Arctic to tropic - adaptation and response of anaerobic microorganisms to temperature effects in marine sediments

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Zusammenfassung

Mit der vorliegende Dissertation möchte ich einen Beitrag zur Aufklärung der Anpassung anaerober Mikroorganismen an Temperatur und des Effektes von Temperaturänderungen auf die Mikroorganismen in marinen Sedimenten leisten. Temperatur ist ein wichtiger Faktor in der Regulierung von biologischen Prozessen und hat daher einen kontrollierenden Einfluss auf den mikrobiellen Kohlenstoffkreislauf in den Sedimenten. Biogeochemische und molekulare Methoden wurden angewandt um die Reaktionen von Mikroorganismen aus arktischen und gemäßigten Sedimenten auf Temperaturänderungen zu untersuchen. Diese Ansätze erlaubten auch neue Einblicke in die physiologische Anpassung von Mikroorganismen aus verschiedenen geografischen Regionen an Temperaturmodifikationen zu gewinnen.

In einer Studie über die Reaktionen der mikrobiellen Gemeinschaften auf wiederholte Frost-Tau-Bedingungen zeigten wir, dass moderate Frost-Tau-Bedingungen einen geringen Effekt auf den mikrobiell mediierten Abbau von organischem Material in arktischen Sedimenten aus der Gezeitenzone hatten. Offensichtlich konnten die *in situ* Bakteriengemeinschaften drastischen Temperaturschwankungen weitgehend überstehen und ohne Verzögerung reaktiviert werden.

In einem Temperatur-Gradienten-Block verglichen wir die Temperaturreaktionen von Sulfatreduktionsraten (SRR) in Schelf- und Kontinentalhangsedimenten aus dem Südwest- und Südostatlantik mit den Reaktionen von SRR in Sedimenten aus arktischen Fjorden. Ziel dieser Studie war es festzustellen, ob die Reaktion der mikrobiellen Gemeinschaften auf Temperatur auf eine enge Anpassung an die Umgebungstemperatur zurückzuführen ist, oder ob sie gemischte Gemeinschaften unterschiedlicher Temperaturgruppen widerspiegelt. In den südatlantischen Schelfsedimenten und den Sedimenten aus der Gezeitenzone Svalbards waren psychrotolerante bis mesophile Sulfat-reduzierende Gemeinschaften vorhanden, wohingegen in den Sedimenten vom südatlantischen Kontinentalhang und den arktischen

Schelfsedimenten psychrophile Gemeinschaften dominierten. Das niedrige Temperaturoptimum (T_{opt}) der arktischen Sedimente und der Sedimente des kalten südatlantischen Kontinentalhangs zeigte, wie die *in situ* Temperatur die vorherrschenden Temperaturgruppen der Sulfat-reduzierenden Gemeinschaften bestimmte. Hohe Raten bei T_{opt} und ein breiter Temperaturbereich der SRR in mehreren Sedimentproben von südatlantischen Kontinentalhängen zeigten die zusätzliche Präsenz von mesophilen Sulfat-reduzierenden Bakterien (SRB). Diese sind dort vermutlich nicht *in situ* gewachsen, könnten jedoch mit Schelfsediment, indem mesophile Bakterien dominierten, den Kontinentalhang hinunter transportiert worden sein.

Die Temperaturreaktion des Abbaus von organischem Kohlenstoff über bakterielle Sulfatreduktion in polaren, gemäßigten und tropischen marinen Sedimenten wurde untersucht, um die Temperaturanpassung von SRB an die Umgebungstemperatur zu quantifizieren. Relative SRR und Temperaturoptima deuteten auf überwiegend mesophile SRB in wärmeren Breiten hin, während polare Regionen SRB mit psychrophilen Anpassungen aufwiesen.

Wir überprüften arktische und gemäßigte Sedimente die für ein Jahr bei erhöhter Temperatur inkubiert wurden auf Veränderungen der mikrobieller Gemeinschaften. Genetische Fingerabdruckmuster einer denaturierenden Gradientengelelektrophorese beider Sedimente ließen vermuten, dass langfristige Exposition mit erhöhten Temperaturen die Vielfalt der mikrobiellen Gemeinschaft in marinen Sedimenten beeinflusst. Zusammenfassend zeigte diese Studie das die Umgebungstemperatur für die Auswahl adaptiver Physiologien verantwortlich ist und das thermische Gruppen von Mikroorganismen ein globales biogeographisches Muster aufweisen. Diese Arbeit leistet einen Beitrag zum Verständnis des Einflusses von umweltrelevanten Temperaturszenarien (erhöhte Temperatur, Frost-Tau-Effekte) auf den mikrobiell mediierten organischen Kohlenstoffkreislauf.

Summary

The aim of the present work was to investigate the adaptation and response of anaerobic microorganisms to temperature effects in marine sediments. Temperature is an important factor regulating the rate of biological processes and therefore exerts a control on microbial sedimentary carbon cycling. Biogeochemical and molecular methods allowed new insights into the response of microorganisms from Arctic and temperate sediments to temperature effects and into the physiological adaptations of microorganisms from different geographical regions to alternative temperature regimes.

We have gained insights into the freeze-thaw effects on microbially mediated organic carbon mineralization in Arctic intertidal sediment. We determined that moderate freeze-thaw conditions have little effect on the microbially mediated organic carbon degradation in intertidal Arctic sediments. It is apparent that the *in situ* microbial communities can largely withstand drastic temperature fluctuations and are reactivated without delay.

The temperature responses of sulfate reduction rates (SRR) in continental shelf and slope sediments from the southwest and southeast Atlantic were compared with those in sediments from Arctic fjords. We wanted to assess if the temperature response of the microbial communities indicates a narrow adaptation to ambient temperature or rather reflects mixed communities of different temperature groups. In the south Atlantic shelf sediments and in intertidal flat sediment from Svalbard, psychrotolerant to mesophilic sulfate-reducing community were present, whereas in south Atlantic slope sediments and Arctic shelf sediments psychrophilic community dominated. The low temperature optimum (T_{opt}) in Arctic sediment and in cold south Atlantic slope sediments shows how the *in situ* temperature determined the predominant temperature groups of the sulfate-reducing community. High rates at T_{opt} and a broad temperature range of SRR in several south Atlantic slope sediments indicated the additional presence of mesophilic sulfate-reducing bacteria. These had probably

not grown *in situ* but were transported down-slope with sediment from the shelf where mesophilic bacteria dominated.

The temperature response of carbon mineralization via bacterial sulfate reduction of polar, temperate and tropical marine sediments was studied to quantify temperature adaptation of sulfate reducing bacteria (SRB) to ambient temperatures. In temperate and tropical sediments relative SRR and temperature optima indicate mostly mesophilic SRB in warmer latitudes while in polar regions SRB shows psychrophilic adaptations.

We screened for changes in microbial community composition in Arctic and temperate sediments incubated at elevated temperature for a year. Altered denaturing gradient gel electrophoresis fingerprint pattern in both sediments suggests that long term exposure to increased temperature may affect the richness of microbial community in marine sediments.

In summary this study demonstrated that environmental temperature selects for adaptive physiologies and thermal groups of microorganisms exhibit a global biogeographic pattern. This thesis contributes to explaining the influence of environmentally relevant temperature scenarios (increased temperature; freeze-thaw effects) on microbial microbially mediated organic carbon cycle.

CONTENTS

Abbreviations

DAPI	4’6-diamino-2-phenylindole
DGGE	denaturing gradient gel electrophoresis
DIC	dissolved inorganic carbon
DNA	deoxyribonucleic acid
DOC	dissolved organic carbon
PCR	polymerase chain reaction
POM	particulate organic matter
rRNA	ribosomal ribonucleic acid
SR	sulfate reduction
SRB	sulfate reducing bacteria
SRR	sulfate reduction rate
TGB	temperature gradient block
Tmin	minimum temperature
Topt	optimum temperature
Tmax	maximum temperature
VFA	volatile fatty acid

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Preface

This study was funded by Deutsche Forschungsgemeinschaft (DFG) via the Priority Program 1162 'The impact of climate variability on aquatic ecosystems (Aquashift)' and by Max Planck Society. The project has been supervised by Prof. Dr. Bo Barker Jørgensen and Prof. Dr. Volker Brüchert. The research was conducted at Department of Biogeochemistry, Max-Planck-Institute for Marine Microbiology, Bremen, from April 2007 to Mai 2011.

This dissertation focuses on the adaptation and response of anaerobic microorganisms to temperature effects in marine sediments and comprises four parts. The first is an introduction into the topic and the aims of the study. The second is the summary and discussion of the results obtained during the PhD period. The third part of this thesis comprises three manuscripts. The first manuscript has been already published and a journal' PDF file is included in the thesis. Two other manuscripts are included as drafts close to submission. The third part contains also appendix, where preliminary results of a project performed during PhD are described and discussed. Part four is the conclusion of the thesis.

PART I Introduction

1. Introduction

1.1. Marine carbon cycle

The ocean is crucial to the global carbon cycle as the largest active carbon reservoir on Earth (Figure 1.1; Hedges, 1992; Schimel, 1995). Marine carbon exchanges constantly with atmospheric and terrestrial reservoirs of carbon over time scales ranging from hours to millions of years (Falkowski et al., 2000). Short-term marine carbon cycle processes include photosynthesis, respiration, air-sea exchange of carbon dioxide. Carbon cycling between ocean and rocks occurs over longer time scales (Hedges, 1992).

