of 20 cm. At Station 3, C:N ratios is 7 in the top 2 cm of the sediment and increase much steeper throughout the sediment than at Station 5 to reach 10.5 at a depth of 20 cm.

Sediment samples were obtained with a multiple sediment corer (MUC), from which 26 mm diameter sediment sub-cores were taken with a length of 16 cm and 20 cm at Station 3 and 5, respectively. As far as possible, sub-cores were collected from the same cast. Sub-cores were stored at 12°C until they were sectioned within 6 hours after collection. Two parallel cores were sectioned per station. Cores were sectioned in two centimeter intervals, which correspond to a sediment volume of about 10 cm^3. Half of the sediment from each section was stored immediately at -20°C for construction of 16S rRNA clone libraries. The other half was divided into 0.5 cm^3 aliquots that were fixed for 24 hours in 1.5 ml formaldehyde (4% v:v). The sediment was then washed twice with 1x phosphate-buffered saline (PBS; 4 mmol L^{-1} sodium phosphate [pH 7.2], 130 mmol L^{-1} NaCl) and it was finally stored in a mixture of 1x PBS and ethanol (2:3 v:v) at -20°C.

TABLE 1. Locations and sediment characteristics at the sampling stations (26)

	Station 3	Station 5
Coordinates	22°38.3S, 14°18.27E	23°45.08S, 14°18.27E
Depth (m)	70	112
Bottom water temperature (°C)	13.3	11.8
Bottom water oxygen (µmol L^{-1})	< 4.5	< 4.5
Porosity (%)		
0-2 cm	95	93
4-6 cm	90	93
10-12 cm	80	87
POC (dry weight %)		
0-2 cm	6	4
4-6 cm	6	5
10-12 cm	4	7
C:N ratio:		
0-2 cm	7	7
4-6 cm	8	7
10-12 cm	10	8
DOC (mmol L^{-1})		
0-2 cm	1.5	1.3
4-6cm	1.4	1.4
10-12 cm	1.6	2.0
Average sulfate reduction rates: (nmol cm^{-3} d^{-1})		
0-2 cm	162	41
4-6 cm	15	40
10-12 cm	4	28
Sediment description	Dark, olive green mud; first 5cm is soft and fluffy; Below 5 cm depth - mica and fine sand. No bacterial mat visible. Low abundance of *Thiomagarita*	Top 15 cm of sediment extremely soft fluff; Olive green mud; light-colored precipitates at about 15-20 cm depth. Sediment covered by thick layer of *Beggiatoa*

DNA extraction, PCR amplification and construction of 16S rRNA clone library

Total community DNA was extracted directly from 0.5 g wet weight of sediment sample (BIO 101 Fast DNA spin protocol) from three different depths at each station. 16S rRNA clone libraries were constructed for three depth intervals. The depth intervals included the surface of the core 0-2 cm, the middle section 4-6 cm and the bottom of the core 10-12 cm. These depths were chosen because biogeochemical rate measurements indicated the highest

rates of hydrolysis and sulfate reduction occur in the topmost 5 cm of the sediment and there is a steep decrease in rates below 5 cm depth (26). Purified DNA from the sediment was used as a template for the polymerase chain reaction (PCR) amplification. *Bacteria*-specific 16S rRNA primer pairs were used for amplification and included the forward primer GM3F and the reverse primer GM4R (43). Primers were synthesized by Pharmacia Biotech. The PCR mastermix consisted of 5 µL of 10x Taq-polymerase buffer, 4 µL dNTPs (2.5 $mmol.L^{-1}$ each), 5 µL bovine serum albumin (3 $mg.ml^{-1}$), 0.15 µL of Taq polymerase (5 $U.\mu l^{-1}$), 1 µL of each of the primers (50 $\mu mol.L^{-1}$), 2 µL of DNA extract and 31.8 µL ultrapure, sterile water. The PCR was run on a Master-cycler (Eppendorf) at the following cycling conditions: 1 cycle at 96°C for 3 minutes followed by 30 cycles consisting of three steps, the first step at 96°C for 1 minute followed by an annealing step at 50°C for 1 minute and a third step at 72°C for 3 minutes. The final cycle was run at 72°C for 10 minutes. PCR products were inspected by gel electrophoresis on 1% (w:v) agarose gels. PCR products were purified with the QIAquick PCR purification kit (Qiagen). Purified PCR products were ligated and cloned into high efficiency competent cells of *Escherichia coli* (JM109) using the TOPO TA cloning kit (Invitrogen). The transformed cells were plated on Luria-Berani (LB) selective agar plates which contained ampicilin (100 µg ml^{-1}), isopropyl-ß-D-thiogalactopyranoside (IPTG, 0.5 mmol L^{-1}), and 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-Gal, 80 µg ml^{-1}) and incubated overnight at 37°C. After incubation white colonies were transferred into LB culture medium (10 g L^{-1} Bacto-tryptone, 5 g L^{-1} NaCl, 5 g L^{-1} Bacto-yeast extract, 15 g L^{-1} agar, 100 µg mL^{-1} ampicilin, 80 µg mL^{-1} X-Gal, and 0.5 mmol L^{-1} IPTG) for 18 hours to allow amplification. Plasmid was extracted from clones with correctly sized inserts using the Montage Plasmid $Miniprep_{96}$ kit (Millipore, Bedford, Mass). Plasmid quality was checked with gel electrophoresis.

For sequencing of plasmid inserts, 1 µL of plasmid DNA (100 ng μL^{-1}) was used and sequencing was performed with ABI BigDye on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Partial sequencing of 300-500 base pairs (bp) was performed using the forward and reverse16S rRNA primers GM1F and GM1R (42), the forward primer GM13F and the reverse primer GM13R (38). Sequences were aligned and compared to 16S rRNA sequences that were present in the dataset of the Ribosomal Database Project (RDP) in January 2004 (~95 000 rRNA sequences, http://rdp.cme.msu.edu) by using the automatic aligning tool of the ARB software (34) and the results were corrected manually where necessary. Sequences were compared to sequences that were present A subset of sequences were selected for nearly full-length sequencing (>1400 bp). Nearly full length sequences were deposited in GENBANK (http://www.ncbi.nlm.nih.gov/Genbank/index.html) and the accession numbers (EU290675-EU290740) are shown in Table 2.

Phylogenetic analysis

Phylogenetic distances were calculated using the Jukes-Cantor model. Tree topologies were evaluated by the neighbor joining, maximum parsimony and maximum likelihood method. For this study we defined that >97% similarity in sequences represented the same rRNA clone type. Sequences were analyzed for chimeras using the Bellerophon software program (21). Sequences that showed potential chimeric origin were excluded from further analysis (n = 1).

Analysis of coverage by clone libraries

A total of six different clone libraries were constructed. At each of the two sampling stations, three different clone libraries were constructed for the depths 0-2 cm (top), 4-6 cm (middle) and 10-12 cm (bottom). The proportion of phylotypes that were represented in the

different clone libraries were estimated using rarefaction analysis (20). Rarefaction curves and coverage estimates are commonly used to assess whether most of the diversity in the environment has been captured in clone libraries (27). Additionally, coverage of the diversity within the sediment by the clone libraries was estimated by the non-parametric estimator of sample coverage *(C_{ACE})* (5), which estimates coverage as the proportion of individuals in relatively rare phylotypes (≤10 clones) that occur more than once in a library.

$$C_{ACE} = 1 - \frac{F_1}{Nrare}$$

where F_1 is the number of phylotypes occurring only once in the library, and N_{rare} is the total number of individuals in phylotypes occurring 10 or fewer times. The advantages and disadvantages of this estimator are reviewed elsewhere (27).

Phylogenetic richness, diversity and evenness

Species richness, which refers to the number of species, and species evenness, which refers to the distribution of individuals among species are the two statistical properties used to quantify species diversity (23). In addition phylotype richness estimators provide a less subjective alternative to estimate the efficiency of clone library coverage (27), in particular the S_{chaol} estimator of phylotype richness which is the least biased estimate of richness at small sample sizes (9). The richness of phylotypes in each clone library was estimated by using the S_{chaol} estimators (6, 7).

$$S_{chaol} = S_{obs} + \frac{(F_1)^2}{2(F_2+1)} - \frac{F_1 F_2}{2(F_2+1)^2}$$

S_{obs} is the number of different phylotypes in a clone library, F_1 is the number of phylotypes that occurred once and F_2 is the number of phylotypes that occurred twice.

Phylogenetic diversity was estimated by calculating the Shannon-Weaver diversity index, H’ (56), which is a common estimator of species diversity (52, 23):

$$H' = -\sum_{i=1}^{S} p_i \log p_i$$

Where s is the number of phylotypes, $p_i = n_i/N$, n_i is the number of individuals in the ith phylotype, and N is the total number of individuals.

The evenness index of phylotypes (J') was estimated using the equation (48):

$$J' = \frac{H'}{\log S}$$

where H' is the Shannon-Weaver diversity index and S is the number of phylotypes.

Total microbial cell counts and Catalyzed Reporter Deposition Fluorescent In Situ Hybridization (CARD-FISH)

Duplicate sub-cores were sectioned in two centimeter intervals. The fixation, preservation, sonication, hybridization and staining of bacterial cells in the sediment and subsequent microscopic evaluation are described in our previous study at these two sampling stations (26). Information on the probes and conditions used during hybridization to quantify active members of the *γ-proteobacteria,* SRB, and *Bacteroidetes* phylum are also summarized in our previous study (26). In this study, we determined the abundance of active members of the *α-proteobacteria* with probe Alf968 (45), *Planctomycetes* with probe Pla46 (46), and *Actinobacteria* with probe HGC69a (53) at Station 5. The probe HGC69a targets part of the 23S rRNA, while all the other probes used target part of the 16S rRNA. All nucleotide probes used were purchased from Interactiva (Ulm, Germany).

Results

Coverage by clone libraries, phylogenetic- richness, diversity and evenness

More than 50% of the diversity in the environment has been covered by the different clone libraries as revealed by the C_{ACE} estimator of coverage (Table 2).

TABLE 2. Estimation of the coverage of diversity by the clone libraries (C_{ACE}). Estimation of phylotype richness by Chao's index (S_{chao1}); phylotype diversity by the Shannon-Weaver index (H') and phylotype evenness (J') for the different clone libraries constructed.

Clone Library	Estimation of coverage (C_{ACE})	Phylotype richness (S_{chao1})	Phylotype diversity (H')	Phylotype evenness (J')
St. 3: 0-2 cm	0.79	9.5	0.99	0.81
St. 3: 4-6 cm	0.8	14	0.79	0.74
St. 3: 10-12 cm	0.64	22	0.93	0.81
St. 5: 0-2 cm	1	7	0.27	0.32
St. 5: 4-6 cm	0.5	5	0.13	0.18
St. 5: 10-12 cm	0.43	16	0.43	0.48

The clone library from the bottom sediment at Station 5 had a low coverage of only 43% of the diversity. Rarefaction analysis indicated that the size of the clone library from Station 3 was sufficiently large to cover most of the diversity in the sediment (Fig. 1). The clone library at Station 5 failed to cover a representative portion of the diversity as revealed by the linear shape of the curves (Fig. 1). For non-asymptotic curves such as the rarefaction curves obtained for Station 5, the clone library size is considered too small to provide an accurate estimate of phylogenetic richness (4). Phylogenetic richness can only be compared when asymptotic maxima have been reached by rarefaction curves (18). However, phylotypes were classified arbitrarily and since DNA-DNA pairing values of more than 70% do not necessarily produce groups of bacteria that correspond to real ecological units. Therefore, phylogenetic richness and diversity were probably underestimated.

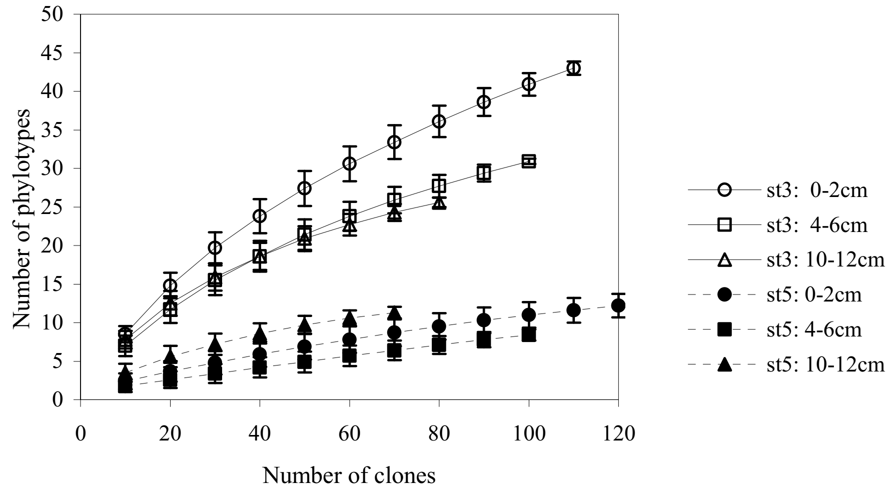

Fig. 1: Rarefaction analysis of the clone libraries obtained from Station 3 and Station 5 was performed with the free online analytic rarefaction 1.3 software (20). The error bars are 99% confidence levels.

At both sampling stations phylotype richness as determined by the S_{chaol} estimate increased with depth (Table 2). At Station 5 phylotype richness was comparable to that of Station 3 in the surface 2 cm of the sediment, but almost two times lower in the bottom sediment (Table 2). Both phylotype diversity (H') and evenness (J') were high at Station 3, exceeding 0.8 (Table 2). There was no trend with depth in phylotype diversity and evenness at Station 3. At Station 5 phylotype diversity and evenness were approximately 2-4 times lower than at Station 3. Phylotype diversity and evenness were higher for the clone library from the bottom sediment compared to the top (0-2 cm) and middle (4-6 cm) clone libraries at Station 5.

TABLE 3: Affiliation of clone sequences. The number of sequences is the sum of sequences from both sampling stations and all three depths.