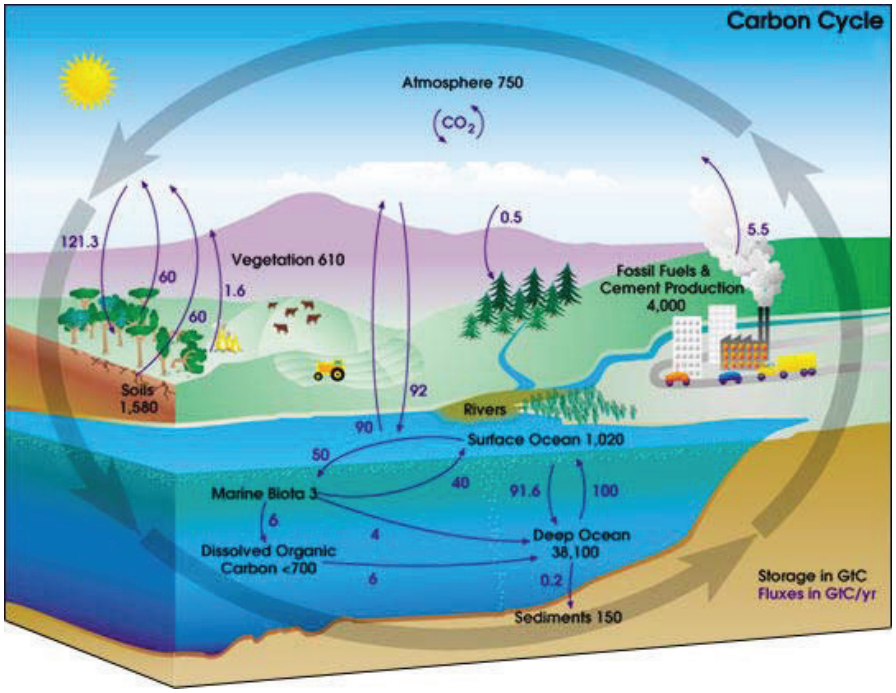

Figure 1.1 The global carbon cycle
Source:http://earthobservatory.nasa.gov/Library/CarbonCycle/carbon_cycle4.html

Marine carbon cycling is predominantly catalyzed by microorganisms. Photosynthetic organisms, mainly eukaryotic phytoplankton and cyanobacteria, convert inorganic carbon (CO_2) to organic carbon as biomass in the upper water column. This particulate organic matter

(POM) sinks through the water column and a large portion becomes dissolved organic matter (DOM). Both POM and DOM are subject to microbial mineralization, and most of the organic carbon is recycled to dissolved inorganic carbon (DIC) in the water column (Azam and Malfatti, 2007). A fraction of organic matter is not respired in the water column and reaches the sediments. The size of this fraction is largely dependent on the water depth (Canfield, 1991). It is estimated that for coastal environments 25 to 50% of the carbon fixed by the pelagic primary producers reaches the sediment surface. With increasing distance from the coast, primary production decreases and organic matter is oxidized as it sinks through the water column such that the amount reaching the deep sea floor and sedimentary microbial community is low (Arnosti and Jørgensen, 2003; Kasten and Jørgensen, 2006).

1.2. Carbon degradation in marine sediments

Most of the organic carbon that reaches the sediments is degraded by sediment-dwelling microorganisms either aerobically or anaerobically, releasing CO_2 and nutrients into the pore water and the overlying water column (Canfield, 1994). Particulate organic matter comprised of carbohydrate, protein, nucleic acid and lipid molecules is remineralised in different steps by different microorganisms as depicted in Figure 1.2 (Arnosti, 2004; Arnosti, 2011). Initial degradation of sedimentary POM involves microbial production of extracellular ezymes and occurs via extracellular hydrolysis of macromolecules. Hydrolysis begins a cascade of remineralization reactions and results in smaller products for direct microbial uptake (Arnosti and Jørgensen, 2003; Brüchert and Arnosti, 2003). The products of hydrolysis are mono-,di- and small oligomers, e.g. sugars, amino acids, long chain fatty acids, which are taken up mostly by fermentative bacteria. Fermenters oxidize these compounds further to volatile fatty acids (VFAs) and hydrogen, which can be taken up by terminal oxidizers.

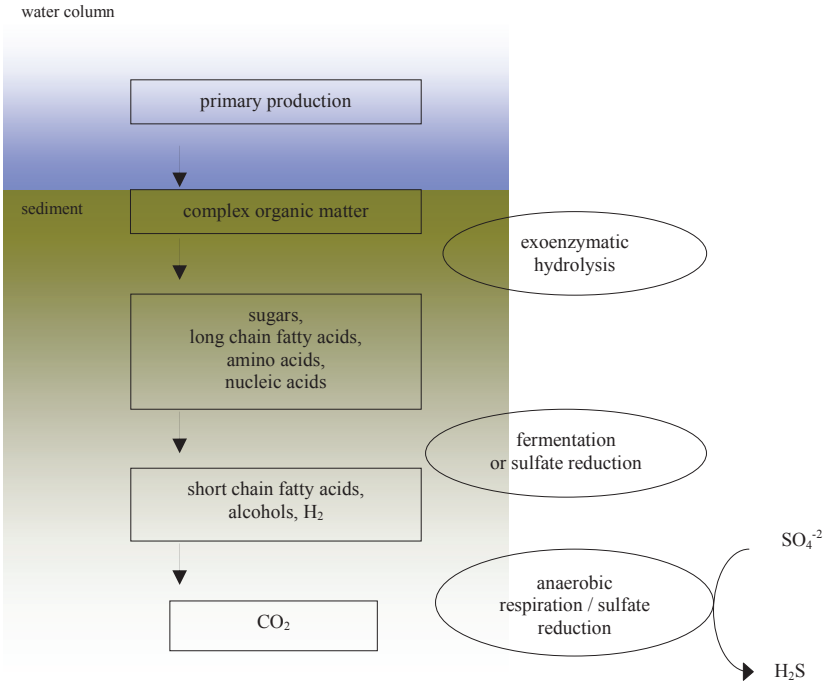

Figure 1.2 Scheme showing some major processes in the sedimentary carbon cycling, including sulfate reduction as an example for anaerobic respiration (modified from Hubert et al., 2010).

Microorganisms respire organic matter to CO_2 using oxidants in the sequence corresponding to a gradual decrease in redox potential of the oxidant. Thus, free energy yield is decreasing with the different electron acceptors. Oxygen, when present, is energetically favored over other oxidants and is the first to be consumed. Next, nitrate, Mn/Fe oxides and sulfate are successively consumed in the degradation of any remaining organic matter (Schulz et al., 2006). Depending on the primary productivity in the overlying water column, oxygen can be depleted within the first upper centimeters of sediments. Oxygen is the most important electron acceptor in the oligotrophic seafloor of the deep ocean. In upwelling areas and oxygen minimum zones present at continental margins, sulfate is the main electron acceptor accounting for up to 50% of carbon mineralization (Jørgensen, 1982). Once sulfate is depleted,

terminal oxidation is performed by methanogenic archaea producing methane and/or CO_2, depending on the substrate.

Sulfate-reducing bacteria (SRB) consume organic carbon often in the form of volatile fatty acids (VFAs), such as acetic acid. Under *in situ* conditions, sulfate reduction and fermentation are usually well balanced as evidenced by low concentrations of the intermediates, VFAs and H_2 (Finke and Jørgensen, 2008). Under steady state conditions, H_2 concentrations are kept at a minimum, controlled thermodynamically, while VFAs concentrations do not appear to be thermodynamically controlled but usually remain in the lower micromolar range (Christensen and Blackburn, 1982; Wu and Scranton, 1994; Wellsbury and Parkes, 1995). The actual mechanisms controlling VFA concentrations in marine pore waters remain poorly understood. The extent to which organic matter reaching the sediment is remineralised depends largely on its quality, thus if the microorganisms can access and degrade organic matter deposited on the seafloor (Canfield, 1994).

2. Temperature influence on carbon cycling in marine sediments

2.1. Temperature as controlling factor for sedimentary organic carbon mineralization

Temperature is an important environmental factor influencing most biochemical reactions; it affects the rates of bacterial growth and respiration, and therefore exerts a selective pressure in the environment. Increasing the temperature usually causes a chemical reaction to proceed at more rapid rates while the reactions are slowed down when temperature decreases (Arrhenius, 1908). Field studies on temperate sediments revealed the seasonality of respiration rates and showed that temperature is the main controlling factor as metabolic rates increase in summer season and are low in winter (Moeslundi et al., 1994; Rysgaard et al., 2004; Al-Raei et al., 2009). Decreases in respiration rates in the winter season in temperate

environments led to the suggestion that microbial metabolism is low in cold environments (Pfannkuche and Thiel, 1987). However, in polar regions during summer season, after the spring phytoplankton bloom when permanently cold sediments receive a large amount of organic matter, the efficiencies and rates of benthic mineralization can be as high as those measured in tropical and temperate environments (Arnosti et al., 1998; Kostka, 1999; Robador et al., 2009). The amount of carbon that is preserved in cold sediments through burial is similar to temperate coastal sediments with similar sedimentation rates (Kostka, 1999). Clearly, low temperature does not inhibit microbial activity in cold sediments and organic carbon input exerts major control on the rate of organic matter mineralization (Kostka, 1999).

2.2. Thermal adaptation groups of bacteria and their adaptive strategies

Microorganisms have responded to selective pressure imposed by temperature by evolving various thermal adaptations that allow them to degrade organic matter efficiently in different climate zones. Based on the temperature response of growth, different temperature groups of bacteria are defined according to their cardinal temperatures, i.e., the minimum temperature (T_{min}) or maximum temperature (T_{max}) that limits bacterial activity, and the optimum temperature (T_{opt}) at which the highest rates are supported. Above T_{opt} process rates drop steeply, which is due to enzymatic denaturation and other physiological malfunctioning of the cells and shows that this is a biologically catalyzed process (Feller and Gerday, 2003). According to Morita (1975), psychrophilic bacteria have $T_{min}<0°C$, $T_{opt}\leq15°C$, and $T_{max}\leq20°C$. Psychrotolerant bacteria have $T_{min}\leq0°C$, $T_{opt}\leq25°C$, and $T_{max}\leq35°C$. Mesophilic bacteria have $T_{min}>0°C$, T_{opt} at 25-40°C, and T_{max} at 35°C-40°C.

As is typical for microbial processes, T_{opt} for respiration and growth of microorganisms is generally found to be well above the *in situ* temperature (Isaksen and

Jørgensen, 1996; Knoblauch and Jørgensen, 1999; Dunker et al., 2010). In cold environments the T_{opt} for anaerobic respiration is up to 10°C higher than the T_{opt} for growth. In temperate environments, dominated by mesophilic microorganisms, the discrepancy between T_{opt} of microbial metabolism and the *in situ* temperature of the sediments is smaller. Around hydrothermal vents and in coastal areas with volcanic activity T_{opt} of microbial metabolisms was found to be near the *in situ* temperature (Jørgensen et al., 1992; Stetter et al., 1993). These observations indicate that microbial metabolism is better adapted to the *in situ* temperature in extremely hot environments rather than permanently cold conditions.