Cluster	No. of sequences Full	Partial	Next Relative	Accession no.	Similarity (%)
Alphaproteobacteria					
	2	2	*Ahrensia kieliense*	EU290708 EU290679	96 96
	1	1	Uncultured	EU290695	94
Epsilonproteobacteria					
	1	1	uncultured	EU290712	93
Beta-gamma-proteo-bacteria					
	1	7	Uncultured *Piscickettsiaceae*	EU290725	93
	1		*Methylococcaceae*	EU290681	
		26	uncultured sulfur-oxidizing symbiont		96
	22	200	*Gammaproteobacteria-2* uncultured	Rest of the accession numbers not listed here	75-88
	1	7	*Beggiatoa – Nevskia - Thioploca* –uncultured	EU290675	93
		1	*Aquicella et rel.-Ifremeria*		
	1	2	*Gammaproteobacteria-1-Alteromodales-1-Colwellia*	EU290739	92
	1	1	*Cellvibrio et rel.*-uncultured	EU290692	97
	3	7	*Saccharospirillaceae-Kangiella*-uncultured	EU290714; EU290698; EU290683	99
		1	*Vibrionales-Photobacterium*		
		1	*Oleiphilus*		
Deltaproteobacteria					
	2	6	Uncultured soil *Bacteria*	EU290680 EU290707	95 95
		13	*Desulfobulbaceae*-uncultured		
	1	1	*Desulfocapsa*	EU290696	99
	2	2	*Desulfobacteriaceae Desulfatibacillum alkenivorans*, Iron-sulfide containing	EU290686 EU290687	91 94
	1	1	*Desulfosarcina*	EU290724	97
	1	2	*Desulfobacula*	EU290701	99
	2	53	uncultured SRB	EU290703 EU290705	92 92
		14	*Desulfobacterium anillini*		
Fusobacteria					
	1		uncultured	EU290699	91
Gemmatinodales					
		1	Uncultured		

TABLE 3 (continue)

Spirochaetes	1	2	*Spirochaetaceae-Spirochaeta-1-Spirochaeta* species	EU290731	89
Bacteroidetes	1	4	*Anaerophaga et rel.-Cytophaga fermentans*	EU290711	94
	1	5	*Flavobactereaceae-1-Gelidibacter-Psychroserpens – Gelidibacter* species	EU290693	89
	1	1	*Polaribacter-Tenacibaculum-* uncultured *Cytophagales*	EU290727	90
	1	1	*Sphingobacteriaceae-Eubostrichs dianae*	EU290717	90
		4	uncultured		
		3	*Saprospiraceae*		
Chlorobi		4	uncultured		
Actinobacteria	2	72	uncultured	EU290722;EU 290733	85 87
Acidobacteria	2	12	*Acidobacteria_10-* uncultured	EU290737 EU290691	95 99
Planctomycetes	1	2	*Pirellula*-uncultured	EU290716	95
	1		*Candidatus* ”Scalindua brodae”	EU290720	79
	3	10	uncultured	EU290719 EU290690 EU290728	95 99 95
OP8	3	17	*Candidatus* Bacteria - uncultured	EU290730; EU290736; EU290738	94-96
Uncultured bacteria	2	19	uncultured bacteria	EU290732 EU290713	99 95
Betaprotebacteria		1	uncultured		
WS3		7	uncultured		
Chaldithrix		2	*Chaldithrix abyssi*		
Firmicutes		2	*Acetivibria et rel.*		
Thermomicrobia		7	uncultured		
OD1-OP11-WS6-TM17		2	uncultured		
TM6		3	uncultured		

Diversity and vertical zonation of bacteria

Station 3

A total of 296 bacterial clones (114 clones from the top, 100 clones from the middle and 82 clones from the bottom) were sequenced. The clone library consisted of 260 partial and 36

full to almost full length sequences. The bacterial 16S rRNA clone libraries were diverse at Station 3 and included sequences affiliated with most phyla previously detected in marine sediments (Fig. 2 and Table 3). Members of the *proteobacteria* dominated the clone libraries, in particular members from the *δ-proteobacteria* (30% of the clones at the top, 41% of the clones at the middle and 17% of the clones at the bottom), and the *γ-proteobacteria* (25%of the clones at the top, 32% of the clones at the middle and 12% of the clones at the bottom). Members from the *α-proteobacteria, ε-proteobacteria* and *β-proteobacteria* were only observed in the top sediment with a frequency of occurrence of 3%, 2% and 1% respectively.

The frequency of occurrence of sequences affiliated to the *Bacteroidetes* group was higher in the top sediment (18% of the clones), compared to the deeper sediment (5% of the clones in the middle and 9% of the clones in the bottom). In contrast the frequency of occurrence of sequences affiliated to the *Actinobacteria* and *Planctomycetes* increased in the deeper sediment compared to the top and middle depth intervals. In the top and middle depth intervals the frequency of occurrence of these groups varied between 1-2%, but in the bottom sediment their frequency of occurrence was 10-13%. The *Fusobacteria, Spirochaetes, Acidobacteria, Chaldithrix, Thermomicrobia, Firmicutes*, OD1-OP11-WS6-TM17 divisions, TM6 and the *Candidatus* “WS3” each accounted for less than 5% of all the clones.

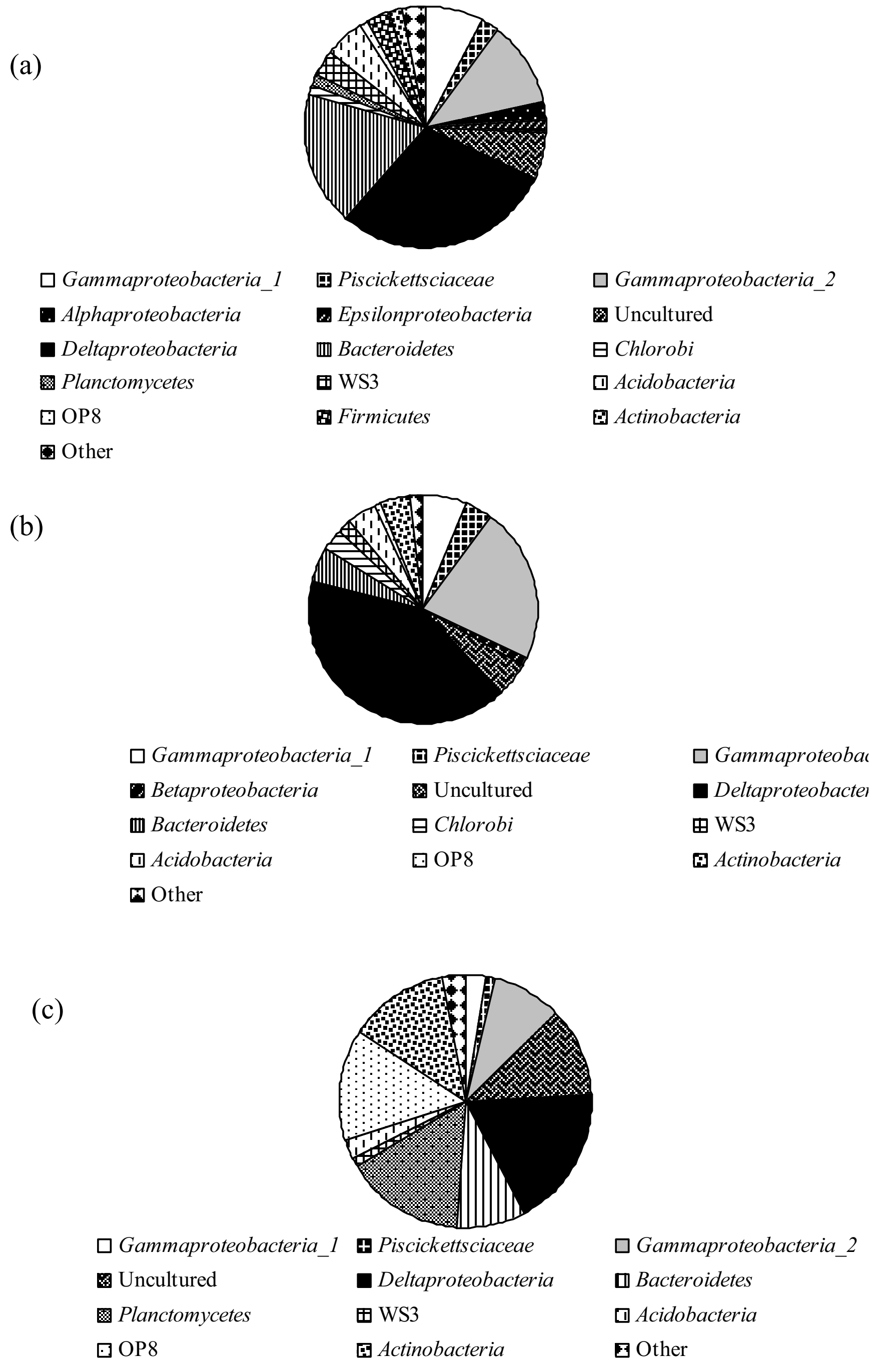

Fig. 2: Frequencies of different bacterial phylogenetic lineages in 16S rRNA clone libraries derived from Station 3 in: (a) 0-2 cm, (b) 4-6 cm and (c) 10-12 cm.

Station 5

A total of 386 bacterial clones (202 clones from the top, 108 clones from the middle and 76 clones from the bottom) were sequenced. The clone library consisted of 359 partial and 26 full to almost full length sequences. The bacterial 16S rRNA clone libraries at Station 5 represented fewer phylotypes (Fig. 3), compared to Station 3 (Fig. 2). The clone libraries in the top and middle sediment were dominated by clones from *γ-Gammaproteobacteria* (87% of the clones in the top and 96% of the clones in the bottom) (Fig. 3a and b). In the bottom sediment *γ-Gammaproteobacteria* contributed only 5% of the clones to the total clone library (Fig. 3c). The clone library in the bottom sediment was dominated by sequences that affiliated to *Actinobacteria* (76% of the clones). In contrast the *Actinobacteria* were not represented at all by cloned sequences in the clone libraries from the top and middle sediment. The frequency of occurrence of clones of the *δ-proteobacteria* was 10%, 2% and 8% in the top, middle and bottom sediment, respectively. Cloned sequences affiliated to the *Bacteroidetes* bacteria were only observed in the clone library of the top sediment (6%). The frequency of occurrence of the other phylotypes represented in the clone libraries at Station 5 was less than 5% and included members from the *Firmicutes* bacteria, the OP8 division, *Acidobacteria* and *α-proteobacteria*.

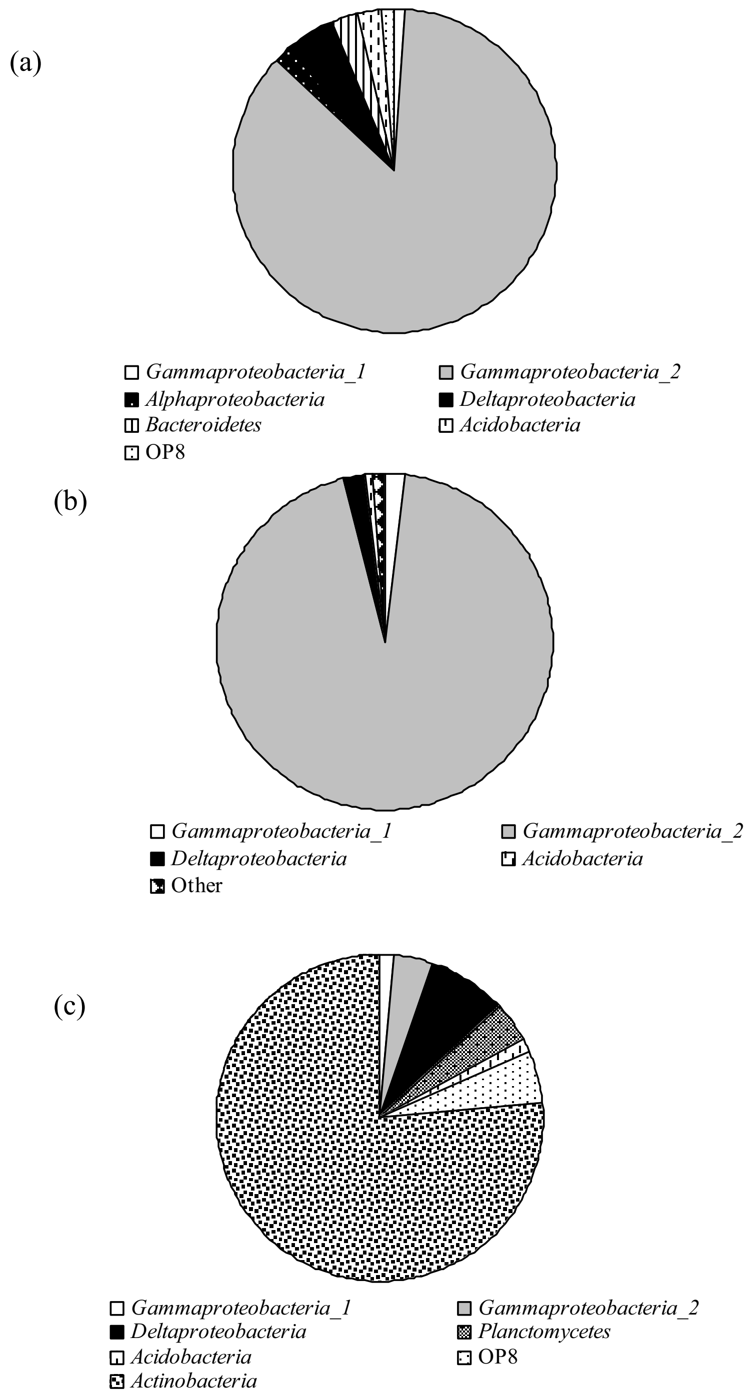

Fig. 3: Frequencies of different bacterial phylogenetic lineages in 16S rRNA clone libraries derived from Station 5 in: (a) 0-2 cm, (b) 4-6 cm and (c) 10-12 cm.

Distribution and abundance of Bacteria and Archaea

More than 80% of the total DAPI cell counts hybridized to the bacterial probe, EUB I-III, in the top 2 cm of the sediment (Fig. 4a,b), indicating high amounts of 16S rRNA in bacterial cells and reveals high metabolic activity of microorganisms (26). We did not detect archaeal sequences in our 16S rRNA clone libraries. This can be explained by the few gene copies of the 16S rRNA gene within the domain *Archaea* (36). However, we assessed the abundance of *Archaea* in the sediment, using CARD-FISH. Archaeal abundance was low at both sampling stations and only about 4-25% of the DAPI cell counts hybridized to the archaeal probe ARCH915 (Fig. 4a,b). At Station 3, archaeal abundance varied between 5-17% of the DAPI cell counts (5×10^6 cells ml^{-1} wet sediment - 2×10^7 cells.ml^{-1} wet sediment) (Fig. 4a). However, at Station 5, archaeal abundance varies between 4-10% of the DAPI cell counts and only increases below a depth of 10 cm to 15-25% of the DAPI cell counts (Fig. 4b). The relative abundance of the metabolically active SRB, *Bacteroidetes* bacteria, and *γ-proteobacteria* was determined with CARD-FISH in our previous study at these two sampling stations (26) and the results are briefly summarized here in Figure 4 (c and d) for the purpose of comparison to our clone library results. Due to the detection of *Planctomycetes, Actinobacteria* and *α-proteobacteria* in the clone libraries, specific probes targeting these bacteria were used to establish their relative abundance. The relative abundance of active *Planctomycetes, Actinobacteria,* and *α-proteobacteria* at Station 5 decreases with depth from ~24%, 3%, and 23%, respectively of the total DAPI stained cells in the top 2-4 cm of the sediment to ~7%, 1%, and 4%, respectively of the total DAPI stained cells at a depth of 12-14 cm (Fig. 4d).

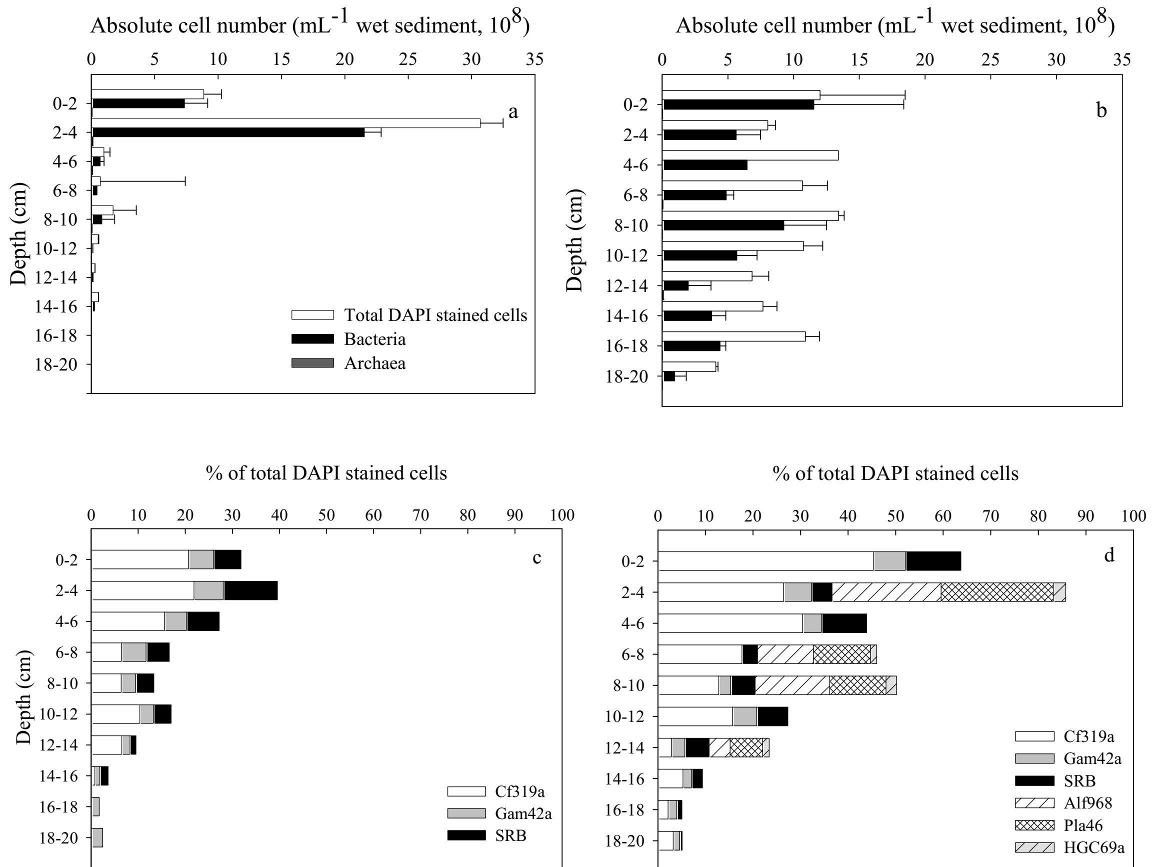

Figure 4: (a and b) Abundance of *Bacteria* and *Archaea* at a) Station 3, and b) Station 5. The data is from duplicate cores. (c and d) Relative abundance of the different groups of *Bacteria* at c) Station 3, and d) Station 5. The relative abundance of SRB is the total abundance established with the probes DSR 651, DSS 658, and DSB 985. The relative abundance of the different phylogenetic groups is the averages from duplicate cores.

Discussion

Shift in microbial community structure with depth

In the upper few millimeters of Namibian shelf sediments the role of O_2, NO_3^-, Mn(IV) and Fe(III) as electron acceptors in organic carbon mineralization can be neglected, since most of the sediment is reduced, having a negative redox potential (3) and sulfate reduction is

the dominant pathway during organic carbon mineralization, despite the lack of steep sulfate gradients within the same zone (26, 3, 2). In these sediments there is an increasing substrate limitation of the active bacteria with depth, because dissolved organic carbon (DOC), including dissolved carbohydrates becomes increasingly unavailable to the microorganisms with increasing depth (26). This results in vertical depth gradients of organic compounds that serve as energy sources for microorganisms. We examined the effect of these gradients on the composition of the microbial community by constructing clone libraries at the two sampling stations from three vertical depths within which the biogeochemical environment and availability of DOC differ (26). This study revealed a significant shift in the microbial community structure with depth.

The increase in phylogenetic richness with depth at both sampling stations is surprising, since the availability of electron acceptors and energy sources decrease with depth. In contrast, the abundance of active bacteria is directly related to the availability of electron acceptors and energy sources within the sediment and decreases with depth. Microorganisms that are adapted to thrive in environments with abundant, readily-available carbon sources, which occur in the top 2 cm of the sediment, are called copiotrophs and they are “zymogenous”, which means they have the ability to ferment carbohydrates (29). These microorganisms will exhibit r-strategists life histories, and have a high growth rate (13), which agrees with the higher abundance of *Bacteria* in the top 6 cm.

In contrast, in the bottom sediment (10-12 cm depth) where DOC becomes increasingly unavailable to microorganisms, species richness was high and bacterial abundance low. Microorganisms whose activity are adapted to low amounts of readily-degradable carbon sources or to compounds that are not readily-biodegradable are referred to as oligotrophs and they have an allochthonous mode of feeding (29). In Namibian shelf sediments carbon sources for allochthonous organisms will be chemically or biologically transformed carbon

within the sediment. They exhibit K-strategist life histories with a low growth-rate (13), which agrees with the lower bacterial abundance observed in the sediment below 10 cm depth. An ecological classification of bacterial phyla in soil into copiotrophic and oligotrophic categories has been applied in a few studies (13). The absence of certain phylotypes from deeper sediments (10-12 cm depth) in our study provides initial hints to the applicability of this classification system to marine sediments. However, detailed correlation studies and cultivation of isolated microorganisms need to be performed to confirm its applicability. We recognize that not all microorganisms within the top 6 cm of the sediment are copiotrophs and similarly not all microorganisms deeper than 10 cm depth are oligotrophs. We also recognize that the species richness of microorganisms within the environment is controlled by a multitude of physical and biological factors. However, our results suggest that the amount and accessibility of DOC throughout the sediment depth is an important determining factor for the life strategies adopted by bacteria and ultimately affects the bacterial composition and phylogenetic richness throughout the sediment depth in Namibian shelf sediments.

DOC is labile to degradation in the surface sediment and become increasingly resistant to degradation below a depth of 10 cm, probably due to the formation of complex polysaccharides and polysulfides. Microorganisms adapt to exploit the various pools of organic carbon throughout the sediment depth indicating a vertical niche separation. A few phylotypes were detected only in the clone libraries of the top and middle depths and were absent in the bottom. These phylotypes include *α-, β-* and *ε-proteobacteria.* This reflects the specialization of certain phylotypes to specific ecological niches within the sediment. The partitioning of resources within the sediment creates specific niches, enhancing microbial specialization and division into distinct ecological guilds (59). Phylotype niche specificity was also observed in gas hydrates from the Gulf of Mexico (39). Only one partial sequence in

the middle sediment was closely affiliated to the *β-proteobacteria. α-proteobacteria* was detected in the water column of other oxygen minimum zone areas, where their relative abundance may reach up to 20%, but their abundance decrease with depth (14). This explains their low contribution to clone libraries in Namibian shelf sediments and other marine sediments (32). The sequences affiliated to the class *α-proteobacteria* were closely related to uncultured bacteria and *Agrobacterium kieliense* which has been re-named to *Ahrensia kielensis* after taxonomic revision by Uchino et al. (60). These bacteria are capable of thiosulfate oxidation (58).

ε-proteobacteria is metabolically diverse and can utilize various electron acceptors, including oxygen, nitrate and different sulfur species (31, 44). *ε-proteobacteria* may live in deep anoxic waters as obligate or facultative chemoorganotrophs by fermenting organic matter (31). They can act as sulfur reducers and/or oxidizers and have been detected in the 16S rRNA gene libraries from redoxclines of anoxic waters of the Cariaco Basin and the Black Sea (31). The restriction of *α-* and *ε-proteobacteria* to the top 2 cm of the sediment may therefore be explained by the high sulfate reduction rates (100-220 $nmol.cm^{-3}.d^{-1}$) in the top 2 cm of the sediment (26). Another possible explanation for the detection of clones from *α-β-* and *ε-proteobacteria* only in the surface 2 cm of the sediment is mixing with the bottom water because of sediment re-suspension. Evidence for this is provided by the higher frequency of occurrence of these bacteria in the clone libraries of Station 3 than at Station 5. At Station 3 a greater amount of re-suspension of sediment occurs compared to Station 5 (26). The 5 times higher abundance of active *α-proteobacteria* in the top 2-4 cm compared to the sediment at a depth of 12-14 cm depth at Station 5 also suggest that these bacteria originate from the water column.

Further evidence for phylotype niche specificity that exists within these sediments is the almost absence of *Actinobacteria* in the top 6 cm of the sediment and the significantly higher

frequency of *Actinobacteria* clones in the bottom sediment, especially at Station 5. The high frequency of occurrence of *Actinobacteria* clones in the sediment at Station 5 was surprising, since *Actinobacteria* were believed to contribute to only a small fraction of the bacterial communities in marine sediments (17). However, CARD-FISH data revealed that the number of active *Actinobacteria* within the sediment at Station 5 is low (1-3% of the total DAPI stained cells). Recently a few studies reported a significant contribution of these bacteria to clone libraries from marine sediments (61, 37, 57). *Actinobacteria* are known for their ability to degrade complex polymers such as alginates, laminarin and hydrocarbons (17) and for their production of bioactive compounds (57). The physiological abilities of *Actinobacteria* allow active members of these bacteria to thrive in organic-rich Namibian shelf sediments where an increase in the complexity of DOC, due to possible chemical polymerization and sulfurization occurs with increasing depth (26).

***Bacteriodetes**:* CARD-FISH data revealed the presence of active members of *Bacteroidetes* bacteria throughout the sediment at both sampling stations (26). The majority of the sequenced clones of the *Bacteroidetes* phylum in the clone libraries were closely related to *Cytophaga* and *Flavobacteria* species. The *Bacteroidetes* typically contains bacteria with hydrolytic and fermenting abilities (62) and active members were previously detected in anoxic sediments (40, 50, 32). The presence of *Bacteroidetes* bacteria in clone libraries and low amounts of active members of these bacteria detected by CARD-FISH in anaerobic sediment as deep as 14-16 cm (26), suggest that anaerobic members from the *Bacteroidetes* play an important role in hydrolytic and fermenting processes in anoxic, organic-rich sediments. The decreasing trend with depth in frequency of occurrence and relative abundance of *Bacteroidetes* bacteria with potential hydrolytic and fermentative capabilities matches the decreasing hydrolysis rates of polysaccharides with depth (26). *Bacteroidetes* bacteria can be classified as copiotrophs and are dominant in environments with high carbon

availability and re-mineralization rates (13). Such an environment exists in the top 10 cm of Namibian shelf sediment.

Gammaproteobacteria: *γ-proteobacteria* occurred throughout the sediment and the clone libraries were dominated by the *γ-2-proteobacteria*, followed by *γ-1-proteobacteria*. These groups included cultured and uncultured representatives with diverse metabolic abilities. At Station 3 the clone libraries were dominated by *γ-proteobacteria* capable of sulfide oxidation, including representatives from the *Beggiatoa-Nevskia-Thioploca* clade and uncultured sulfide oxidizing symbionts. At Station 5 the majority of the *γ-2-proteobacteria* clones were closely affiliated to uncultured members that belong to the sulfide oxidizing family *Ectohiorhodospiraceae*. Up to 80% of the sulfate reduction occurs within the first six centimeters of the sediment and produces hydrogen sulfide (3, 2). A fraction of the sulfide is oxidized by large sulfur bacteria (2, 55), but occasionally, in particular during periods of extreme bottom water nitrate depletion hydrogen sulfide spreads over thousand of square kilometers of the bottom water on the continental shelf.

The higher frequencies of clones (this study) and abundance of active members of *γ-proteobacteria* (26) in the top 10 cm of the sediment can be explained by higher rates of sulfate reduction and thus hydrogen sulfide formation in the surface sediment, which will support a larger population of sulfide-oxidizing *γ-proteobacteria*. Hydrogen sulfide concentrations of 85.6 $\mu mol\ L^{-1}$ were measured in the top 0.5 cm, while higher concentrations of 1100 $\mu mol.L^{-1}$ were measured at a depth of 6 cm (data not shown). This indicates substantial oxidation of hydrogen sulfide. No bacterial mat was observed at Station 3 and a low abundance of large sulfur bacteria, capable of sulfide oxidation were recorded (26). However, previous recordings were made of *Beggiatoa* and *Thiomargarita* mats at these two stations (3, 2). Thus, bacterial sulfide oxidation in the surface sediment is performed by both the large-sulfur bacteria and sulfide oxidizing γ-*proteobacteria* and their degree of

involvement in sulfide oxidation depends on environmental factors such as nitrate and oxygen availability.

Other *γ-proteobacteria* included facultative aerobic fermenters (*Vibrionales* and *Photobacterium*), bacteria degrading high-molecular-weight organic compounds, such as *Colwellia* (24), and methanotrophs (representatives from the *methylococcaceae*).

Deltaproteobacteria: The majority of the *δ-proteobacteria* in the clone libraries was sulfate reducing bacteria, belonging to two families: *Desulfobulbaceae* and *Desulfobacteriaceae*. Active members of these two families were also detected in clone libraries of other marine sediment (41, 11, 51). Full length sequenced clones affiliated to a variety of genera, including *Desulfocapsa, Desulfatibacillum, Desulfosarcina, Desulfobacula* and *Desulfobacterium*. CARD-FISH results with specific probes, targeting these genera, indicated a high activity of these bacteria (26). The high species diversity of SRB in the clone libraries can be explained by their diverse metabolic capabilities (10, 49, 41). They can use a variety of carbon sources, such as different volatile or long-chain fatty acids, alcohols or aromatic compounds (8, 33, 63). The diversity of fatty acids utilized by SRB is reflected in the low concentrations of all the volatile fatty acids (VFA) measured in these sediments, indicating rapid turnover of VFA. The high diversity of SRB throughout the sediment depth is a result of niche partitioning, which is the way in which the different species distribute the available resources in the environment. According to the “niche overlap hypothesis” (47), highly diverse communities arise in environments which are stable over long periods of time as a result of competition-maintained niche diversification.

The diversity of sulfate reducing *δ-proteobacteria* is the same throughout the depth of the sediment, even though up to 80% of the sulfate reduction occurs within the first six centimeters of the sediment, while there is a direct relationship between the abundance of SRB and sulfate reduction rates with depth. The disparity between the diversity and

abundance of SRB with depth indicate that the sulfate reduction rates measured in our earlier study (Table 1, 26), is more related to the abundance of the SRB with depth, than to the diversity of the SRB. The direct relationship between the abundance of SRB and sulfate reduction rates with depth is reflected in the cell specific sulfate reduction rates calculated and discussed in our earlier study (26). The diverse physiological abilities of SRB allow them to occur throughout the sediment, while their absolute abundance is limited by the quantity and quality of substrate available.

Only a fraction of the total population capable of sulfate reduction was counted by CARD-FISH, because sulfate reducing prokaryotes are phylogenetically diverse and are found in both the *Archaea* and *Bacteria* domains (49). It is possible that sulfate reducing *Archaea* also occurred in the top sediment, because the relative abundance of *Archaea* varied between 5-17% of the total DAPI cell counts. This is higher than recorded for other marine sediments (50, 32). We propose that *Archaea* with sulfate reducing abilities will play a greater role in the sediment deeper than 10-12 cm, where the abundance of active SRB decrease, since SRB and *Archaea* compete for the available substrate. The diversity and role of sulfate reducing *Archaea* in upwelling sediments should be assessed.

Planctomycetes: Blast results from cloned full length sequences that are affiliated to the *Planctomycetes* revealed that they are related to uncultured members and the genera *Pirellula* and *Candidatus* "Scalindua brodae" (AY254883.1). *Planctomycetes* can oxidize organic substrates through nitrate reduction (15) and *Pirellula* is capable of heterolactic acid fermentation (16). *Candidatus* "Scalindua brodae" is capable of anaerobic ammonium oxidation (ANAMMOX) (28). Although cloned sequences of this genus in the water column of the Namibian shelf were previously reported (64, 30), this is the first report of sequences from bacteria related to the genus *Candidatus* "Scalindua brodae" in Namibian shelf sediments. The high abundance of active *Planctomycetes* in the top 2-4 cm of the sediment

(24% of the total DAPI stained cells) suggests that these bacteria play a significant role in the oxidation of organic substrates, and are probably also involved in ANAMMOX within the top sediment. Further investigations to establish the identity, abundance and ecological role of these bacteria are required.

Hydrographic influence on community richness and diversity

Both sampling stations occur in a highly productive part of the Benguela upwelling system, but differences in the metabolic activity of the microorganisms involved in organic carbon mineralization were observed (26). The differences in biogeochemical rates and bacterial abundance between the two sampling stations were attributed to differences in hydrographic features, such as current strength and direction, which cause enhanced sediment reworking at the shallower Station 3 (26). This has an effect on bulk sediment composition, affecting the amount of particulate organic carbon and nitrogen within the sediment at the two stations. At both sampling stations the top 5 cm of the sediment was well mixed where the highest rates of hydrolysis and sulfate reduction were observed (Table 1), but the sediment composition differed between the two stations below a depth of 5 cm. The effect of different hydrographic conditions at the two stations is also reflected by the results from the clone libraries, revealing a difference in microbial community structure between the two stations.