The influence of environmental temperatures controls the presence of thermally adapted, active microorganisms. The bacteria living in different climate regions evolved adaptations to a broad range of temperature to overcome the purely chemical effect of varying temperatures. Cold adapted, psychrophilic and psychrotolerant microorganisms posses physiological adaptations to cope with low temperature. Compared to mesophilic and thermophilic bacteria, psychrophilic bacteria synthesise a higher proportion of short-chained unsaturated fatty acids and shorten chains in the membrane lipids to increase cell membrane fluidity (D'Amico et al., 2006). Enzymes of psychrophilic bacteria have high specific activities at low temperatures, often up to an order of magnitude higher than the enzymes of their mesophilic counterparts (Feller, 2003). Also, the cellular transcription and translation apparatus in psychrophiles is modified to aid protein synthesis at low temperatures (D'Amico et al., 2006). The evolution of cold adaptations allow psychrophilic and psychrotolerant microorganisms to maintain high metabolic activity despite constraints imposed by low temperature or drastic temperature fluctuations that accompany freeze–thaw conditions. Numerous studies have reported that in soils microorganisms thrive and are capable of growth below freezing point, at temperatures as low as -20°C (Junge et al., 2003; Rivkina et al., 2004;

Gilichinsky et al., 2005). It remains unresolved, however, whether marine microorganisms are active when marine sediment is frozen. In Manuscript 1 of this thesis activity of sulfate-reducing bacteria was measured in frozen sediments.

2.3. Temperature sensitivity of functional groups within microbial food chains

Microbial functional groups involved in the sequential remineralization of organic carbon, e.g., hydrolyzers, fermenters, terminal oxidizers have different temperature sensitivities. Consequently, carbohydrate hydrolysis was found to have a different temperature response than sulfate reduction, with similar T_{opt} but a higher T_{max} in both permanently cold and temperate environments (Arnosti et al., 1998). Acetate production from complex organic matter in coastal sediment showed a higher temperature optimum and maximum than sulfate reduction from comparable temperate sites (Wellsbury et al., 1997; Arnosti et al., 1998). In temperate environments Weston and Joye (2005) observed a greater temperature sensitivity of the sulfate reduction process than for the processes of hydrolysis and fermentation of complex organic matter. While hydrolysis and fermentation were not disturbed by low temperature, the activity of SRB was inhibited because they could not uptake VFAs, produced by fermenters, hence VFAs accumulated. In the summer season VFAs were quickly consumed by sulfate reducing bacteria again and SRB activity was limited by low concentrations of VFAs (Weston and Joye, 2005). This transient uncoupling between sulfate reduction and fermentation by a temperature increase remains poorly understood. Different temperature sensitivity could lead to altered carbon flow under increased temperature conditions. Further studies on the response of sequential processes involved in organic matter remineralization to environmentally relevant temperature scenarios will help predict the effects of climate change on sedimentary

carbon cycling. In the Manuscript 1 of this thesis the freeze-thaw effects on fermentation and sulfate reduction processes was measured.

2.4. Different thermal groups of microorganisms on a global scale

Microorganisms are able to thrive and even grow at temperatures from below freezing to greater than boiling (Morita, 1975; Kashefi and Lovley, 2003). In different climatic regions the dominance of thermally adapted groups of bacteria dictate, together with the supply of labile organic matter, the rate of organic matter degradation in sediments (Kostka, 1999). Hence, psychrophiles and psychrotolerant have been found in permanently cold sediments and mesophiles predominantly in warmer shelf sediments at lower latitudes (Arnosti et al., 1998; Kostka, 1999). However, the coexistence of microorganisms with unexpected thermal adaptations is often reported, e.g., in permanently cold or temperate environments which shows that the temperature of the habitats is not always the last factor determining the presence of different temperature classes of microorganisms (Isaksen et al., 1994; Hubert et al., 2009). Bacteria that grow effectively in temperate environments and function at temperatures extending into the mesophilic range can be isolated from the cold deep-sea floor (Finster and Bak, 1993; Chen et al., 2003; Stein and Macdonald, 2004; Aono et al., 2010). Interestingly, the cold Arctic sea-bed includes thermophilic bacteria that are not metabolically active there (Isaksen et al., 1994; Hubert et al., 2009). Hubert and colleagues suggest that thermophiles are delivered to the cold sediments by seabed fluid flow from warm subsurface petroleum reservoir and ocean crust ecosystems (Hubert et al., 2009).

The Baas-Becking hypothesis “the environment selects” explains spatial distribution of microbial diversity. It can also be used when studying the distribution of thermal groups of microorganisms (De Wit and Bouvier, 2006). Similar to biogeography of microorganisms –

my studies aimed to establish the patterns of occurrence of thermal groups of microorganisms on a global scale. In the Manuscripts 2 and 3 the distribution of thermal adaptation groups of microorganisms was surveyed in sediments derived from different climatic regions.

3. Global change affects carbon flow and microorganisms distribution

Physical, chemical and biological characteristics of Earth are determined mainly by the oceans (Falkowski et al., 2000). Climate change is altering ocean ecosystems (Hoegh-Guldberg and Bruno, 2010). Particularly affected are high latitude environments. The Arctic Ocean accounts for 20% of the world's continental shelves and burial of organic carbon in the Arctic Ocean may account for ca. 7 to 11% of the global budget (Stein and Macdonald, 2004; Rachold et al., 2005). It is suggested that a warming of Arctic surface waters by even a few degrees could lead to substantially more carbon and other elements being processed by the microbial loop resulting in lower incorporation to higher trophic levels and therefore decreased export to the deep sea and the benthos (Kirchman et al., 2009). In the Bering Sea region decreased organic carbon fluxes to the seafloor and lower benthic respiration rates might be observed due to diminishing ice shelves and disappearance of associated unique microbial ecosystems (Grebmeier et al., 2006).

Robador et al. (2009) showed that two-year incubation at increased temperature (10°C and 20°C) had a pronounced effect on rates of sulfate reduction as well as on the composition of the sulfate-reducing community (Robador et al., 2009). Follow-up studies revealed that after two years of incubation at increased temperature (10°C and 20°C) DOC concentration in sediments increased whereas VFA levels were low and sulfate reduction rates were comparable to the rates measured before the incubation started. Robador and colleagues suggested that over time DOC becomes refractory and unavailable to microorganisms (Robador et al., 2010). It was hypothesized that the net accumulation of DOC in warming

marine sediments could be related to a change in the composition of the microbial community in response to the permanent temperature increase. The effects of increased temperature during a one-year incubation on microbial community composition in Arctic and temperate sediments are described in the Appendix in this thesis.

It is predicted that not only will carbon flow be affected by global warming, but also spatial distributions of psychrophilic and psychrotolerant microorganisms in marine environments. Microbial 'generalists' may displace many of the resident 'specialists' and the decline in different cold habitats (ice types etc.) will limit the number of potential niches for them. It is likely that in the future in the presently warming regions such as the Arctic, psychrotolerant bacteria with broad thermal tolerances will flourish at the expense of psychrophiles partially due to the temperature increase, but also due to the ice decline and loss of niches (Vincent, 2010). In Manuscript 2 of this thesis we suggest that mesophilic sulfate-reducing bacteria are transported along the continental slope down to the deep-sea and leave their signature in the temperature profile of sulfate reduction.

4. Aims and outline of the present study

Most climate change scenarios predict not only a general warming trend, but also an increased variability in weather conditions (IPCC, 2007), including alterations in precipitation and thawing patterns, which will lead to more variable soil and sediment conditions (Groffman et al., 2001). The rapid climate change causes the Arctic Ocean to shift towards new states, with implications for food webs and biogeochemical fluxes. The impact of rising temperature on microbial community composition needs to be addressed, as it is unclear whether altered microbial communities influence the rate of carbon remineralization. Another significant component of the global carbon cycle is coastal permafrost. Many Arctic coastlines are currently in transition as rising sea level inundates and thaws coastal permafrost

(Rachold et al., 2000). At present, organic matter mineralization rates in thawing permafrost are not well quantified, but are likely critically dependent on the reactivation and recovery of bacteria. The effects of freeze-thaw on the anaerobic carbon mineralization in marine sediments need to be investigated to quantify the rate organic matter degradation under these conditions.

1) Manuscript I The purpose of this study was to examine the effect of freeze-thaw on anaerobic carbon mineralization processes by subjecting natural communities of marine bacteria in seasonally freezing arctic sediment to different freeze-thaw treatments. The study was based on the hypothesis that moderate freeze-thaw treatment does not affect organic matter degradation, whereas drastic freeze-thaw scenarios decrease rates of organic matter degradation.

2) Manuscript II The second study assessed a) to which extent the temperature responses of the microbial communities reflect the *in situ* temperature and b) whether their cardinal temperatures are the result of a narrow adaptation to *in situ* temperature or rather reflect mixed communities of different temperature groups. The temperature dependence of ^{35}S-sulfate reduction rates (SRR) in shelf and slope sediments from the South Atlantic (SA) off the coast of Namibia, Uruguay and Argentina were compared with those in permanently cold shelf sediments of Svalbard in the Arctic Ocean. The study hypothesized that a mixed microbial community of different temperature groups would be present in all studied sediments.

3) Manuscript III The aim of the third study was to investigate the physiological adaptation of the sulfate-reducing bacterial community to environmental sediment temperatures, expressed in sulfate reduction rates as an important mechanism controlling ultimately, the efficiency of carbon cycling. In order to understand the effect that ambient temperatures may have on the microbial carbon cycling in marine sediments, the temperature dependencies of the SRB

community in sediments from different latitudes were compared using temperature gradient incubation experiments. It was hypothesized that the temperature response of sulfate reduction is correlated with the temperature of the environment.