The phylogenetic richness, diversity and evenness at the two sampling stations differed significantly. In contrast to Station 5, phylotype evenness was higher at Station 3 with no significant dominance of a particular group of *Bacteria*. Therefore, at Station 3 bacterial diversity was influenced more by the number of different phylotypes than by the distribution of individuals among species. We believe that the differences in phylogenetic richness, diversity and evenness between the two sampling stations result from real differences in the

microbial community structure and activity as well as the quantity and quality of DOC in the sediments. The higher phylogenetic diversity in the top 6 cm of the sediment at Station 3 can be explained by a greater sharing of the available carbon sources, resulting in niche diversification and therefore a higher bacterial diversity. At Station 5 the available resources are shared less efficiently, resulting in a lower amount of ecological niches and therefore a lower diversity. There exist a de-coupling of initial and terminal processes involved in organic carbon mineralization and a greater accumulation of increasingly refractory DOC and carbohydrates with depth at Station 3 compared to Station 5 (26). The higher amount of refractory organic carbon in the deeper sediment (10-12 cm) at Station 3 makes it an environment with limited carbon resources. Competition for the limited, accessible DOC within the deeper sediment at Station 3 may lead to niche specialization and diversification, resulting in higher phylogenetic diversity. Microbial response to the available DOC in the sediment depends on the physiological capabilities of the microorganisms (36), which ultimately determines which microorganisms can co-exist successfully within the environment.

In contrast to phylogenetic diversity and richness, the absolute prokaryote abundance in the top 4 cm of the sediment was similar at both sampling stations. The difference in absolute cell number between the two stations was only observed for the sediment below 4 cm depth, where Station 5 had a higher abundance of prokaryote cells. The more gradual decrease in absolute abundance of prokaryotes at Station 5 compared to Station3, match the gradual decreasing biogeochemical rates at Station 5, indicating that the rate of biogeochemical reactions involved in organic carbon mineralization is directly related to the absolute abundance of bacteria.

Conclusions

Microbial communities in organic-rich sediments on the Namibian shelf are highly diverse and microbial community structure varies horizontally on the shelf and with depth. Hydrographic factors affect the microbial community structure within Namibian shelf sediments indirectly through its effect on sedimentation, the sediment composition and characteristics, and ultimately the quantity, quality and accessibility of DOC to microorganisms. The differences in microbial community structure and diversity between the two sampling stations are a manifestation of ecophysiological requirements of the bacteria, in particular with regard to the availability of DOC substrates.

Variations in organic carbon substrate variability with depth produce vertical gradients, presenting niche variability, which dictates how the available energy resources are distributed, resulting in niche diversification by microorganisms. This results in vertical stratification of taxonomic lineages with depth, where some phylotypes are restricted to the top 2 cm of the sediment, while others occur only in the bottom 10-12 cm. Most of the phylotypes occurred throughout the sediment depth, but their dominance within the clone libraries change with depth. This vertical stratification was greater at the distal station (Station 5), which had lower phylogenetic diversity and evenness compared to the shallower Station 3. We propose that organisms with copiotrophic characteristics thrive in the surface 6 cm of the sediment, whereas oligotrophic-type organisms thrive in the deeper sediments.

Our study revealed the identity and abundance of microorganisms involved in the different steps of organic carbon mineralization and in the oxidation of sulfide in these sediments. The organisms involved in hydrolytic and fermenting processes include members from the *γ-proteobacteria, Bacteroidetes, Actinobacteria, Firmicutes*, *Acidobacteria, Thermomicrobia* and the OP8, WS3 and TM6 clusters. An interesting finding of our work is the presence of active members of *Actinobacteria* in the sediment, since they may be critical

role players in controlling the degradation of complex organic molecules and ultimately organic carbon burial sediments.

Organisms involved in sulfate reduction were also diverse and involved mainly cloned sequences affiliated to the *δ-proteobacteria*, but probably also members from the domain *Archaea*. We propose that there is a direct relationship between the abundance of SRB and sulfate reduction rates with depth, while there is no relationship between the diversity of SRB and sulfate reduction rates with depth. This needs to be verified by correlations studies, which is beyond the scope of our study.

Microorganisms in the clone libraries with sulfide-oxidizing capabilities included representatives from heterotrophic *α-proteobacteria* and *γ-proteobacteria*. In particular clone libraries in the top and middle sediment were dominated by uncultured sulfide oxidizing representatives from the *γ-2-proteobacteria*. Previous observations of large sulfur bacteria at the two sampling stations suggest that both the smaller *γ-proteobacteria* and the large sulfur bacteria play a role in sulfide oxidation and the extent to which they are active may be related to the amount of sulfide, nitrate and oxygen present in the bottom water.

Our study revealed that microbial communities in organic rich sediments are structured by environmental factors, such as hydrographic factors, sediment composition and the quantity and quality of DOC as well as biological factors, such as the physiological abilities of organisms and competition among organisms, resulting in niche partitioning. We recognize that the physiology of most of the microorganisms are poorly understood and that other factors such as predation may also be selective factors structuring microbial communities, however, this initial survey of microbial community diversity in Namibian upwelling sediments is the first attempt to improve our understanding of the microbial ecology of such organic-rich sediments.

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Chapter 4

Microbial response to substrate addition during anaerobic degradation of organic carbon in Namibian shelf sediment

(In preparation)

Julies Elsabé M. *[1,4], Le Loup Julie[1,3], Kohls Katharina[1], and Brüchert Volker[1,2]

[1]Max Planck Institute for Marine Microbiology, Bremen, Germany

[2]Department of Geology and Geochemistry, Stockholm University, Sweden

[3]Laboratoire d'Ecologie et Biogéochimie des Millieux Continentaux, France

[4]Department of Biology, University of Namibia, Windhoek, Namibia

Abstract

We investigated the carbon degradation rates, response time, and temporal evolution of anaerobic bacteria in anoxic organic-rich sediment from the Namibian shelf when supplemented with high molecular weight (HMW) carbohydrates and low molecular weight (LMW) carboxylic acids. A continuous flow-through setup was used to amend sediment with laminarin as model polysaccharide. Denaturing gradient gel electrophoresis (DGGE) allowed us to monitor the temporal evolution of the microbial community composition. In a different experiment, different concentrations of the carboxylic acids acetate and lactate (25 μM, 50 μM, 100 μM, 500 μM, 1 mM, 5 mM, and 10 mM) were injected into undisturbed sediment cores and metabolic turnover was studied by measuring ^{35}S-sulfate reduction rates. Addition of laminarin resulted in a significant (up to 6 fold) stimulation of the metabolic activity of microorganisms. The fermenting bacteria that responded the fastest to laminarin addition belonged to the *Bacteroidetes* phylum. Both the addition of LMW and HMW organic substrates stimulated sulfate reduction rates, but the response was weaker with increasing depth. A DNA clone library based on the DSR sequence bands suggested the dominance of comparably fast-growing incomplete oxidizing SRB in the top 2 cm, while slower growing SRB become dominant in the deeper parts of the sediment. The combined information from these experiments suggests a differential response of the microbial community to substrate addition. Whereas the surface sediment is dominated by bacteria capable of accelerating hydrolysis and carbon oxidation rates in response to enhanced organic matter flux, bacteria present in sediment buried to only 14 cm depth exhibit a slower response.

Introduction

Particulate organic matter (POM) that reaches the sediment after settling from the water column is converted into high molecular weight (HMW) dissolved organic matter (DOM) by exoenzymatic hydrolysis and eventually to low-molecular weight (LMW) - DOM by further hydrolysis and fermentation. The products of hydrolysis and fermentation are LMW dissolved organic carbon (DOC) and serve as energy and carbon sources for sulfate reducing bacteria (SRB). These include volatile fatty acids (VFA) such as lactate, acetate, formate, propionate, butyrate, valerate and other end products such as alcohols, aromatic compounds and, hydrogen (Middelburg et al. 1993; Parkes et al. 1989). The degradability of DOM declines with sediment depth with increasing decomposition and diagenetic alteration (Cowie and Hedges 1994). The progressive decrease of organic carbon reactivity is described by the multi-G model (Berner 1980), which describes the first order decomposition of organic matter in marine sediments and suggests that the in situ rate of sulfate reduction is organic-matter limited. Sulfate reduction reflects the rate of organic carbon turnover and represents a direct measure of the availability of DOC to microbial degradation (Westrich and Berner 1984). Biopolymeric materials, such as cellulose, lignin, carbohydrates and proteins differ in their reactivity with regard to bacterial decomposition and their biodegradability also depends on the freshness of the organic matter (Westrich and Berner 1984).

In areas of high productivity, which includes the Benguela upwelling system off the Namibian coast, brief pulses of increased sedimentation of highly reactive organic matter is experienced after the die out of phytoplankton blooms (Takii et al. 2002). Occasional pulses of highly reactive, complex organic substrates associated with algae blooms affect microbial activity in marine sediments (Takii et al. 2002; Roselló-Mora et al. 1999; Graf et al. 1983). The faster turnover of organic carbon by hydrolysis compared to turnover by sulfate reduction in the deeper sediments (below 10 cm depth) in Namibian shelf sediments was

explained by different modes of response of hydrolytic-enzyme producing bacteria and SRB to carbon input into the sediment at different depth intervals (Julies et al. submitted – Chapter 2). Up to 50% of the organic carbon remineralization in marine sediments occurs through sulfate reduction (Canfield et al. 1993; Jørgensen 1982). Although most SRB belong to the *δ-proteobacteria* subclass, they can be classified into two broad groups based on their nutrition. Incompletely oxidizing SRB utilizes lactate, hydrogen, and propionate as typical substrates, producing acetate as an end-product, while completely oxidizing SRB are capable of utilizing a variety of VFA which they can oxidize to acetate (Knoblauch et al. 1999).

The aim of this study is to investigate the potential response of microorganisms to a sudden high input of HMW- DOC into the sediment and the subsequent increase in LMW-DOC. In Namibian shelf sediments, up to 90% of the sulfate reduction occurs in the top 5-10 cm of the sediment (Julies et al. submitted, Chapter 2; Brüchert et al. 2006). We hypothesize that the SRB communities in the top 10 cm of the sediment consist of fast growing organisms capable of a rapid response to a sudden increase in substrate availability. Since incomplete oxidizing SRB are faster growing organisms compared to complete oxidizers (Rabus et al. 2006; Gräber and Milazzo 1997), the SRB in the top 10 cm of the sediment include incomplete oxidizing bacteria whilst, the SRB community in the sediment below 10 cm depth contain mainly slow growing SRB, capable of complete oxidation. We tested this hypothesis by the separate addition of lactate and acetate, at different concentrations to intact cores, in the top sediment (1 cm and 5 cm depth) and the bottom sediment (15 cm depth). Lactate is a typical substrate for active incompletely oxidizing SRB, while acetate is a substrate for completely oxidizing SRB (Rabus et al. 2006; Knoblauch et al. 1999).

In addition we examined the response of microorganisms at two different depth intervals, 0-6 cm (surface) and 14-16 cm (bottom), to a high input of dissolved carbohydrates, which simulates a high input of highly reactive organic carbon after a phytoplankton bloom that

follows an upwelling event. The emphasis was on carbohydrates, because they constitute a large fraction (64-97%) of the HMW-DOM in surface coastal marine sediments (Julies et al. in prep.; Arnosti and Holmer 1999; Cowie and Hedges 1994). The polysaccharide laminarin is a soluble β-1,3-D-glucose polymer, the main storage glucan of diatoms (Alderkamp 2006) and laminarinase activity has been detected in Namibian shelf sediments (Julies et al. submitted – Chapter 2), other marine sediments (Arnosti 1995; Hoppe et al. 2002; Arnosti and Jørgensen 2006) and in marine bacteria (Wainwright 1981; Brown et al. 1990; Antranikian 1992; Arnosti and Repeta 1994; Alderkamp et al. 2007). Therefore, we added laminarin as a model substrate to simulate a high input of carbohydrates into the sediment.

Materials and methods

Study site and sediment sampling

Sediment was collected with a multi-corer (MUC) on board the *RV Alexander von Humboldt* in March 2004 from Station 5 (23°45.08S, 14°18.27E) for the experiment investigating the effects of LMW-DOC addition, and from Station 13 (23°05.150 S, 14°16.13 E) for the experiment investigating the effects of HMW-DOC addition. The characteristics and in-situ measurements of concentrations and rates at the sampling stations are shown in Table 1.

At Station 5 sub-cores were taken from the MUC as described by Brüchert et al. (2003) and LMW-DOC (lactate and acetate) was injected into intact cores. Sub-cores were collected from the same cast. At Station 13 sediment at the depth intervals of 0-6cm and 14-16 cm were collected from several MUC cores into separate gas-tight jars and stored at 4°C. The sediment from one depth interval was pooled and transferred into the flow cells in a glove bag with a nitrogen atmosphere.

Table 1: Locations and characteristics of sampling stations.

		Station 5	Station 13
Coordinates		23°45.08S, 14°18.27E	23°05.150 S, 14°16.13 E
Depth (m)		112	100
Sampling time		6 March 2004, 9:00 h	12 March 2004, 15:h00
Bottom water temperature (°C)		11.8	13.2
Bottom water oxygen (ml L^{-1})		< 0.1	0.1
Porosity (%):			
	0-2 cm	93	92
	4-6 cm	93	89
	10-12 cm	87	89
DOC (mM):			Not measured
	0-2 cm	1.6	
	4-6 cm	1.5	
	10-12 cm	1.8	
Sulfate conc. (mM):			
	0-2 cm	21.7	24.9
	4-6 cm	15.2	18.4
	10-12 cm	16.2	3.2
Average sulfate reduction rate (nmol cm^{-3} d^{-1})			
	1 cm	34.3	124.1
	0-2 cm	49.4	128
	4-6 cm	15	67
	5 cm	42	78.8
	10-12 cm	4	47
	15 cm	9.6	17.1
VFA (µM)			Not measured
lactate:	1 cm	3	
	5 cm	2	
	15 cm	close to detection limit	
acetate:	1 cm	13	
	5 cm	6	
	15 cm	5	

Experiment 1: The effect of LMW-DOC addition on microbial activity at Station 5

A total of 15 sub-cores were collected at Station 5. Into 14 of these sub-cores substrate (acetate or lactate) was injected into 3 different depths, 1 cm, 5 cm, and 15 cm. One core served as control into which no substrate was injected to measure in situ sulfate reduction rates. Into 7 of the cores acetate was injected at seven different concentrations: 0.025 mM,

0.05 mM, 0.1 mM, 0.5 mM, 1 mM, 5 mM, and 10 mM. In the remaining 7 cores lactate was injected at the same concentrations as for acetate. After substrate injection, the cores were pre-incubated for 24 h at 12°C. Thereafter, the effect of substrate addition on sulfate reduction rates was measured using the whole core $^{35}SO_4^{2-}$ radiotracer incubation method (Jørgensen 1978). Into each of the three depths, 2 µL $^{35}SO_4^{2-}$ radiotracer (80 kBq) was injected through silicon sealed ports. The cores were then incubated for a further 5 h at 12°C in the dark and then sectioned in 1 cm intervals and the sediment was preserved as described by Brüchert et al. (2003). Sulfate reduction rates were measured by the one-step acidic chromium reduction method of Kallmeyer et al. (2004). Reduced ^{35}S and $^{35}SO_4^{2-}$ were counted on a Canberra-Packard 2400 TR liquid scintillation counter (Packard Ultima Gold XR scintillation fluid).