4) Appendix The fourth study provides preliminary results on the response of sedimentary microbial community composition from permanently cold and temperate sediments to one year exposure to increased temperature (4°C, 10°C and 20°). This study is based on the hypothesis that elevated temperature affects microbial community composition in Arctic sediment, but may have no effect on the community composition in temperate sediment.

PART II Results and discussion

5. Freeze-thaw effects

5.1. Reactivation under freeze-thaw conditions and tolerance of different physiological groups to freeze-thaw cycles

Freeze-thaw events affect the activity and population dynamics of microorganisms in sediments and soils because strong fluctuations in temperature can damage or destroy microbial cells and disrupt cell aggregates (Mountfort et al., 2003; Schimel and Mikan, 2005; Sharma et al., 2006; Walker et al., 2006; Yergeau and Kowalchuk, 2008; Männistö et al., 2009). Our results demonstrate that freezing temperatures in freeze-thaw regimes temporarily eliminate bacterial activity, but that sulfate-reducing microorganisms can resume active carbon cycling shortly after thawing of the sediment (Figure 2.1 a). Several studies on soil bacteria have demonstrated detrimental effects of freeze-thaw event on microbial communities (DeLuca et al., 1992). Such a treatment killed up to 50% of the microbes in the first freeze-thaw cycle and irreversibly reduced the soil DNA content by 33% (Pesaro et al., 2003). Arctic soil mesocosm studies showed that microbial respiration remained at a high level in multiple diurnal freeze-thaw cycles although the microbial biomass declined (Larsen et al., 2002). Continued activity in cyclical freeze-thaw experiments seems to be influenced by the ability of the microbial community to metabolize substrates released by the freeze-thaw treatments (Schimel and Mikan, 2005). In our experiments sulfate-reducing bacteria survived in the sediment that was gradually frozen and thawed, as SRR changed with each successive temperature shift between 4ºC and -5 ºC (Figure 2.1 a). Low concentrations of VFA during gradual freeze-thaw incubations (Figure 2.1 b) suggest that the coupling between fermentation and sulfate reduction was maintained during this experiment. These incubation conditions, therefore, seemed to recreate the normal situation in arctic sediment, that is, low

concentrations of VFAs (Figure 2.1 b) due to close coupling between fermentation and terminal oxidation processes (Finke and Jørgensen, 2008; Robador et al., 2009).

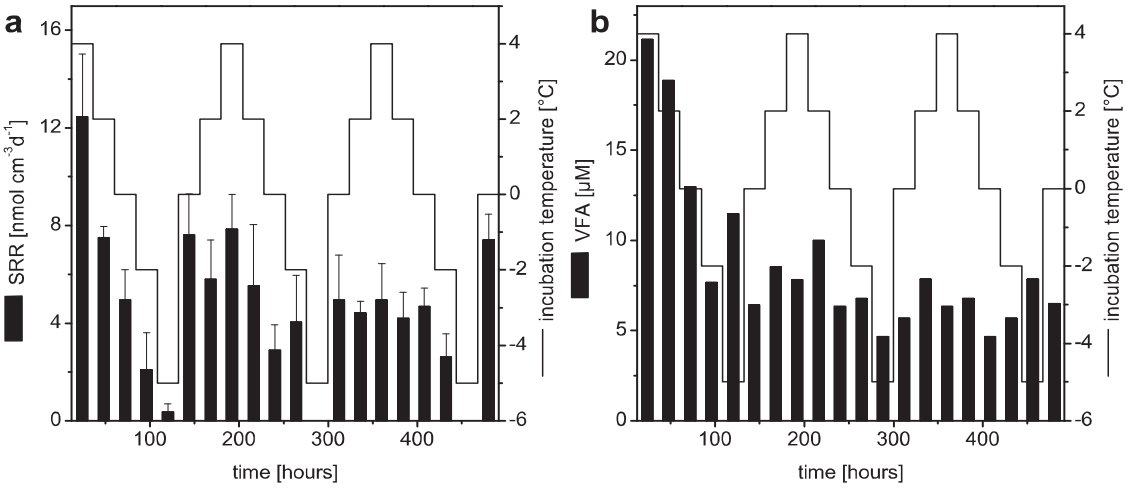

Figure 2.1 a) The sulfate reduction rates (SRRs) during the gradual freeze-thaw experiment. The black line corresponds to different temperatures applied during the experiment. Black bars correspond to SRR measured every 24 h, at respective temperatures. b) Concentrations of volatile fatty acids (VFAs) determined in the gradual freeze-thaw experiment. The analytical error for the summed VFAs concentration is about 6%. The black lines correspond to different temperatures applied during the experiment. Black bars correspond to VFAs levels measured every 24 h, at respective temperatures.

In our multiple freeze-thaw incubations, three successive freeze-thaw cycles, resulted in a decrease in SRR by 80%, however, those sulfate reducers which survived this treatment were able to resume without delay when the sediment was thawed again (Figure 2.2 a).

During multiple freeze-thaw cycles concentrations of VFAs gradually increased during the experiment (Figure 2.2 b). This suggests that detrimental effects of freeze-thaw cycles may have been greater for sulfate reducers than for fermentative microorganisms. These effects suggest major changes in the pathways of carbon processing under repeated freeze-thaw cycles. However, the long-term fate of VFAs that accumulate after freezing periods requires further exploration.

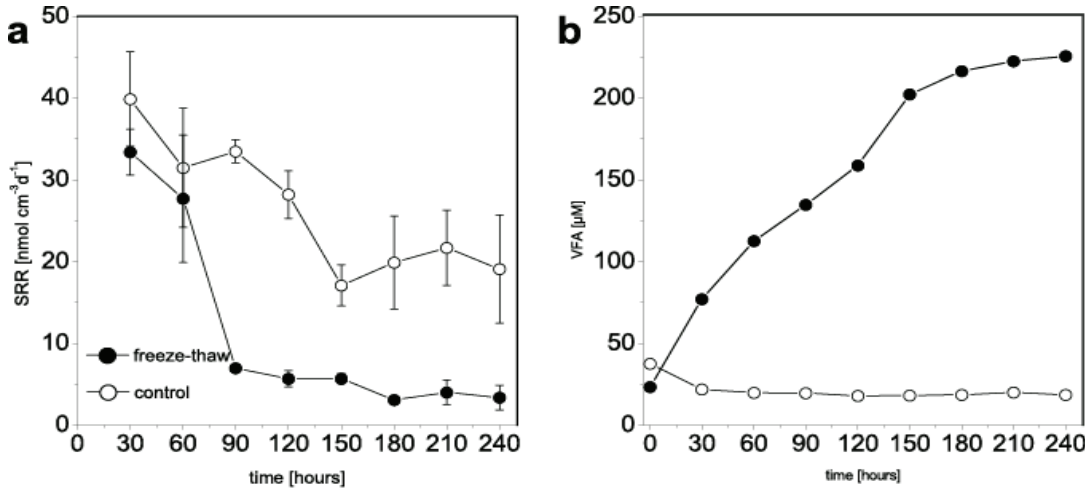

Figure 2.2 a) The sulfate reduction rates (SRR) associated with eight successive 30-h freeze-thaw cycles. Closed symbols correspond to SRR values measured during 18-h thaw phases (at 10°C) that followed 12-h frozen phases (at -20°C). Open symbols correspond to SRR values measured at the same corresponding time points in a control that was constantly maintained at 10°C. **b)** Volatile fatty acids (VFA) associated with eight successive 30-h freeze-thaw cycles. Closed symbols correspond to concentrations of VFA measured at same times as in the control experiment. The analytical error for the summed VFA concentration is about 9% as described in the text.

5.2. Microbial activity in the frozen state

Various studies have reported that microorganisms are capable of growth below the freezing point of water down to temperatures of -20°C (Gilichinsky et al., 1995). Our studies showed no clear evidence that sulfate reduction remained active at -20ºC. During the gradual freeze-thaw experiment rates of sulfate reduction was only detected in the first freeze cycle at -5ºC. This suggests that part of the microbial community, active initially during the first freeze period, was damaged during the subsequent freeze-thaw cycle. The lack of activity of the fraction of microbial community that was not damaged during the freeze-thaw event could be also related to changes in cell structure such as stiffen of membrane lipids with a consequent decrease of the efficiency of embedded protein transport (Nedwell, 1999; Ponder et al., 2005). At Antarctic sites Mountfourt et al. (2003) observed sulfate reduction decoupled from acetate oxidation and a shift of the carbon flow towards methanogenesis under freezing

conditions. The authors suggested that freezing provides a physical barrier that prevents access of the microbes to sulfate or reduces the affinity of sulfate reducers for their substrate.

6. Temperature response of SRR in different geographical regions

6.1. Temperature response of SRR in shelf and slope sediments depends on *in situ* T°C and water depth

Microorganisms adapt to local environmental temperatures. Our results demonstrate how the ambient temperature regime selects for different physiological temperature groups among the sulfate-reducing community. The temperature response of SRR in sediments from the shelf and slope off Argentina, Uruguay and Namibia and in Arctic intertidial flat and seafloor sediments was studied. The sediment temperatures of the Uruguay and Namibia shelf are controlled by ocean currents of different thermohaline characteristics that maintained sediment temperatures of 7-10°C (Hensen et al., 2003; Lass and Mohrholz, 2005; Ortega and Martinez, 2007). Similar temperatures were recorded during the Arctic summer for an intertidal mud flat of Svalbard where the air temperature during low tide may heat the surface sediments up to 9°C (Nørdli 2005). Accordingly, the temperature response of SRR in these sediments was in the psychrotolerant to mesophilic range, the T_{opt} was 25-30ºC, and the activity declined above 35ºC (Figure 2.3 a, b, j).

The *in situ* temperature of the South Atlantic sediments from greater water depths is lower compared to shelf sediments, and thus the T_{opt} and T_{max} decreases with increasing water depth (Figure 2.3 b, c, d, f). Temperature profiles from those deep stations indicate a predominance of psychrotolerant and even psychrophilic bacteria. In sediments from the Argentine Basin at approximately 3000 m water depth we measured a T_{opt} of 12°C after 36 hour incubation (Figure 2.3 e). A comparably low T_{opt} of 12.5°C for sulfate reduction had

been observed in Antarctic sediments from Kap Norvegia in the Weddell Sea (Isaksen and Jørgensen, 1996). This temperature optimum is the lowest published temperature optimum for an anaerobic microbial process in nature. The authors reasoned that the temperature profile resembled the response of psychrophilic isolates and likely reflected also the growth rate optimum of a predominantly psychrophilic community (Isaksen and Jørgensen, 1996).