Experiment 2: The effect of HMW-DOC addition on microbial activity at Station 13

Flow-through cells through which artificial seawater (ASW), amended with the polysaccharide laminarin was pumped, were used to investigate the effect of HMW-DOC addition on the microbial community at two depth intervals (0-6 cm and 14-16 cm) in sediment collected from Station 13. The major advantages of using flow-through cells include less manipulation of the sediment structure and easy sampling of the inflow and outflow (Brüchert and Arnosti 2003; Roychoudhury et al. 1998; Dixit et al. 2001). The flow cells were polycarbonate cylinders (diameter 47 mm) with a removable cap at each end. The total volume of each cell was 34.8 ml. The influent entered the flow cell through a 1 mm-wide hole in the center of the one cap. It was then distributed in radial grooves from the center of the cell across the whole diameter of the cell. Then it passes through 0.45 µm pre-combusted Whatman GF/C filters and then through the Teflon frits before it reaches the sediment. The effluent left on the opposite side in a similar path. The outflow was collected

in pre-combusted glass vials for VFA, total dissolved carbohydrates, and DOC analysis. For sulfate and sulfide determination, the outflow was collected in 100 mL cryo vials containing 5 mL of 5% (w/v) zinc acetate to precipitate hydrogen sulfide.

The effect of laminarin addition was tested for the two different depth intervals in two separate experimental runs. During the first experimental run sediment from the depth interval 0-6 cm (top sediment) was placed in four separate flow cells. Effluent from two of the flow cells were collected for sulfate and sulfide measurements (cells c and d, Fig. 1), while the effluent from the remaining two flow cells were collected for measurements of the other pore water constituents (cells a and b, Fig. 1). During the second experimental run the same setup was followed, but the four flow cells were filled with sediment from the 14-16 cm depth interval (bottom sediment).

Filtered, autoclaved artificial seawater (ASW) medium was pumped through the sediment cells with a Sykam 1021 HPLC pump with PEEK pump head. The composition of the ASW medium was after Widdel and Bak (1992). Anoxic conditions were maintained throughout the experiment by a N_2 overpressure in the influent. The pH of the influent was measured before and after each experimental run and was 7.2. The cells were placed in a thermostat water bath that maintained the temperature at 12°C. Each cell was connected to the ASW medium with PEEK tubing. The flow rate maintained was 2-3 $ml.h^{-1}$. Initially only ASW was pumped through the cells for a period of 72 h. After 72 h, laminarin was added to the ASW to a final concentration of 8 mM C monomer equivalents. Samples were taken from the inflow at the beginning of the experiment to determine the concentrations of sulfate, sulfide, dissolved carbohydrates, DOC and volatile fatty acids (VFA).

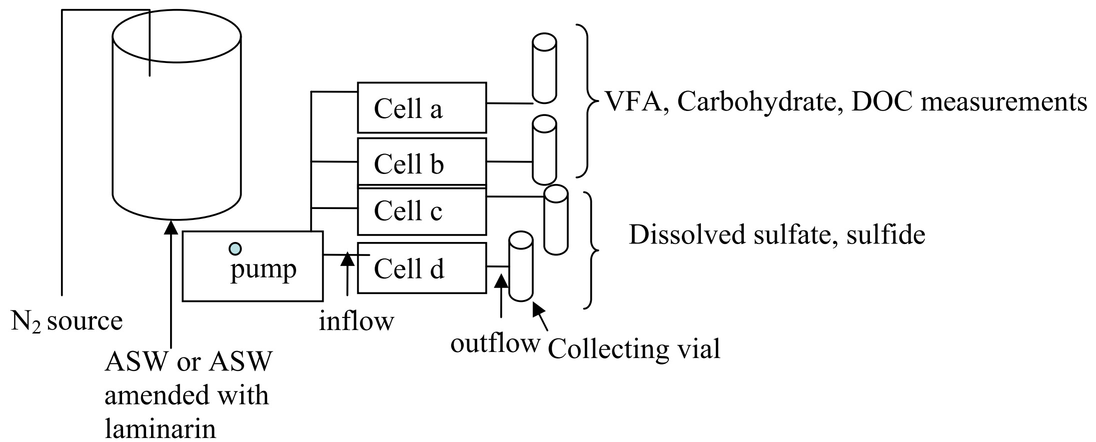

Fig 1: Diagram illustrating the experimental setup during both experimental runs. 4 cells were used per run. Two of the collecting vials were cryo vials used to collect outflow for sulfate and sulfide measurements from cells c and d, while two vials were pre-combusted glass vials to collect outflow from cells a and b for the measurement of the other pore water constituents.

Samples were taken from the cumulative effluent collected over a 24 h period. Table 2 shows the sampling strategy from the flow cells. The total volume of outflow was measured at the time of sampling to calculate the exact flow rate. After samples were taken from the outflow, new vials were used for collecting the outflow for the next 24 h period. The preservation and analytical methods used to measure the concentration of the various pore water constituents are summarized in Table 3.

Table 2: Sampling strategy for the flow cell experimental setup with sediment from Station 13.

Sediment depth (cm)	Time points during experimental run (h)	Flow cells from which samples were taken	Analysis performed on sample
0-6	0	Taken before the start of the experiment.	Pore water for VFA, DOC, carbohydrates, sulfate, and sulfide analysis. Sediment for DGGE
	3, 6, 9	Cells a and b	VFA, DOC, carbohydrates
		Cells c and d	Sulfate and sulfide
	24	Cells a and b	VFA, DOC, carbohydrates
		Cells c and d	Sulfate and sulfide
		Cell b (terminated)	Sediment for DGGE
	48, 72, 96, 108, 132	Cell a	VFA, DOC, carbohydrates
		Cells c and d	Sulfate and sulfide
	156	Cell a	VFA, DOC, carbohydrates
		Cells c and d	Sulfate and sulfide
		Cell c (terminated)	Sediment for DGGE
	180	Cell a	VFA, DOC, carbohydrates
		Cell d	Sulfate and sulfide
	204	Cell a	VFA, DOC, carbohydrates
		Cell d	Sulfate and sulfide
		Cells a and d (terminated)	Sediment for DGGE
14-16	0	Taken before the start of the experiment.	Pore water for VFA, DOC, carbohydrates, sulfate, and sulfide analysis.
	24, 48, 72	Cells a and b	VFA, DOC, carbohydrates
		Cells c and d	Sulfate and sulfide
	96	Cells a and b	VFA, DOC, carbohydrates
		Cells c and d	Sulfate and sulfide
		Cell b (terminated)	Sediment for DGGE
	168	Cell a	VFA, DOC, carbohydrates
		Cells c and d	Sulfate and sulfide
		Cell c (terminated)	Sediment for DGGE
	192, 216	Cell a	VFA, DOC, carbohydrates
		Cell d	Sulfate and sulfide
	240	Cell a	VFA, DOC, carbohydrates
		Cell d	Sulfate and sulfide
		Cell a and d (terminated)	Sediment for DGGE

Table 3: Preservation and analytical methods used during the determination of different pore water constituents collected in the outflow.

Pore water constituents	Preservation of outflow collected	Analytical method	Reference
VFA	-20°C	2-nitophenylhydrazide derivatization and HPLC	Albert and Martens (1997) modified by Arnosti et al. (2005)
SO_4^{2-}	Outflow collected in 5 mL ZnAc (5% w/v) – kept at 4°C	Non-suppressed anion exchange chromatography	Ferdelman (et al., 1997)
H_2S	Outflow collected in 5 mL ZnAc (5% w/v) – kept at 4°C	Spectro-photometrically	Cline (1969)
Dissolved Carbohydrates	-20°C	Spectro-photometrically with the phenol-sulfuric acid method.	Chaplin and Kennedy (1986)
DOC	-20°C	High temperature catalytic oxidation with a total organic carbon analyzer (TOC-5050A – Shimadzu) and infra red detection of evolved CO_2	
Br-	4°C	HPLC	Brüchert and Arnosti (2003)

Determination of break-through time

The exchange time for the pore water volume in the sediment was determined by the addition of 2 mM sodium bromide to the influent of two of the flow cells during the experimental run with sediment from the 14-16cm depth interval. Samples were taken every 24 h and concentrations of bromide were measured by HPLC as described in Brüchert and Arnosti (2003).

Biogeochemical rate calculations

Rates (μM d^{-1}) of the various biogeochemical reactions involved in initial and terminal metabolism of carbon remineralization during the two experimental runs were calculated from the linear change in concentration over time with the following equation:

$$\mathbf{R_p = Q/V \times (C_{out} - C_{in})} \quad \mathbf{(1)}$$

$$\mathbf{R_c = Q/V \times (C_{in} - C_{out})} \quad \mathbf{(2)}$$

Where: R_p = Rate of VFA, H_2S, and DOC production (μM d^{-1})

R_c = Rate of SO_4^{2-} consumption (μM d^{-1})

Q = Flow rate per day (ml d^{-1})

V = Pore water volume per cell (mL) = (volume of cell) x (porosity)

C_{out} = Concentration in outflow (μM)

C_{in} = Concentration in inflow (μM)

Molecular analysis of the response of microorganisms to substrate addition

Sediment samples for molecular analysis were taken from the flow cells at various time intervals and the sediment was immediately frozen until DNA extraction was performed. For the sediment from the depth interval (0-6 cm), samples were taken at the beginning of the experiment (0 h), and then from the flow cells after 24, 156 and 204 h. For the experimental run with sediment from the bottom (14-16 cm), samples were taken after 96, 168 and 240 h. This meant that at the time of taking the sediment sample from the flow cell, the experiment for that particular flow cell was stopped (Table 2).

Denaturing gradient gel electrophoresis (DGGE) was used as the genetic fingerprinting technique to study the change in bacterial genetic diversity during the two experimental runs, because it is a rapid method that allows for the analysis of numerous samples (Muyzer 1999; Muyzer and Smalla 1998). Total community DNA was extracted (BIO 101 Fast DNA spin

protocol) directly from 0.5 g wet weight of sediment from the different samples that were taken at different time points.

Two separate PCR reactions were run with:

(a) The bacterial primers GM5F (with GC-clamp) and 907 RC (Muyzer et al. 1995)

The amplification was performed in a total volume of 100 µL (~ 80 ng), containing 1 U of Taq polymerase (5 U/µL, Eppendorf), TaqMaster PCR Enhancer solution (5x, Eppendorf), Taq buffer (10x Eppendorf), dNTP (0.2 mM final, Eppendorf), BSA (120 µM final, Eppendorf), and 50 µM final concentration of each primer. The PCR was run on a Master-cycler (Eppendorf) and a touchdown program with a total number of 20 cycles was used in order to minimize nonspecific amplification (Don et al. 1991; Muyzer et al. 1995). Annealing temperature for the primer GM5F (with GC-clamp) was 55°C and for the primer 907 RC it was 58°C.

(b) The primers Dsr-1-F-GC clamp (5'-ACS-CAC-TGG-AAG-CAC-G-3') and Dsr1-DGGE-Rev (5'-CGG TGM AGY TCR TCC TG-3') (adapted from Dhillon et al 2003).

The PCR mastermix consisted of 2.5 µL of 10 x Taq-polymerase buffer, 2.5 µL dNTPs, 1 µL bovine serum albumin (3 $mg.ml^{-1}$), 2 µL $MgCl_2$, 0.3 µL of Taq polymerase (5 $U.\mu l^{-1}$), 5 µL PCR enhancer (5 x), 0.25 µL of each of the primers (10 µM), 2 µL (50 ng) of DNA extract and 9.2 µL ultrapure, sterile water per reaction. The primers Dsr-1-F-GC clamp (5'-ACS-CAC-TGG-AAG-CAC-G-3') and Dsr1-DGGE-Rev (5'-CGG TGM AGY TCR TCC TG-3') amplify the dissimilatory sulfite reductase (Dsr) gene of sulfate reducing bacteria (SRB). The PCR was run on a Master-cycler (Eppendorf) the conditions were: an initial cycle at 94°C for 3 minutes followed by 30 cycles consisting of three steps, the first step at 94°C for 40 seconds then an annealing step at 55°C for 40 seconds and a third step at 72°C for 1 minute. The final cycle was run at 72°C for 10 minutes. Primers were synthesized by Biomers Company. PCR products were inspected by gel electrophoresis on 1% (w/v) agarose gels.

A maximum of 76 µL (~500 ng) of the PCR products with the primers Dsr-1-F-GC clamp (5'-ACS-CAC-TGG-AAG-CAC-G-3') and Dsr1-DGGE-Rev (5'-CGG TGM AGY TCR TCC TG-3') was mixed with 10 µL loading buffer and then loaded on the gel prepared for DGGE. Between 20 and 100 µL, (~800 ng) of the PCR products with the primers GM5F (with GC-clamp) and 907 RC was mixed with 10 µL loading buffer and loaded on the gel for DGGE. DGGE was performed as described by Muyzer et al. (1993; 1995), using a Bio-Rad D-Code system and was run at 60°C at a constant voltage of 200 V for 3.5 h. A denaturant gradient containing 20-80% urea-formamide was used. After electrophoresis and staining with ethidium bromide (0.5 mg/L), bands were manually excised and eluted in 30 µL sterile water. The bands were re-amplified using the same primers mentioned above without a GC-clamp. Sequences of re-amplified bands were submitted to the EMBL nucleotide sequence database (www.ebi.ac.uk/embl) with accession numbers EUO35605 - EUO35610 and AM697649 - AM697665.

Results

Experiment 1: The effect of LMW-DOC addition on microbial activity at Station 5

In this experiment we compared the absolute sulfate reduction rates at 29 h after substrate addition. In the top sediment (1 cm depth) lactate addition resulted in a greater stimulation of sulfate reduction compared to acetate addition (Fig. 2 a). The addition of low concentrations of lactate (0.025 mM) resulted in sulfate reduction rate that is 10% higher than in the control. The stimulation of sulfate reduction increased with increasing concentrations of lactate and the maximum stimulation of sulfate reduction was observed at the highest concentration of lactate added (10 mM). At 5 cm depth the stimulation effect of sulfate reduction by acetate and lactate addition did not differ significantly (Fig. 2b). Higher concentrations (10 mM) of both LMW-DOC compounds were required to increase sulfate reduction rate by 10-20% of

that in the control. At 15 cm depth a greater stimulation of SRB by acetate addition compared to the addition of lactate was observed (Fig. 2c). The addition of 0.5 mM acetate produced a 6 fold increase in sulfate reduction rates relative to the control. The maximum stimulation effect of sulfate reduction by acetate was observed with the addition of 1 mM acetate. Concentrations of acetate exceeding 1 mM have an inhibitory effect on SRB, which is reflected in the decrease in sulfate reduction rates (Fig. 2c).