The dependence of the temperature optima and temperature response on the water depth was not observed in the Arctic fjord sediments as the bottom water of the Svalbard fjords is permanently near 0°C. In our study with sediments from Smeerenburgfjorden a broad temperature profile was observed with T_{opt} at 27°C after 8 hour incubation (Figure 2.3 j). In an earlier study of these sediments the T_{opt} had been observed to drop with incubation time and was 21°C after 4.5 day incubation (Arnosti et al., 1998). A similar shift in temperature response was found by Finke and Jørgensen (2008) in Arctic sediment where the T_{opt} dropped from 27°C after 0.3 days to 18°C after 8 days of incubation.

The temperature characteristics of SRR imply the presence of mixed SRB communities composed of mesophilic, psychrotolerant and psychrophilic members in South Atlantic and Arctic sediments (Arnosti et al., 1998; Sahm et al., 1999; Rysgaard et al., 2004). In South Atlantic sediments the spatial distribution of distinct thermal groups was related to the *in situ* temperature of the sediment and consequently to the water depth. Thus, high T_{opt} of SRR on the shelf indicates a predominance of mesophilic and psychrotolerant SRB, whereas in the deeper sediments T_{opt} was lower and hence indicates the presence of SRB adapted to permanently cold conditions typical for psychrotolerant and psychrophilic microorganisms. Predominant temperature responses of SRR in South Atlantic sediments were psychrotolerant, which is consistent with earlier reports that psychrophiles do not prevail in permanently cold sediments (Nedwell, 1989).

6.2. Sediment transport effects on temperature-activity relationships

Deposited labile organic matter from the Benguela upwelling system at the Namibia shelf undergoes suspension and re-deposition leading to a net down-slope transport. The shelf material accumulates in depo-centers at 1000-1500 m water depth where the sediment is consequently rich in organic matter (Inthorn et al., 2006). This down-slope transport of sediment material from the warm shelf may explain the relatively high SRR and the presence of mesophilic SRB in the cold slope sediments (Figure 2.3 a, c). Although psychrotolerant or psychrophilic SRB may be better adapted to live in the slope sediments at the prevailing temperature of 3°C, the continuous downslope transport of SRB from the shelf enables the mesophilic community to be maintained.

A mesophilic signature was also observed in the temperature response of SRR from upper slope sediments off Argentina (Figure 2.3 d) whereas sediments from greater water depths had rather a psychrotolerant to psychrophilic signature (Figure 2.3 e, f). The slope sediments off Uruguay and Argentina are characterized by dynamic depositional conditions with generally high sedimentation rates, including gravity mass flows, and strong surface currents (Riedinger et al., 2005). Thus, these prevailing depositional conditions may be responsible for a redeposition of mesophilic SRB from the shelf and thus explain the occurrence of the microbes in the slope sediments.

6.3. Correlation between environmental temperatures and cardinal temperatures of sulfate reduction

Our results suggest a direct relationship between the ambient environmental temperature and sedimentary bacterial energy metabolism of sulfate-reducers reflected in the T_{opt} (Figure 2.4 A). Although T_{opt} generally exceeds the *in situ* temperatures experienced by the microbial

communities, the proportional increase with the mean ambient temperatures (Figure 2.4 A) implies diverse temperature sensitivities of the dominant microbial community studied in various environments. Arctic and Antarctic sediments exhibited T_{opt} for sulfate reduction of 24-26°C similar to those previously reported for some psychrophilic SRB isolates (Knoblauch et al., 1999). The T_{opt} observed in warmer temperate and tropical sediments, however, are in the range of those reported for nominal mesophiles (Isaksen and Jørgensen, 1996). Sediments from temperate latitudes showed broader thermal ranges than polar sediments and sulfate reduction could be measured from temperatures below 0°C up to the T_{opt} at 35°C (Figure 2.4 B). In tropical sediments T_{opt} for sulfate reduction of 38-44°C was in the higher thermal range (Figure 2.4 B). The difference between environmental temperatures and T_{opt} of bacterial sulfate reduction, however, varied between the sediments implying discrepancies in the adaptation of respiration to ambient temperatures. At *in situ* temperatures of 0°C in polar regions the difference was approximately 27°C, while at *in situ* temperatures of 30°C in tropical habitats this difference was reduced to 15°C (Figure 2.5 B).

Larger differences in the cardinal temperatures can be explained by composition of the active SRB in the sediment. In addition, around hydrothermal vents and in coastal areas with volcanic activity T_{opt} was found to be near the *in situ* temperature (Jørgensen et al., 1992; Stetter et al., 1990). These observations indicate that microbial metabolism may be better adapted to the *in situ* temperature in extremely hot environments rather than permanently cold conditions.

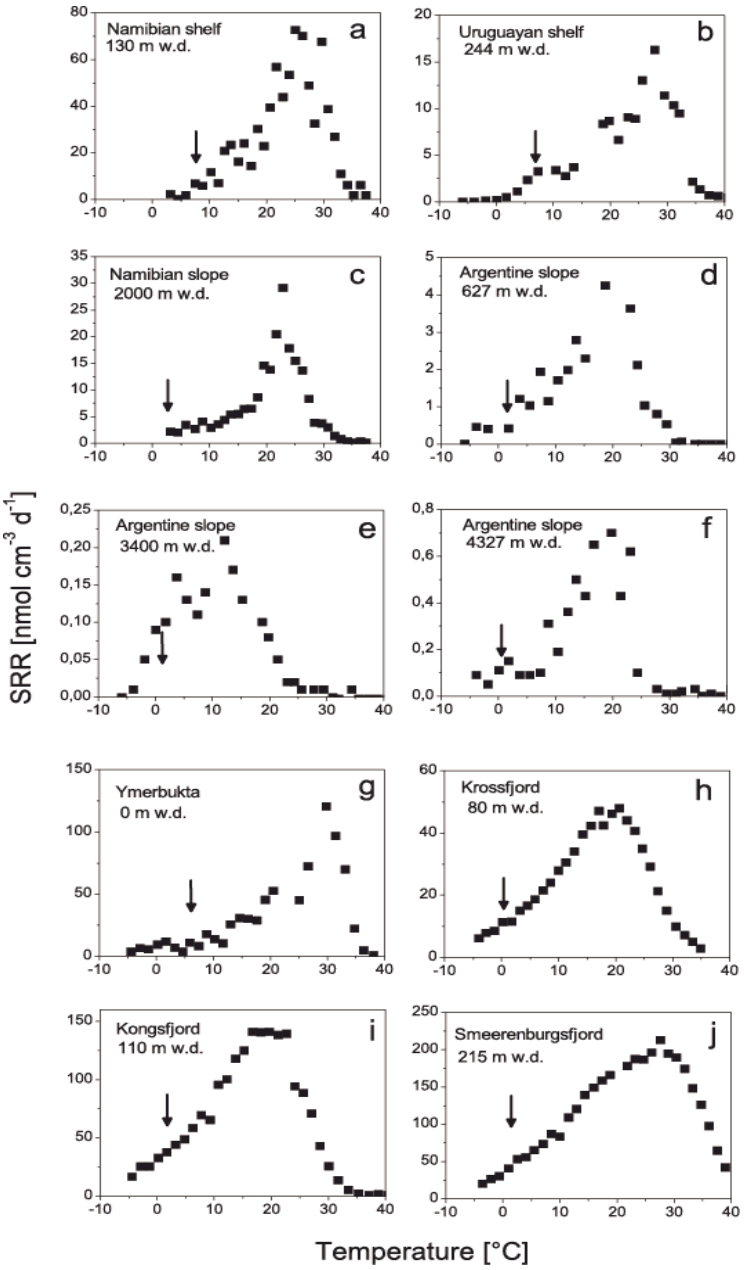

Figure 2.3 SRR measured in temperature-gradient incubation experiments of sediment slurries from the different sampling sites.

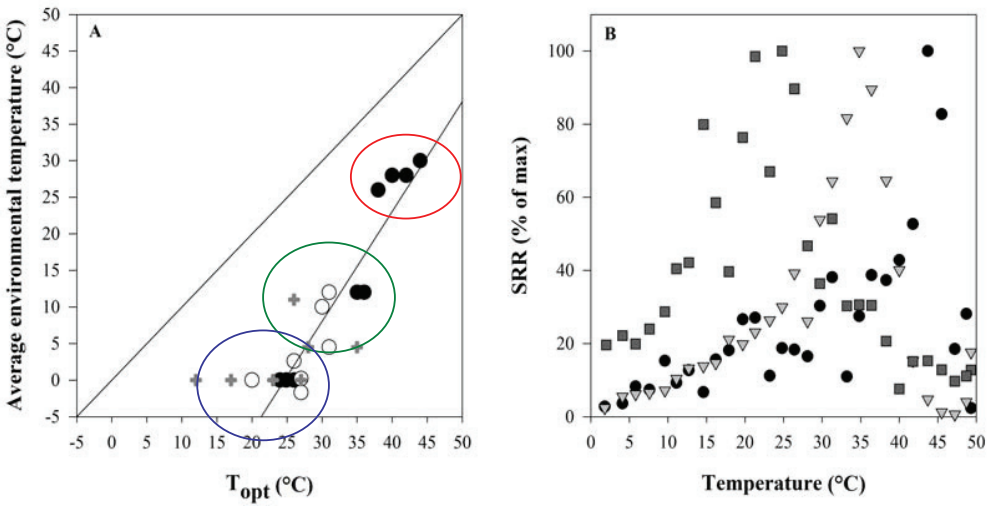

Figure 2.4 (A) Relations between average environmental temperatures and T_{opt} for sulfate reduction in marine sediments grouped according to sampling latitude: Polar regions, blue line; Temperate regions, green line; Tropical regions, red line. The plot is based on: data presented in this study, full circles; data compiled from Isaksen et al. (1994), Isaksen and Jørgensen (1996), Arnosti et al. (1998) and Sagemann et al. (1998), open circles. The straight line passing through the origin is the theoretical curve if environmental temperatures and T_{opt} for SRR were the same. The regression line indicates the empirical relation between environmental temperatures and T_{opt} for SRR. (B) SRR expressed as percentage of maximum rates. Squares: Arctic permanently cold sediment from Svalbard fjords; triangles: Wadden Sea sediment from estuary system subjected to strong seasonal temperature changes; circles: South Chine Sea permanently warm sediment. Profiles were selected to represent the characteristic temperature responses of each group in panel A.

7. The effect of increased temperature on microbial community composition in Arctic and temperate sediments

Increasing temperature may cause changes in microbial community composition and thus strongly influence microbial carbon cycling in the Arctic Ocean. In our studies we observed shifts in microbial community composition as a result of prolonged incubation at elevated temperatures (4°C, 10°C and 20°C), both in permanently cold (annual *in situ* temperature, 2°C) and temperate sediments (average annual temperature, 15°). The disappearance of some species in the Arctic sediments and appearance of new ones after a year of incubation at elevated temperature suggest that richness of microbial community might change due to perturbations. Previous studies demonstrated the steady decrease of microbial cell numbers and specific groups of SRB to the total microbial numbers when Arctic sediment was exposed

to two year incubation at increased (10°C and 20°C) temperature (Robador et al., 2009). This implies that a large fraction of the community was negatively affected by the 10°C and 20°C long-term incubation temperatures. In contrast such a change was not observed in temperate sediment samples (Robador et al., 2009).

It is unknown whether compositional shifts forced by increased temperature will affect ecosystem processes and whether the disturbed community will be functionally similar to the original community (Reed and Martiny, 2007; Allison and Martiny, 2008). The loss of an entire functional group would clearly impact the functioning of an ecosystem (Reed and Martiny, 2007). On the other hand, some species in a microbial community can be functionally redundant, thus the functioning of ecosystem might not be affected by their disappearance (Reed and Martiny, 2007; Allison and Martiny, 2008). To help predict carbon cycling under changing environmental conditions long term studies on the microbial community composition are needed.

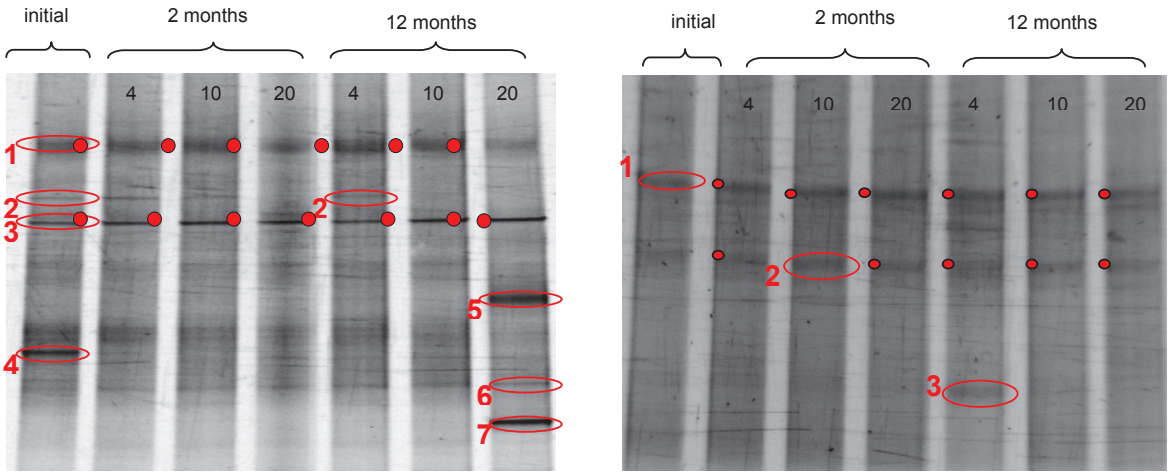

Figure 2.5 DGGE profiles for 16S rRNA gene fragments obtained from DNA extracted from Arctic and temperate sediment samples incubated for different times at increased temperatures. Numbers on the lanes are temperatures at which the sediments were incubated (4°C, 10°C, and 20°C). Circles and numbers mark bands for potential further analysis.

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PART III Manuscripts

Resulting Manuscripts from this thesis work and contributions

1. **Effects of freeze-thaw cycles on anaerobic microbial processes in an Arctic intertidal mud flat.**
   JE. Sawicka, A. Robador, C. Hubert, BB. Jørgensen, V. Brüchert
   This study was designed by JE. Sawicka, A. Robador, BB. Jørgensen and V. Brüchert; sediment incubation experiments and measurements were conducted by JE. Sawicka. The manuscript was written by JE. Sawicka with input from other coauthors.
2. **Temperature characteristics of bacterial sulfate reduction in continental shelf and slope sediments.**
   JE. Sawicka, BB. Jørgensen, V. Brüchert
   This study was designed by JE. Sawicka, BB. Jørgensen and V. Brüchert; sediment incubation experiments and estimation of SRR were conducted by JE. Sawicka and BB. Jørgensen. The manuscript was written by JE. Sawicka with input from other coauthors.
3. **Adaptation of sulfate-reducing bacteria to ambient sediments temperatures in polar, temperate and tropical marine environments.**
   A. Robador, V. Brüchert, JE. Sawicka, BB. Jørgensen
   Original idea by BB. Jørgensen; this study was designed by A. Robador, V. Brüchert and BB. Jørgensen; sediment incubation experiments and estimation of SRR were conducted by A. Robador. Elemental analyses of sediments were conducted by JE. Sawicka. The manuscript was written by A. Robador with input from other coauthors.
4. **Temperature effects on the microbial community composition in Arctic and temperate marine sediments.**
   JE. Sawicka, V. Brüchert.
   The study was designed by V. Brüchert. The sediment incubations and measurements were conducted by JE. Sawicka. The manuscript was written by JE. Sawicka.

Manuscript I

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ORIGINAL ARTICLE

Effects of freeze–thaw cycles on anaerobic microbial processes in an Arctic intertidal mud flat

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Insight into the effects of repeated freezing and thawing on microbial processes in sediments and soils is important for understanding sediment carbon cycling at high latitudes acutely affected by global warming. Microbial responses to repeated freeze–thaw conditions were studied in three complementary experiments using arctic sediment collected from an intertidal flat that is exposed to seasonal freeze–thaw conditions (Ymerbukta, Svalbard, Arctic Ocean). The sediment was subjected to oscillating freeze–thaw incubations, either gradual, from −5 to 4 °C, or abrupt, from −20 to 10 °C. Concentrations of low-molecular weight carboxylic acids (volatile fatty acids) were measured and sulfate reduction was assessed by measuring ^{35}S sulfate reduction rates (SRRs). Gradual freeze–thaw incubation decreased microbial activity in the frozen state to 0.25 % of initial levels at 4 °C, but activity resumed rapidly reaching >60 % of initial activity in the thawed state. Exposure of sediments to successive large temperature changes (−20 versus 10 °C) decreased SRR by 80% of the initial activity, suggesting that a fraction of the bacterial community recovered rapidly from extreme temperature fluctuations. This is supported by 16S rRNA gene-based denaturing gradient gel electrophoresis profiles that revealed persistence of the dominant microbial taxa under repeated freeze–thaw cycles. The fast recovery of the SRRs suggests that carbon mineralization in thawing arctic sediment can resume without delay or substantial growth of microbial populations.
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Introduction

Annual freezing and thawing are common features of high-latitude sediments and soils. Arctic marine coastal environments, such as intertidal mud flats, are exposed to freeze–thaw events in spring and in fall. Shallow-water shelf sediments cover more than 50% of the Arctic Ocean (Jakobsson *et al.*, 2002)—a region sensitive to temperature increases due to climate change. It has been predicted that the warming of arctic environments will thaw terrestrial and drowned submarine permafrost, which may lead to substantial activation of resident microbiota (Schuur *et al.*, 2009). Studies on freeze–thaw cycling and its effects on marine microbial processes have not been conducted for coastal marine sediments. The Svalbard archipelago contains intertidal sediments that freeze periodically at the turn of seasons. Sediments from this archipelago have been the subject of extensive microbial ecology and biogeochemical studies on temperature adaptation (for example, Arnosti *et al.*, 1998; Sagemann *et al.*, 1998; Ravenschlag *et al.*, 2000; Finke and Jørgensen, 2008); however, the effect of freeze–thaw cycles on microbial communities in this environment is unknown.

Freeze–thaw events affect the activity and population dynamics of microorganisms in sediments and soils because strong fluctuations in temperature can damage or destroy microbial cells and disrupt cell aggregates (for example, Schimel and Clein, 1996; Eriksson *et al.*, 2001; Sharma *et al.*, 2006; Mountfort *et al.*, 2003; Schimel and Mikan, 2005; Walker *et al.*, 2006; Yergeau and Kowalchuk, 2008; Männistö *et al.*, 2009). This phenomenon has been studied in soils, in which freezing elevates the salinity while lowering water and nutrient availability (Eriksson *et al.*, 2001; Sharma *et al.*, 2006; Yergeau and Kowalchuk, 2008). Nutrients that are released from aggregates during thawing become

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available to microorganisms that survive freezing, resulting in temporary stimulation of microbial activity that had been low or negligible in the frozen state (Schimel and Clein, 1996; Pesaro *et al*., 2003; Grogan *et al*., 2004). Sharma *et al*. (2006) showed that at the turn of winter to spring, freeze–thaw cycles enhanced denitrification and caused a surge in N_2O and CO_2 emissions from soil. However, experiments designed to simulate the effect of intermittent warm Chinook winds in western Canadian soils indicated that repeated freeze–thaw cycles substantially decreased the viability of microorganisms (Walker *et al*., 2006). Statistical analysis of DNA- and RNA-based molecular fingerprinting of Antarctic soil microbial community also showed that frequent freeze–thaw cycles decreased the abundance of 16S rRNA genes and changed the microbial community diversity (Yergeau and Kowalchuk, 2008).