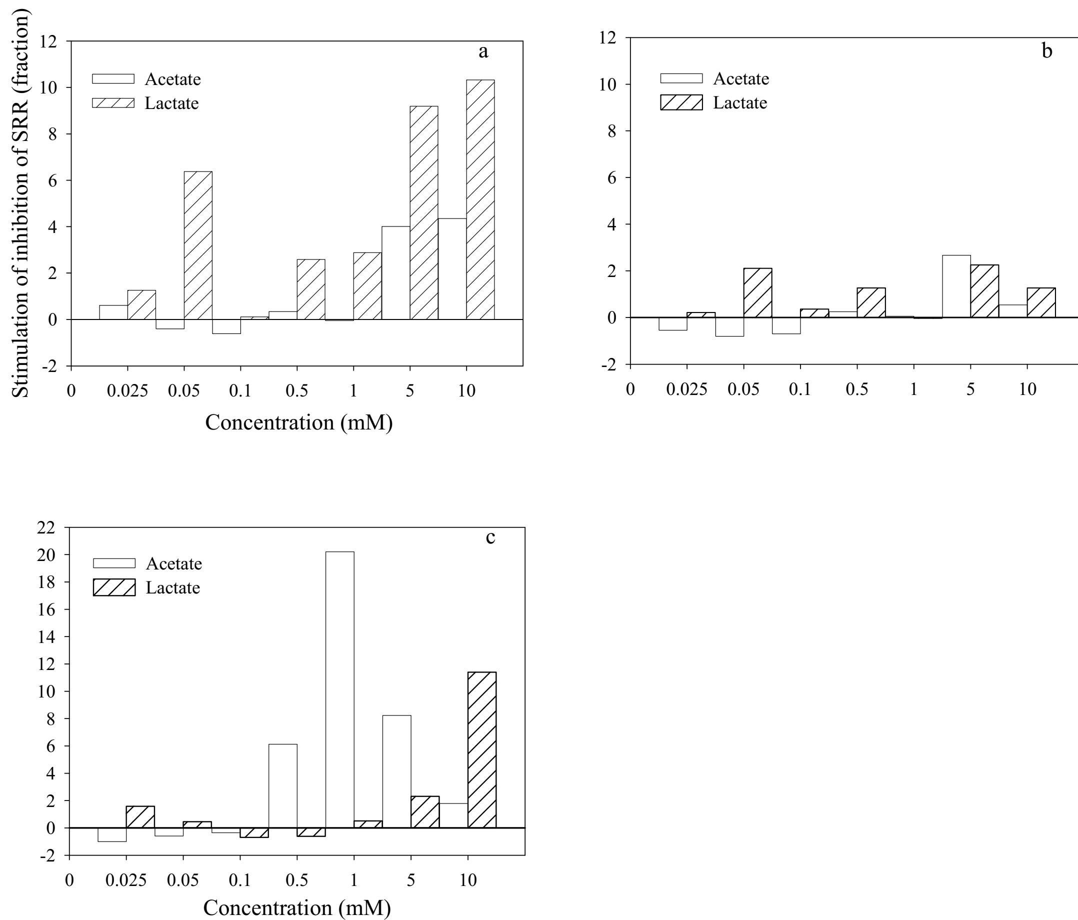

Fig. 2: The stimulation of sulfate reduction at different depths, a) 1 cm, b) 5 cm, and c) 15 cm, by the addition of different concentrations of lactate and acetate expressed as a fraction that has been calculated by dividing the sulfate reduction rate after substrate addition by the in-situ sulfate reduction rate measured in the control core.

Core heterogeneity may be responsible for the lower than in situ sulfate reduction rates when low substrate concentrations (less than 0.1 mM) were added and is not due to

inhibitory effects on sulfate reduction as it appears in Fig. 2a-c. We assume that the leakage of substrate into adjacent sections that were not injected is low and can be neglected, based on results from previous substrate amendment experiments using intact cores (Julies submitted, Chapter 1). In addition the sulfate reduction rates in un-injected sections of the core did not differ significantly from those in the control core (results not shown).

Experiment 2: The effect of HMW-DOC addition on microbial activity at Station 13

Although VFA, DOC, carbohydrates, dissolved sulfate, and sulfide were measured in the outflow of all the flow cells in the initial stages of the experiment, we only report the data for cells a and d, because the other two flow cells (b and c) were terminated during the course of the experiment to sample sediment for molecular analysis (Table 2). Averages were calculated for measurements performed on duplicate cells (cells a and b for VFA, DOC, and carbohydrate analysis, and cells c and d for dissolved sulfate and sulfide analysis) during the first 24 h and 96 h in the 0-6 cm and 14-16 cm depth intervals, respectively. The standard deviations were low (between 1% and 16%), indicating homogeneity of the sediment within the different flow cells.

Break-through time in the flow cells

The flow rate maintained throughout both experimental runs varied from 2 to 3 ml h^{-1}. At this flow rate the pore water in the sediment from the bottom sediment was replaced after 96 h with the influent (Fig. 3). A similar breakthrough time can be expected for the top sediment, because the porosity of the sediment, and therefore the amount of pore water was similar at both depth intervals (Table 1). Between the times the experiment was started (0 h) and 96 h after the ASW started flowing into the cells, there was a mixture of ASW and the original pore water in the cells. Laminarin as substrate was added after 72 h and therefore it

can be expected that the ASW amended with laminarin replaced the pore water, consisting mainly of ASW in the cells after an additional 96 h. In between the times of laminarin addition and the 168 h time point, there was a mixture of ASW and laminarin in the flow cells. Thus, the concentration of laminarin in the cells gradually increased. Therefore, it is sensible to compare only the metabolic rates after the 168 h time point, and rates are shown only for the time period after the 168 h time point. Bromide concentration in the outflow was measured only for 96 h in cell b (Fig. 3), because the sediment was removed from this cell at the 96 h time point for molecular analysis.

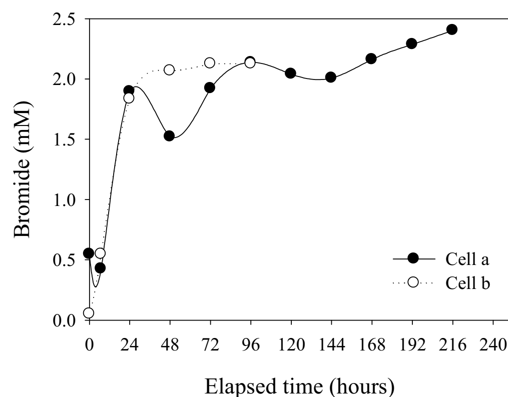

Fig. 3: Bromide breakthrough time for a flow rate between 2-3 $ml.h^{-1}$. 2 mM Bromide was added to the inflow and collected from the outflow of cells a and b during the second experimental run with sediment from a depth of 14-16 cm at Station 13.

Volatile fatty acids (VFA)

In the top sediment (0-6 cm) the concentration of acetate was 18 µM at the start of the experiment, while the concentrations of all the other VFA were below detection limit. The concentrations of individual VFA started increasing 24 h after laminarin was added. The most abundant VFA in the outflow were acetate and formate. The rate of formation of all the other VFA remained below 15 µM d^{-1}. Acetate concentrations after laminarin addition reached up to 20 µM, initially and then started declining after the 180 h time point (108 h after laminarin

addition) (Fig. 4a). Since no acetate was detected in the inflow, concentrations reflect the difference between production and consumption by the microbial populations in the sediment.

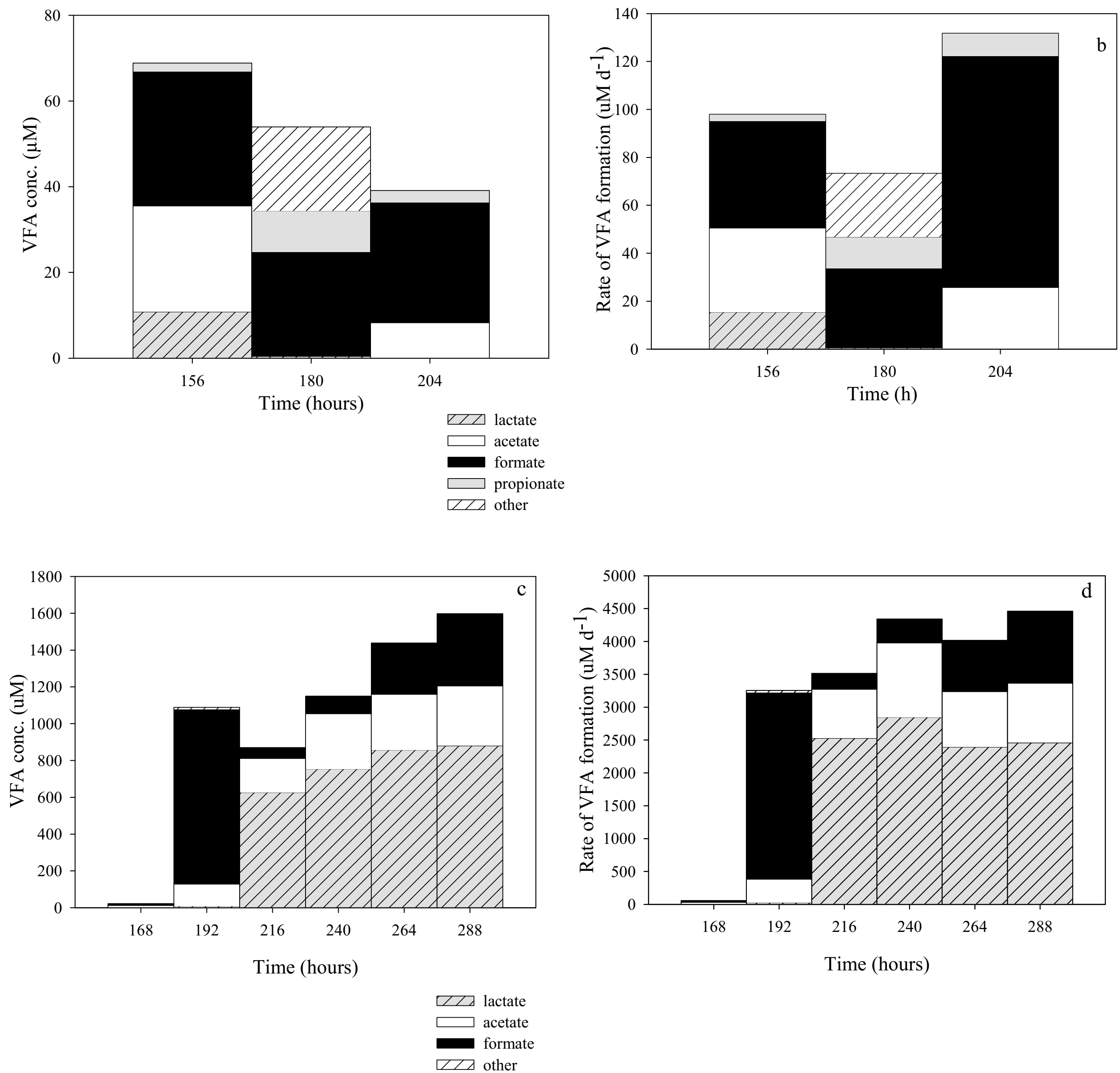

Fig. 4: VFA concentrations (concentration in inflow- concentration in outflow) with sediment from a) 0-6 cm depth, and c) 14-16 cm depth at Station 13. (b) and (d) show the rate of VFA formation in the sediment from 0-6 cm, and 14-16 cm depths, respectively.

In the bottom sediment (14-16 cm), an increase in VFA concentrations and therefore, VFA formation rate was observed after the 192 h time point (120 h after laminarin addition) (Fig. 4c,d). This increase was 20 times higher than in the surface sediment. Formate, lactate and acetate had the highest rates of formation after the addition of laminarin (Fig. 4d). While concentrations of lactate in the top sediment were lower than 10 µM, it was much higher in the depth interval 14-16 cm (6-8 mM) (Fig 4a,c). The concentration of all the VFA in the outflow before the addition of laminarin did not exceed 2 µM.

Dissolved organic carbon and total dissolved carbohydrates

Carbohydrate concentrations (difference between concentration in the inflow and outflow) in the controls, before laminarin addition did not exceed 1 mM monomer equivalents (~0.2 mM C), which is approximately 20% of the DOC concentration. Following laminarin addition, the contribution of dissolved carbohydrates to the DOC concentration in the top 6 cm increased gradually to reach ~88%, while it was only 0.1% in the 14-16 cm depth interval (Fig 5 a,b). After breakthrough DOC concentrations in the top 6 cm decreased gradually from ~28 mM monomer equivalents (4.7 mM C), indicating a net consumption of DOC (Fig. 5a). In contrast the DOC concentrations in the 14-16 cm depth interval increased slowly from 58 mM monomer equivalents to ~69 mM monomer equivalents, indicating a lower consumption of DOC and an accumulation of DOC in the pore water (Fig. 5b).

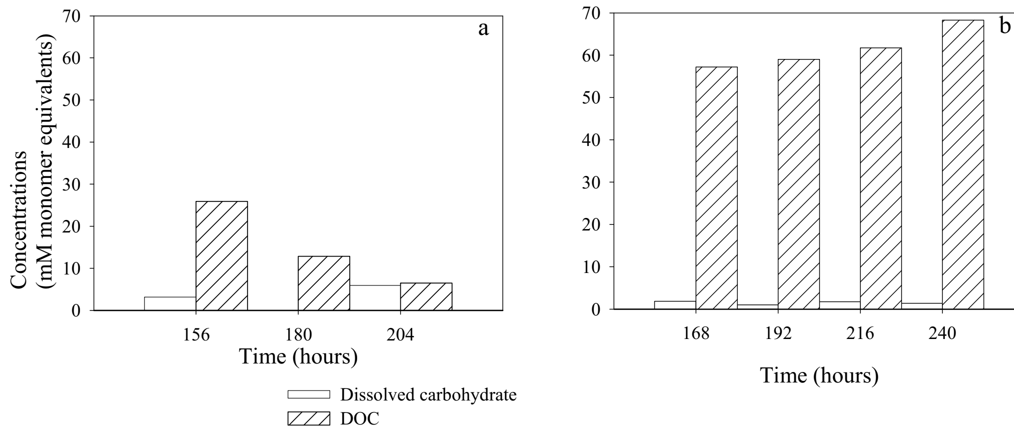

Fig.5: a) Carbohydrate and b) DOC concentrations (difference between concentration in the inflow and outflow) after laminarin addition in a) 0-6 cm and b) 14-16 cm sediment depth.

Dissolved sulfate and sulfide

Dissolved sulfate concentration in the top 6 cm of the sediment was ten times higher than in the sediment from 14-16 cm depth at the start of the experiment. During the first 96 h of the experiment prior to the addition of laminarin to the inflow, sulfate concentrations remained at ~1 mM, even though a continuous sulfate concentration of 2 mM was flowing through the cells, indicating sulfate reducing activity in the cells. After laminarin addition, the consumption of sulfate was initially 10 times higher in the top sediment compared to the sediment at 14-16 cm depth. However, at the end of the experiments the sulfate consumption rate was ~1.5 times higher in the sediment from 14-16 cm depth compared to the top sediment (Fig 6a).

Since no sulfide was detected in the inflow, the sulfide concentrations measured corresponded to the sulfide formed in the cells by sulfate reduction. The increase in sulfide concentration and sulfide formation rates after amendment of the inflow with laminarin reveal a stimulation of sulfate reduction rates (Fig. 6 c,d). This stimulation of sulfate reduction was significantly greater ($p < 0.05$) in the top sediment (20 fold increase) compared

to the sediment in the 14-16 cm depth interval (9 fold increase). The maximum sulfide formation rate was 1.8 mM d^{-1} that was reached 108 h after substrate addition in the top sediment (Fig. 6b).

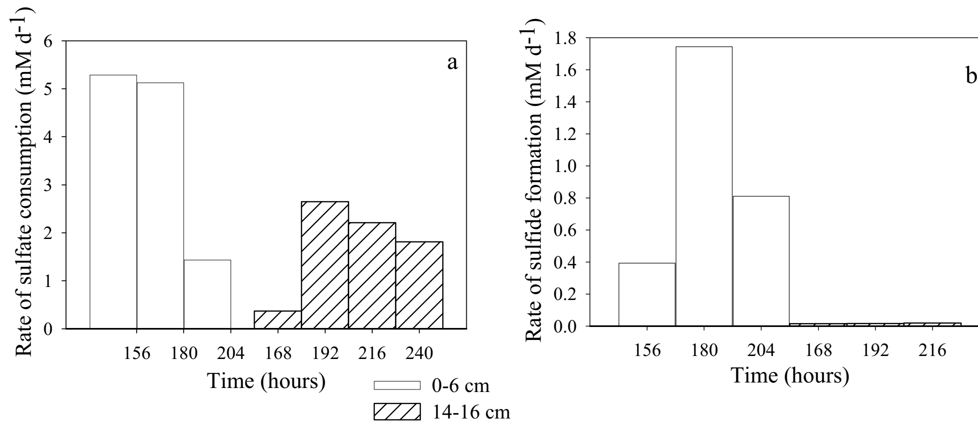

Fig. 6: (a) Sulfate consumption rates and b) rates of sulfide formation during the flow cell experiment with sediment from Station 13.