The purpose of this study was to subject natural communities of marine bacteria in seasonally freezing arctic sediment to different freeze–thaw treatments and to examine the effect on anaerobic carbon mineralization processes. Incubation experiments were conducted using sediment from an intertidal flat in the Svalbard archipelago to measure microbial sulfate reduction rates (SRRs) and concentrations of volatile fatty acids (VFAs). Different temperature regimes and freeze–thaw gradients were applied to simulate different scenarios experienced by this sediment annually.

Materials and methods

Sampling site

Sediment was collected from Ymerbukta, an intertidal flat in a shallow embayment in Isfjorden (78°16′61N, 014°02′69E) on the west coast of the Svalbard archipelago. This site freezes in the fall when the air temperature in Isfjorden drops to as low as −20 °C, and thaws in the following summer, when it reaches temperatures as high as 9 °C (Nordli, 2005). Samples were collected in August 2006 and August 2007. The temperature of the sediment at the time of samplings was 6.5 °C and the air temperature was 6.5 °C. For *in situ* measurements, 26-mm diameter sediment cores penetrating to 16 cm depth were sealed at both ends with rubber stoppers, leaving air in the headspace, and stored at 4 °C. For sediment and slurry incubation experiments, samples were collected at low tide from the zone of highest sulfate reduction (3–9 cm depth; Figure 1a). Sediment was stored in gas-tight polyethylene bags at 4 °C until further processing in the laboratory.

Freeze–thaw experiments

To simulate freeze–thaw cycles, sediment was subjected to three different time course experiments with different temperature amplitudes and time periods.

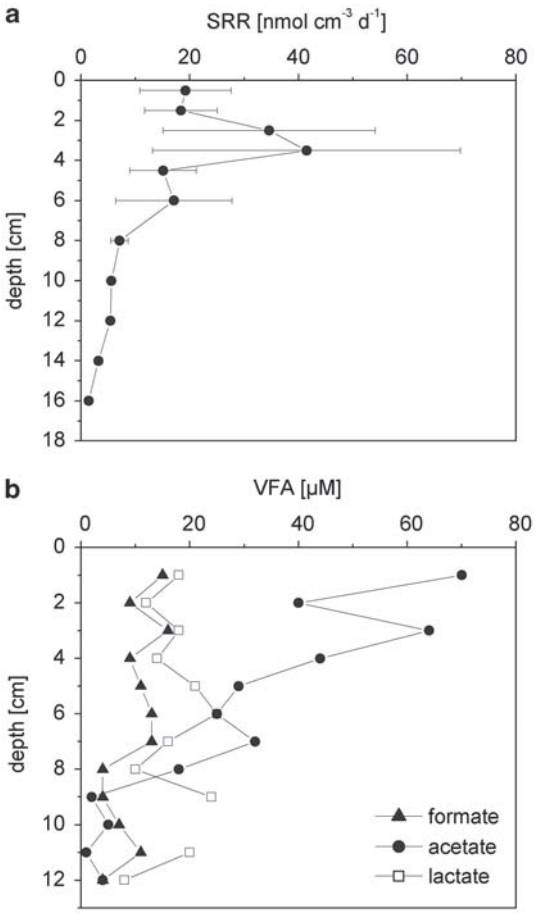

Figure 1 (**a**) Depth profile of sulfate reduction rates (SRRs) in Ymerbukta sediment. The SRR values were determined at 7 °C, error bars correspond to SRR values measured in duplicate cores. (**b**) Concentrations of volatile fatty acids (VFAs) in Ymerbukta sediment.

Gradual freeze–thaw incubation

The purpose of this experiment was to simulate a natural freeze–thaw process in the sediments. At the turn of seasons, sediments are likely to experience freeze–thaw event that happens over 24-h cycles. A total of 150 g of sediment was mixed with anoxic seawater medium (Widdel and Hansen, 1991) in a weight ratio of 1:2. The slurry was distributed under N_2 into 15-ml Hungate tubes (3 ml) (Ochs GmBH, Bovenden/Lenglern, Germany) and 200-ml Duran culture bottles (100 ml) (DURAN Group GmbH, Wertheim/Main, Germany) and sealed with butyl rubber stoppers. To simulate slow freezing and subsequent thawing conditions, sediment slurries were subjected to stepwise temperature changes over 20 days. The incubation temperature was consecutively lowered in 24-h intervals from 4 to 2, 0, −2 and −5 °C, and was subsequently increased from −5 to 0, 2 and 4 °C in the thawing phase. The SRR values were determined for each time interval.

The Hungate tubes and culture bottles were incubated in a temperature-regulated water bath filled with dilute antifreeze liquid for convenient

temperature manipulation. To determine the SRR value, 20 µl of ^{35}S-sulfate tracer (100 kBq) was injected into triplicate Hungate tubes that were incubated for 24 h before the change in temperature. The SRR value, therefore, represents the average rate for a 24-h interval. In addition, VFA concentrations were measured in 4-ml sub-samples removed from the 200-ml culture bottles at the end of each 24-h time interval.

Long-term freeze–thaw incubation

This experiment mimicked mid-winter to mid-summer difference (frozen and thawed, respectively) and explored the scale and rate of recovery of bacteria from frozen state. Undiluted, homogenized sediment was incubated for 12 weeks alternately at −20 °C for 3 weeks and at 10 °C for 3 weeks. Sediment was sub-sampled from the polyethylene bag into 5-ml glass cylinders in a nitrogen-filled Two-hand AtmosBag (Sigma-Aldrich, Steinheim, Germany) to maintain anoxic conditions. The cylinders contained ca. 3 ml sediment and were sealed at both ends with butyl rubber stoppers. Before each sediment extraction, the bag was homogenized for 10 min by manual kneading. After the sub-samples were taken the sediment tubes were kept for a week at 0 °C before the start of the 12-week incubation. This period was necessary to allow SRR to decrease again after a temporary stimulation due to sediment mixing—a phenomenon that has been observed previously (Finke and Jørgensen, 2008). After each freeze and thaw period, three tubes were removed to determine sulfate concentration, SRR and the total cell number by 4,6-Diaminodino-2-phenylindole (DAPI) staining. All analyses were determined in triplicates. Additional sub-samples were incubated under the same conditions for denaturing gradient gel electrophoresis (DGGE) profiling of the bacterial community. To determine the SRR value in this particular experiment, 5 µl of ^{35}S-sulfate tracer containing 500 kBq was injected into sub-samples at the start of the 12-week incubation period. Samples were incubated with tracer for three, six, nine and twelve weeks, respectively. As a control assay, sediment was sterilized by autoclaving for 25 min at 120 °C and was then incubated with the same amount of tracer at 10 °C. Four additional control incubation experiments were initiated with sulfate radiotracer added to sediments that were immediately frozen, incubated frozen for 1, 2, 5 and 10 weeks, and thawed only before fixation in zinc acetate. Incubations were terminated by extruding the sediment from glass tubes into centrifuge tubes containing 10 ml of 20% (w/v) zinc acetate solution, then homogenizing it with a vortex mixer and freezing it at −20 °C. As tracer was injected at the beginning of the experiment, total reduced inorganic ^{35}sulfide (^{35}S-TRIS) represents cumulative sulfide formed during frozen and thawed periods. Three-week averages of SRR were then calculated as the difference in TRIS activity between the beginning and the end of each experimental period, as exemplified for the first thawed period:

$$\mathrm{SRR} = [\mathrm{SO_4^{-2}}] * \rho_{\mathrm{sed}} * \frac{^{35}\mathrm{TRIS_c} - ^{35}\mathrm{TRIS_b}}{a_{\mathrm{total}}} * \frac{1}{t} * 1.06 * 1000$$

where SRR is the SRR; SO_4^{-2} the sulfate concentration in the porewater of the sediment sample; ρ_{sed} the porosity of the sediment; ^{35}TRIS the radioactivity of total reduced inorganic sulfur (counts per minute, c.p.m.); *b* the first frozen period (first 3 weeks, that is, the incubation time); *c* the sum of frozen and thawed periods (6 weeks); a_{total} the total radioactivity used (c.p.m.); and 1.06 is the correction factor for the expected isotopic fractionation;

The number 1000 is the factor for conversion from nmol l^{-1} to nmol cm^{-3}.

Multiple freeze–thaw incubations

The purpose of this experiment was to test the long-term survival of bacteria under repeated freeze–thaw conditions. This experiment consisted of eight freeze–thaw cycles, each of which covered a 12-h frozen phase at −20 °C and an 18-h thawed phase at 10 °C. The SRR values were determined at the end of each thawed phase. As a control an additional incubation was carried out at 10 °C and rates were determined for the same cycle intervals as for the freeze–thaw treatments.

Sediment slurries (150 ml, 1:2 v/v) were incubated while stirring during the thawed phase. The control slurry was constantly stirred. To measure SRR values, sub-samples of 3 ml were transferred into N_2-flushed glass Hungate tubes and sealed with butyl rubber stoppers. A volume of 20 µl of ^{35}S-sulfate tracer (activity: 100 kBq) was injected into triplicate tubes at each time point. The SRR values were measured for the duration of each thawed phase and at the corresponding times in the control slurry. The VFA concentrations were measured by sub-sampling ca. 4 ml of slurry at the end of each thawed phase and at the corresponding time points from the control slurry.

Sulfate reduction rates

In situ SRR values were measured in two parallel cores using a whole-core incubation method (Jørgensen, 1978) by injecting 5 µl of carrier-free, $^{35}SO_4^{2-}$ tracer solution in 4% NaCl (~100 kBq per injection) in 1-cm intervals to a depth of 16 cm. Incubations were carried out for 8 h at 7 °C. All samples were distilled using the low-blank cold chromium distillation method described in the study by Kallmeyer *et al.* (2004). Briefly, centrifuged sediment was diluted with 10 ml dimethylformamide

and placed in a distillation flask. Total reduced inorganic sulfide (TRIS) was acid-distilled under nitrogen at room temperature by adding 12 ml 6 N HCl and 12 ml 1 M chromium chloride to the solution. The TRIS was recovered as zinc sulfide in traps containing 7 ml of 5% zinc acetate solution and ^{35}S was counted in a liquid scintillation counter (Packard, Tricarb 2500 TR, Packard-Becker BV, Groningen, The Netherlands) without luminescence correction and the high sensitivity mode turned off; energy range 4—167 keV. The scintillation cocktail used was Lumasafe Plus (Lumac BV, Groningen, The Netherlands) mixed with the ZnS solution in a ratio of 2:1 (v/v).