Response of the microorganisms to laminarin addition in sediment from Station 13

DGGE analysis with *Bacteria* primers GM5F (with GC-clamp) and 907 RC revealed that in both depth intervals the addition of laminarin caused a change in the DGGE-pattern after the experiments have been running for 168 h, which is 96 h after laminarin addition was started (Fig.7). In the top sediment band 1 increased in intensity at the last time point (204 h). This band was also observed in the sediment from the duplicate cell (band 2) that was sampled for DNA extraction at the end of the experiment, indicating homogeneity of the sediment. Sequences from all the excised DGGE bands were aligned with the automatic aligning tool of the ARB software (Ludwig et al. 2004) and compared with sequences present in the Ribosomal Database Project (RDP) in January 2004 (~95 000 rRNA sequences, http://rdp.cme.msu.edu) and in the database of the National Center for Biotechnology Information (NCBI), using their Basic Logical Alignment Search Tool (BLAST)

(www.ncbi.nlm.nih..gov/blast). Band 1 and 2 represent bacteria from the *Bacteroidetes* phylum and are closely related to *Cytophaga fermentans* (M58766.2).

In the 14-16 cm depth interval two DGGE bands increased in intensity after laminarin addition. The one band is labeled 3 and the other one carries the labels 4, 5 and 6 for the different times of sampling (Fig.7). Both bands represent bacteria that belong to the *Bacteroidetes* phylum. Band 3 (accession number EUO35607) has a similarity of 97% with uncultured bacteria in the order *Cytophagales* (AJ535257.1), while bands 4, 5 and 6 (accession numbers EUO35608-EUO35610) have a similarity of 94% with *Cytophaga* species (AB015260.1).

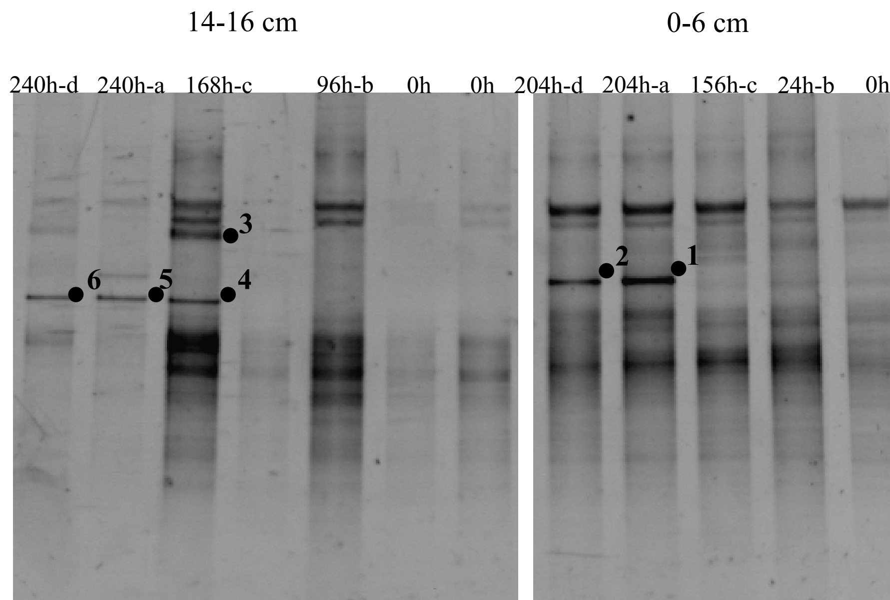

Figure 7: DGGE fingerprints of PCR-amplified 16S rRNA gene fragments with the bacterial primers GM5F (with GC clamp) and 907R. The bands numbered 1-6 were manually excised and re-amplified. The time points at which samples were taken and the flow cells (a, b, c, or d) from which the samples were taken are indicated on top of each band. For the last time points, in both depth intervals DNA was extracted from duplicate flow cells.

Sequences from excised bands after DGGE analysis with the primers Dsr-1-F-GC clamp (5'-ACS-CAC-TGG-AAG-CAC-G-3') and Dsr1-DGGE-Rev (5'-CGG TGM AGY TCR TCC TG-3') were aligned with the ARB software (Ludwig et al. 2004) and a DsrAB

consensus tree was constructed using maximum parsimony, maximum likelihood, and distance matrix treeing methods. The banding pattern for sediment from the top 6 cm and from the 14-16 cm depth interval did not change significantly after laminarin addition, indicating no apparent change in the SRB community for the duration of the experiments (Fig. 8). However, the diversity of SRB in the top sediment was higher than in the 14-16 cm depth interval as judged from the number of visible bands in the DGGE gel (Fig. 8). Three different clusters of SRB were identified from sequences of the amplified bands (Fig. 9). Two of these clusters included *Desulforhabdus*-related (DSR-III) and, *Desulfovibrio*-related (DSR-II) bacteria. The majority (~65%) of the sequences from the amplified bands were in the DSR-II cluster (Fig. 9). The other sequences represented SRB which are *Desulfobacterium*–related (DSR-I).

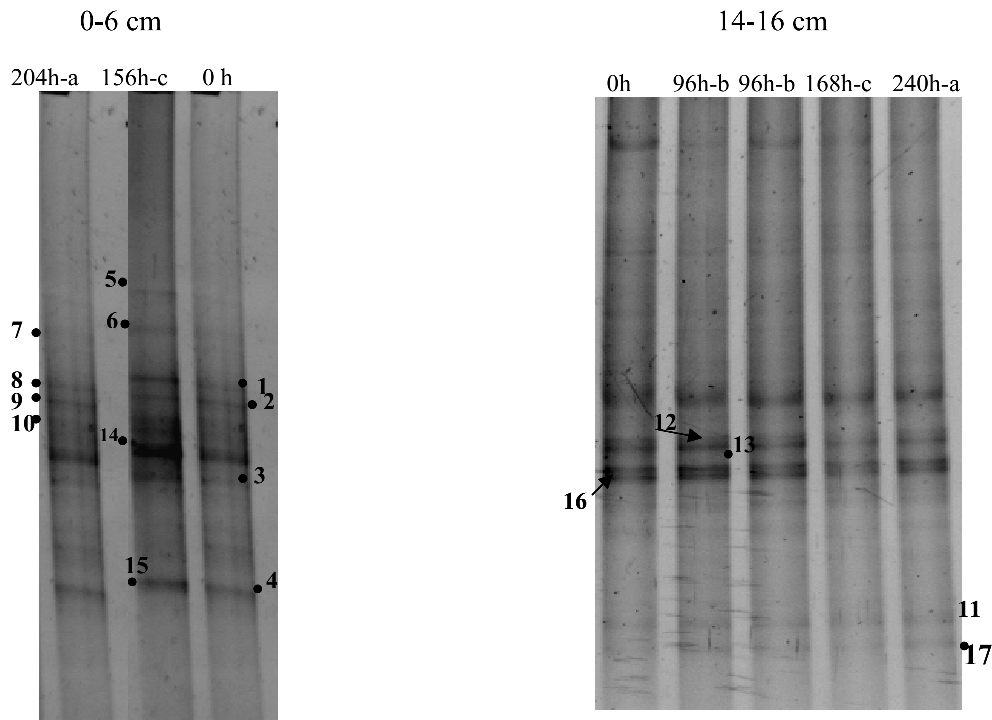

Figure 8: DGGE fingerprints of PCR-amplified Dsr gene fragments for sediment from a) 0-6 cm depth and b) 14-16 cm depth. The bands numbered 1 to 17 were manually excised and re-amplified.

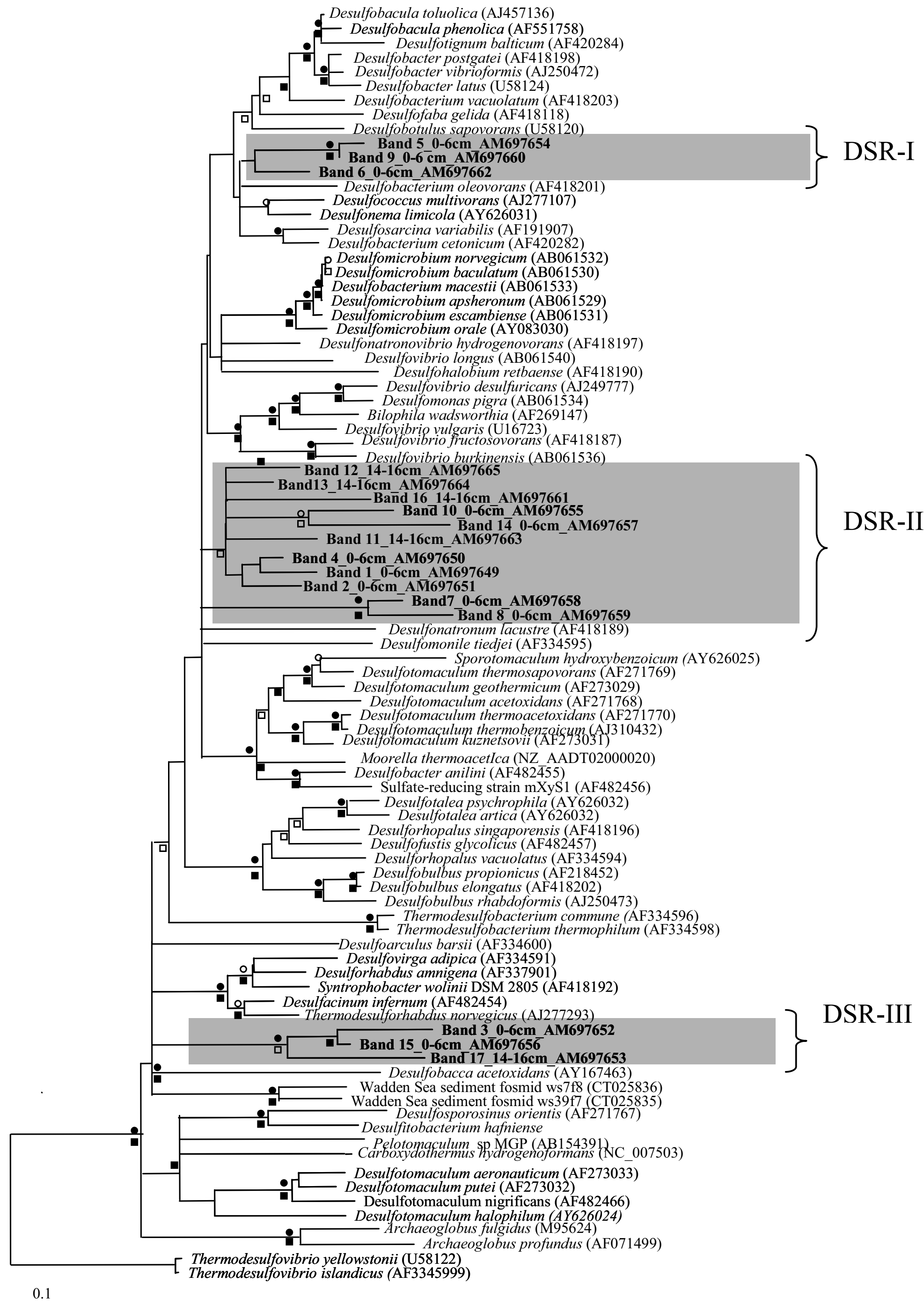

Figure 9: DsrAB consensus tree showing the relationship of excised Dsr-DGGE bands (in boldface) to selected sequences from cultures and environmental sulfate reducing prokaryotes. Polytomic nodes connect branches for which a relative order could not be determined unambiguously by applying distance matrix, maximum parsimony, and maximum likelihood treeing methods. Parsimony (circles) and distance matrix (squares) bootstrap values are indicated by solid (≥90%) and open (75-90%) symbols. The scale bar shows estimated sequence divergence (10%) according to the distance matrix method.

Discussion

Most studies performing substrate addition experiments utilize slurries or bag incubations (Finke et al. 2006; Arnosti et al. 2005; Weston and Joye 2005; Hansen et al. 2000). However, biogeochemical rates are enhanced and overestimated, because of the stimulation of microbial response during the homogenization of the sediment (Arnosti et al. 2005, Hansen et al. 2000). In our study the stimulation of metabolic rates of the microorganisms is due to the addition of substrates and not to the pre-handling of the sediment by utilizing intact sediment cores during the experiment where LMW-DOC was added, and flow-through cells during the experiment where HMW-DOC was added.

The effect of LMW-DOC addition on sulfate reducing bacteria

VFA are intermediate products during organic carbon mineralization therefore the low VFA concentrations measured throughout the sediment depth in a previous study on the Namibian shelf (Julies et al. submitted - Chapter 2) indicate their rapid turnover (Hoehler et al. 1998). Several studies used ^{14}C-labelled VFA to determine their turnover rate in marine sediments (Wellsbury and Parkes 1995; Shaw and McIntosh 1990; Sansone 1986), however these studies do not allow the assessment of the effect of VFA addition on sulfate reduction rates. The inhibition of sulfate reduction with molybdate ($MoO4^{2-}$) to determine the importance of VFA as substrate for SRB has also been widely used (Parkes et al. 1989; Sørensen et al. 1981) however, molybdate complexes VFA and affects the detection of VFA (Finke et al. 2007). In our study we measured sulfate reduction rates in intact cores after acetate or lactate addition. The ten times higher concentrations of acetate required compared to lactate to observe a stimulation of the SRB can be explained by the presence of higher in-situ concentrations of acetate compared to lactate (Table 1). Concentrations of acetate exceeding 10 mM inhibited sulfate reduction and possibly also fermentation processes.

The addition of lactate and acetate to separate cores resulted in depth-specific responses of SRB, revealing the existence of different SRB communities throughout the sediment depth. In the top sediment the higher stimulation the SRB community by lactate addition compared to acetate addition, suggests that most of the SRB are incomplete-oxidizing bacteria, capable of a rapid response and growth. In the sediment at 15 cm depth the SRB community consists of slower growing complete-oxidizing bacteria, which were capable of utilizing the acetate better than lactate. Substrate preference of SRB at different depths of the sediment was also illustrated in other coastal marine sediments (Mudryk et al. 2000; Knoblauch et al. 1999; Gibson et al. 1987). This depth–specific response of SRB suggests that the ecological niches occupied by different SRB throughout the sediment depth are a function of the physiological abilities of the bacteria, especially their ability to utilize different substrates. Clone library results at Station 5 from a previous study did not reveal a difference in the members of the SRB communities with depth (Julies et al. in prep. – Chapter 3). The present study emphasizes the importance of an ecological classification of microbial communities, rather than using phylogenetic classification in isolation to understand the structure and function of microbial communities. The importance of functional diversity over phylogenetic diversity was indicated in a few reviews and studies (Fierer et al. 2007; Cottrell and Kirchmann 2000; Roselló-Mora et al. 1999). It is important to establish the different ecological niches of microorganisms involved in organic carbon mineralization in marine sediments, because carbon cycling within an ecosystem is affected by the functional characteristics of organisms, rather than by their taxonomic identity (Fierer et al. 2007).

The effect of polysaccharide addition on the metabolic activity of microorganisms

A sudden high input of highly reactive, complex organic substrates affects microbial activity in marine sediments (Takii et al. 2002; Roselló-Mora et al. 1999). We examined the

effect of HMW-DOC addition on microbial community activity and structure in a continuous flow-through cell experiment. The polysaccharide, laminarin was added to the sediment as a representative HMW-DOC compound. The immediate increase in VFA formation rates in both depth intervals after the pore water was replaced by the inflow amended with laminarin (after 72 h), indicated a rapid response of hydrolytic and fermentative bacteria. A rapid stimulation of organic carbon mineralization rates by HMW organic carbon addition were also observed in other substrate addition experiments with anaerobic marine sediments (Takii et al. 2002; Roselló-Mora et al. 1999). Brüchert and Arnosti (2003) explain a rapid hydrolytic response to substrate addition by an increased production of extracellular enzymes for the specific polysaccharide. The low (below 70 µM) concentrations of VFA detected in the outflow in the top sediment, compared to the sediment at 14-16 cm depth (below 1.6 mM), suggests a rapid turnover of intermediates in the top sediment. Therefore, the response of microorganisms to a high input of carbon into the sediment is different in the top and bottom (14-16 cm depth) sediment. The higher VFA concentrations from the bottom sediment indicate a lower consumption rate of VFA by SRB, resulting in its accumulation in the pore water. Although there were no significant difference in the sulfate concentrations and sulfate consumption rates after laminarin addition, between the top and the bottom sediment, the rate of sulfide formation was significantly higher in the top sediment compared to the bottom sediment. This reveals a closer coupling of initial hydrolytic and fermentation processes to terminal oxidation in the top sediment, keeping VFA productions low, while there exist a de-coupling of initial and terminal processes in the bottom sediment, so that VFA are produced faster by fermenting bacteria than they can be consumed by SRB. These observations confirm the findings of our previous study in whole sediment cores (Julies et al. submitted – Chapter 2).

Acetate is an important substrate for complete-oxidizing SRB in marine sediments and approximately 50% of the sulfate reduction in marine coastal sediments is driven by acetate (Finke et al. 2006; Sørensen et al. 1981; Balba and Nedwell 1982). In the surface sediment there is a good agreement between the rate at which dissolved carbohydrates are consumed and the rate at which acetate is formed by fermentation after laminarin addition. Acetate concentrations reflect the acetate that is formed in the sediment and was ~30 µM formed in a 24 h period, after laminarin addition and after breakthrough was achieved (156 h time point). This acetate formation by fermentation can theoretically be obtained if ~10 µM monomer equivalent d^{-1} substrate is consumed ($C_6H_{12}O_6 \rightarrow 3\ CH_3COO^- + 3\ H^+$). In our experiment the carbohydrate concentration, which reflect the amount of carbohydrates consumed within the 24 h, was ~5 mM monomer equivalent or 0.8 mM C (at the 156 h time point). Thus, measured carbohydrate consumption was 500 times higher than the theoretical consumption required. The lower than expected acetate formation can be due to its consumption by SRB, which is reflected in the high sulfide concentrations (0.4 mM) at the same time point. Assuming a 2:1 stoichiometry ($2CH_2O + SO_4^{2-} \rightarrow H_2S + 2HCO_3^-$), ~100% of the carbohydrate degradation provided substrate for SRB in the top sediment.

The effect of polysaccharide addition on the composition of the microbial community

DGGE provides a rapid way to obtain qualitative information on the diversity of the microbial population (Muyzer et al. 1993). When we used the bacterial primers GM5F (with GC clamp) and 907RC, we detected more bands with DGGE in the top sediment (0-6 cm) compared to the bottom sediment (14-16 cm) before we started the experiment (0 h time point). This suggests that the diversity of microorganisms at these two depths intervals is significantly different.

We recognize that the microbial community within the sediment that we used for our experiment might not be the same as that which existed originally in the sediment, because of possible changes in the microbial community composition during storage of the sediment, when bacteria did not receive any input of fresh organic matter. The results presented here therefore represent how a starved community will respond to a sudden increase in highly reactive HMW-DOC.

In both depth intervals the DGGE fragments affiliated with sequences of the *Bacteroidetes* phylum. The enrichment of *Bacteroidetes* and *Cytophaga*-related bacteria by particulate organic detritus and HMW-DOC has been illustrated in aerobic marine environments, specifically in the water column (references in Kirchman 2002) and anaerobic marine sediments (Roselló-Mora et al. 1999). The ability of marine *Bacteroidetes* to hydrolyze polysaccharides aerobically is well known (Bauer et al. 2006; Kirchman 2002; Eilers et al. 2001) and their involvement in laminarin degradation has been illustrated (Alderkamp 2006; Pankratov et al. 2006; Julies et al. in prep.). The chief end products of the anaerobic respiration of *Bacteroidetes* are acetate and succinate (Xu et al. 2003; Madigan et al. 2000). In the water column most *Bacteroidetes* are aerobic (Cottrell and Kirchman 2000), but in other environments, they can also be obligate anaerobes and saccharolytic, which means they obtain carbon and energy by hydrolysis of carbohydrates (Xu et al. 2003). They possess beta-1,3-glucosidase activity which is responsible for laminarin degradation (Xu et al. 2003). The rapid increase in the activity of *Bacteroidetes* bacteria explains the initial rapid increase in VFA formation rates. We recognize that the abundance of other heterotrophic bacteria might also have increased after substrate addition if the experiment was run for longer. The rapid response of *Bacteroidetes* bacteria over other heterotrophs, such as the *γ-proteobacteria*, might be explained by a faster growth rate of *Bacteroidetes*. Although net growth rates that were two-fold faster was recorded for *Bacteroidetes* compared to other

bacteria (Jürgens 1999), other studies recorded slower growth rates (references in Kirchman 2001) and there is still uncertainty about the growth rate of these bacteria. In order to obtain a more refined idea of the response of the microbial community to substrate addition, we also need to use RNA-based DGGE, to allow a more reliable assessment of the active members of the community (Rosselló-Mora et al. 1999).

In the top 6cm of the sediment, the response of the SRB community to HMW-DOC addition was detected immediately after breakthrough was reached after laminarin addition, following the increased formation of VFA in the sediment. Due to their polyphyletic nature and consequently the failure of applying a single 16S rRNA gene-targeting probe or primer to detect all SRB simultaneously, the use of primers to detect Dsr functional genes proved useful to study the diversity of SRB (Leloup et al. 2006). Dsr is the key enzyme in anaerobic sulfate respiration, catalyzing the six-electron reduction of (bi)sulfite to sulfide (Odom and Peck 1984). DSR genes, which code for this enzyme, are present in all known SRB (Leloup et al. 2006; Wagner et al. 1998). In our experiment, the use of specific primers for Dsr functional genes during DGGE analysis could detect the major differences in the SRB community before and after laminarin addition. Our study provides an initial glance at the immediate and potential response of the SRB community to substrate addition.

In the bottom sediment the banding pattern did not change after substrate addition, indicating a slower response of SRB in the bottom sediment, compared to the surface sediment. The diversity of SRB in the surface sediment was higher than in the bottom at the start of the experiment, as revealed by the lower number of bands detected in bottom sediment. Lower microbial diversity in deeper sediment compared to the surface sediment was also detected from clone libraries from other two sampling stations on the Namibian shelf in one of our earlier studies (Julies et al. – Chapter 3).

In the top 6 cm of the sediment the bands sequenced represented mostly incompletely oxidizing SRB, including *Desulfovibrio*-related bacteria. Only one of the DGGE fragments sequenced from the top 6 cm was affiliated to completely oxidizing *Desulforhabdus*-related bacteria. Incomplete oxidizers produce acetate as the main end-product of their catabolism and lack the acetyl-CoA enzyme, preventing them to further oxidize acetate (Rabus et al. 2006; Madigan et al. 2000; Knoblauch et al. 1999). These bacteria exhibit faster growth compared to completely oxidizing SRB (Rabus et al. 2006; Gräber and Milazzo 1997) and typically utilize lactate, hydrogen and propionate as substrate (Knoblauch et al. 1999). Their greater presence and activity in the top sediment compared to the 14- 16 cm depth interval, explains the lower lactate concentrations (~10 μM) measured in the top sediment, compared to the lactate concentrations in the 14-16 cm depth (600-800 μM).

In the bottom sediment (14-16 cm depth) both completely oxidizing SRB, including *Desulfobacterium*-related and *Desulforhabdus*-related bacteria, and incompletely oxidizing SRB, including *Desulfovibrio*-related bacteria dominated. Completely oxidizing SRB are capable of oxidizing fatty acids such as acetate, lactate, succinate, certain alcohols and even benzoate completely to CO_2 (Rabus et al. 2006; Madigan et al. 2000). They oxidize acetate to CO_2 via the acetyl-CoA pathway, which is a series of reversible reactions that involves the enzyme *carbon monoxide dehydrogenase* (Rabus et al. 2006). Purdy et al. (2003) illustrated a lack of response of *Desulfobacterium* to single substrate addition, despite the fact that they contributed significantly to the natural microbial community. *Desulfobacterium* are capable of utilizing a wide range of substrates and can be considered as flexible generalists that will co-metabolize a variety of substrates (Purdy et al. 2003). The rapid stimulation of this genus in our study might be explained by the fact that a polysaccharide was added as substrate, which upon hydrolysis and fermentation formed a variety of VFA that could serve as electron donors.

The results from the flow cell experiments are in agreement with our findings from the experiment where LMW-DOC was added to intact cores, even though the sediment for these two experiments are from different sampling stations on the shelf. Both experimental studies revealed that in the top sediment (~top 10 cm) where high sulfate reduction rates are measured, the SRB are dominated by fast growing incomplete oxidizing bacteria, while the role of complete-oxidizing SRB become increasingly dominant in the deeper sediment (~below 10 cm depth) where lower sulfate reduction rates are measured. A selective stimulation of SRB upon carbon source addition was also observed by studies in other marine sediments (Kleikemper et al. 2002; Purdy et al. 1997). The use of fluorescent in situ hybridization (FISH) analysis with specific probes that target the SRB present in the sediment and the use of rRNA DGGE (Teske 1996) will allow for correlation studies to determine the effect of substrate addition on the abundance and activity of different microorganisms involved in organic carbon mineralization and will verify our initial findings from this study.

Conclusions

This study is the first to determine the immediate, short-term response of microorganisms to an input of HMW-DOC and LMW-DOC in organic rich sediments of an upwelling system. The fermenting bacteria that responded the fastest to laminarin addition belonged to the *Bacteroidetes* bacteria, while the SRB belonged to three different clusters. Hydrolytic-enzyme producing bacteria are stimulated to a greater extent by high inputs of HMW-DOC into the sediment compared to SRB, resulting in an accumulation of fermentation products. Microorganisms in the top 6 cm of the sediment and in the bottom sediment (14-16 cm depth) have a differential response to HMW-DOC addition. Faster growing, incompletely oxidizing SRB are responsible for the high sulfate reduction rates measured in the top sediment, while

slower growing, completely oxidizing SRB are responsible for most of the sulfate reduction in the deeper sediment.

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Conclusions and Perspectives

In this thesis the carbon transformation processes within the shelf sediments from the Namibian upwelling system was studied to determine the reactivity of organic carbon in the sediment and investigate the control of microbial community structure and activity by the availability and accessibility of carbon sources. The focus is on carbohydrates as a carbon source.

A tight coupling between initial hydrolytic and fermentation steps and terminal oxidation appears to be controlled by the availability of degradable dissolved organic carbon (DOC). The tight coupling between hydrolysis and fermentation to terminal oxidation is reflected in the low volatile fatty acid concentrations in the sediment, indicating their rapid turnover. Despite the high organic carbon concentrations in sediments of the Namibian upwelling system, the biological accessibility of the organic carbon was found surprisingly limited. The increase in DOC and the persistence of carbohydrates with depth provide evidence for the rapid diagenetic transformation of DOC. This is manifested in the decrease in numbers of active bacteria involved in fermentation and terminal oxidation, and the decrease in cell-specific sulfate reduction rates with depth, suggesting increasing starvation of bacteria with depth. Therefore, the restriction of up to 90% of sulfate reduction to the top 6-10 cm of the sediment and the steep gradients within Namibian shelf sediments is the result of the transformation of labile DOC into low reactive substances within the top sediment. We hypothesize that possible, secondary reactions, such as reactions with sulfides and polysulfides and the formation of organic sulfur compounds may counteract complete carbon oxidation. **The nature of complex dissolved organic molecules in the sediment needs further investigation.**

Bacteria within the sediment are physiologically adapted to the highly reactive organic carbon within the top 6 cm of the sediment and the low reactivity of DOC in the sediment

below 14 cm depth. This is evident from the differential response of bacteria to the addition of low molecular weight and high molecular weight organic substrates to the sediment. Substrate amendment of the sediment stimulated sulfate reduction rates, but the intensity of the response decreased with depth. A clone library based on the Dsr sequence bands suggested the dominance of comparably fast-growing incomplete oxidizing sulfate reducing bacteria in the top 2 cm, while slower growing sulfate reducing bacteria become dominant in the deeper parts of the sediment. The microbial community in the top 6 cm responds faster and to a greater extent to substrate addition compared to the microorganisms in the deeper sediment (below 14 cm depth). Therefore, the different physiological abilities of bacteria results in niche diversification within the sediment, which is also reflected in the high phylogenetic diversity of Bacteria as revealed by 16S rRNA clone libraries. **It is important to asses the various ecological roles of different groups of Bacteria involved in carbon transformation of the sediment.** This thesis demonstrated the importance of using an ecological classification of microbial communities, rather than using phylogenetic classification in isolation to understand the structure and function of microbial communities.

Significant differences were found between the two closely spaced sampling stations in concentrations and gradients of DOC, total carbohydrates, in rates of polysaccharide hydrolysis and sulfate reduction, and in the phylogenetic diversity of Bacteria within the sediment (Chapter 2 and 3). These differences were attributed to differences in hydrographic features between the two stations and emphasize the importance of particle retention and sediment re-suspension in the bottom boundary layer. The results presented in chapters 2 and 3 suggest a strong physical forcing imposed by water column processes on biogeochemical carbon dynamics and that **the bottom boundary layer is a critical compartment in the transformation of organic matter, but it needs verification**.

Abstracts of manuscripts not included in this thesis

Biogeochemical and physical control on shelf anoxia and water column hydrogen sulfide in the Benguela coastal upwelling system off Namibia

Volker Brüchert, Bronwen Currie, Kathleen R. Peard, Ulrich Lass, Rudolf Endler, Arne Dübecke, Elsabé Julies, Thomas Leipe, and Sybille Zitzmann

Abstract

Shelf anoxia and recurring sulfidic water column conditions are characteristic features of the coastal upwelling system off Namibia. The development of oxygen-depleted water column conditions is linked to the relative dominance of South Atlantic Central Water, which flows southward from the Angolan Dome over Eastern South Atlantic Central Water. Inter- and intra-annual variations in the strength of upwelling influence the thickness and stability of the relatively stagnant boundary layer. Hydrogen sulfide accumulation in this boundary layer is mainly driven by the diffusive flux of the hydrogen sulfide from the sediment. The hydrogen sulfide derives from the rapid degradation of organic material by bacterial sulfate reduction in the topmost 2 cm of sediment. Low reactive iron contents in the diatomaceous mud belt limit iron sulfide precipitation and sulfide oxidation by oxidized iron. In the absence of oxygen, iron, and manganese as important electron acceptors, sulfide oxidation proceeds largely by the reduction of nitrate by the large sulfur bacteria *Beggiatoa* and *Thiomargarita*, which cover large areas of the shelf. Regional differences in the distribution of these bacteria affect the development of sulfidic bottom waters. While hydrogen sulfide is quantitatively oxidized in sediments covered by *Beggiatoa* mats, only a fraction of the sulfide is removed by *Thiomargarita*. Aerial estimations of aerobic water column respiration, diffusive fluxes of hydrogen sulfide from the sediment, and rates of bacterial sulfate reduction indicate that

oxidation of sulfide at the sediment-water interface and oxidation of water column sulfide may comprise up to 25% of the total oxygen consumption in the coastal upwelling system. Advective transport of methane and hydrogen sulfide from gas-charged sediments has an intermittent and locally restricted impact on water column sulfide.