Volatile fatty acid measurements

Volatile fatty acids were measured by high-performance liquid chromatography according to the method of Albert and Martens (1997). Measurements of *in situ* VFA concentrations were carried out on sediment core sliced at the following depths: 0–1, 1–2, 2–3, 3–4, 4–5, 5–7, 7–9, 9–10 and 11—12 cm. Sediment and slurry sub-samples were centrifuged in Spinex tubes at 4000 r.p.m. at 4 °C for 15 min and porewater sub-samples were directly filtered into 1-ml brown borosilicate glass vials (pre-combusted at 480 °C for 4 h) to minimize possible contamination. Acids were derivatized with *p*-nitrophenyl hydrazine, separated by high-performance liquid chromatography using a LiChrosphere 80/100 (Knauer, Berlin, Germany) column at 25 °C, and the concentrations were determined by UV absorption with a UV/VIS detector (Linear) at 400 nm and quantified with commercially available software (Chromstar, SES GmbHAQ11, Bechenheim, Germany). Concentrations were determined after calibration with standard mixtures containing glycolate, formate, lactate, acetate, propionate, isobutyrate, butyrate and valerate. A standard was measured after every fifth sample. The detection limits for the different acids were 0.2 μM for glycolate and lactate, 1 μM for acetate and formate (in samples with high acetate concentrations formate occurred as a rider peak on the acetate shoulder; for peak integration, a vertical drop line was used for peak separation, which led to a slight overestimation of the formate peak), 0.5 μM for propionate and isobutyrate and 2 μM for butyrate, valerate and isovalerate. Only lactate, acetate and formate were detected in our samples; other acids were below the detection limits. The s.d. value for replicate analyses is less then 3% for concentrations above 5 μM (Finke, 2003).

Enumeration of total cells

Total cell numbers were counted by epifluorescence microscopy after staining with DAPI. Total cell counts were determined in triplicate in the original sediment bag stored at 0 °C for 1 month (time point 0) after sampling, and in samples taken after each time point for all experiments. For DAPI staining, sediment samples were treated as described previously (Pernthaler *et al.*, 2002). Sediment was sub-sampled (0.5 ml) and fixed in 4% paraformaldehyde (1 part 24% paraformaldehyde and 5 parts 1 × phosphate-buffered saline, PBS) overnight at 4 °C. Fixed samples were washed three times with 1 × PBS, with centrifugation steps at 10 000 r.p.m. for 5 min between washes, and stored in PBS/ethanol (2:3) at −20 °C until further processing. Samples were then diluted (1:2) in PBS/ethanol and sonicated at minimum power for 20 s with a sonication probe (MS73 Sonopulus HD70 Bandelin, Berlin, Germany). Sub-samples of 10 μl of the suspension were added to 8 ml of PBS, filtered onto polycarbonate membrane filters (Isopore, filter code: GTTP; pore size: 0.2 μm; diameter: 21 mm, Millipore, Schwalbach/Ts, Germany) and stored at −20 °C. Before staining, filters were cut into several sections. The stain, DAPI (10 μl of a 1-μg ml^{-1} working solution) was dropped onto the filter sections and incubated in the dark for 5 min. Filters were then washed twice in MilliQ water, finally embedded in Vectashield mixed with Citifluor AF1 antifadent (Plano, Wetzlar, Germany) and covered with a cover slip. For each replicate, at least 1000 DAPI-stained cells were counted. Bacterial counts were converted into cells per ml of sediment.

DNA extraction and PCR amplification

The extraction of DNA from the sediment was done using the Mo Bio Power Soil Kit (Mo Bio Laboratories, Inc., CA, USA). Amplification of 16S rRNA genes was performed as described by Muyzer *et al.* (1997) using the universal primers: 907r (5′-CCGTCAATT CCTTTRAGTTT-3′) and 352f (5′-CCTACGGGAGGCA GCAG-3′) carrying a GC clamp (Muyzer *et al.*, 1997). A PCR protocol was used as described by Muyzer *et al.* (1997) except that 'touchdown' PCR was used to increase the specificity of the amplification and to reduce the formation of by-products, that is, the annealing temperature was set 10 °C above the expected annealing temperature and decreased by 1 °C every two cycles until an annealing temperature of 55 °C was reached at which nine additional cycles were performed. The program started with a hot start at 94 °C for 5 min (20 cycles in total; Muyzer *et al.*, 1997).

Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis was performed using a Bio-Rad D Code system (Bio-Rad, Munich, Germany). Polyacrylamide gel was poured with a gradient pump (Econo Gradient Pump, Bio-Rad) to achieve gradient ranging from 0% to 80% acrylamide. The gel was polymerized by adding 10% ammonium persulfate and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (Bio-Rad, Munich, Germany) before pouring the gel. A volume of 80 μl of each PCR product was applied onto the gel and the DGGE was

then performed at 60 °C and a constant voltage of 200 V for 3.5 h (Nübel *et al.*, 1999). After electrophoresis, the gel was incubated for 30 min in an aqueous ethidium bromide solution (0.5 μg l^{-1}) and visualized on a UV transilluminator (LTF-Labortechnik, Wasserburg, Germany).

Results

Characterization of study site

The sediments were gray bioturbated muds with water content of 40%, an average total organic carbon concentration of 1, 5% dry weight and a C/N ratio of 16. The SRR value increased from 19 nmol cm^{-3} day^{-1} at the sediment surface to 41 nmol cm^{-3} day^{-1} at 3 cm depth and decreased below this depth (Figure 1a). At the study site, only three VFAs were detected: acetate, lactate and formate (Figure 1b). The acetate concentration was highest in the surface centimeter, and decreased from 70 to 4 μM at 12 cm depth. The range of lactate and formate concentrations was 8–25 and 4–16 μM, respectively, and did not show clear trends with depth.

Gradual freeze–thaw incubation

The SRR values are presented for the gradual freeze–thaw incubations in Figure 2a. At the beginning of the experiment at +4 °C, the SRR value was 12 nmol cm^{-3} day^{-1}, which was the highest rate measured in this experiment. Over the next 5 days of stepwise lowered incubation temperature (2, 0, −2 and −5 °C), SRR dropped to 7.5, 5.0, 2.1 and 0.4 nmol cm^{-3} day^{-1}, respectively. The subsequent increase in temperature to 0 °C (that is, the 24-h thawing period) resulted in SRR increasing 20-fold to 7.0 nmol cm^{-3} day^{-1}. During the second temperature cycle (2, 4, 2, 0 and −2 °C), SRR values were in the range of 2.9–7.8 nmol cm^{-3} day^{-1} and did not show any of the trends as observed during the first cycle. Sulfate reduction was not detected during the second freezing interval but resumed at 6.0 nmol cm^{-3} day^{-1} during the 0 °C thawing phase. The third temperature cycle resulted in SRR patterns that were similar to those observed during the second cycle.

The VFA concentrations versus time are presented in Figure 2b. Only acetate and lactate were detected during the gradual freeze–thaw incubation. As there was no significant trend for the individual VFAs, their peak areas were summed together. The sum analytical error is therefore about 6%. At the beginning of the experiment, the sum VFA was 21 μM. The concentrations of VFA decreased as the sediment was cooled down and remained between 4 and 10 μM throughout the remainder of the experiment. The observed fluctuations after the initial temperature decrease were within the range of uncertainty of the method and were insensitive to changes in temperature.

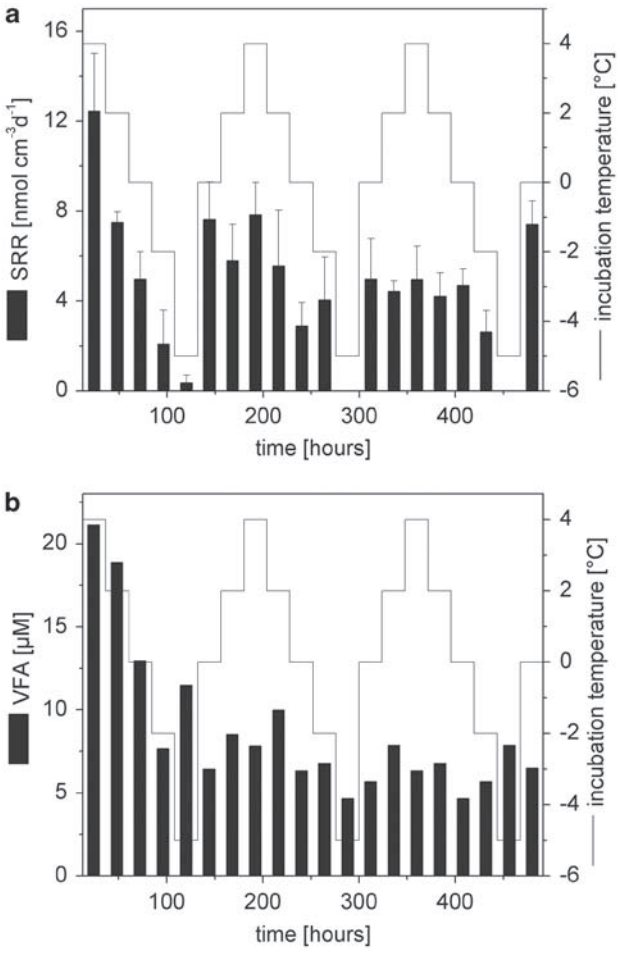

Figure 2 (**a**) The sulfate reduction rates (SRRs) during the gradual freeze–thaw experiment. The black line corresponds to different temperatures applied during the experiment. Black bars correspond to SRRs measured every 24 h, at respective temperatures. (**b**) Concentrations of volatile fatty acids (VFAs) determined in the gradual freeze–thaw experiment. The analytical error for the summed VFA concentration is about 6%, as described in the text. The black line corresponds to different temperatures applied during the experiment. Black bars correspond to VFA levels measured every 24 h, at respective temperatures.

Long-term freeze–thaw incubation

The SRR results for the long-term experiment are presented in Figure 3A. The ^{35}S-TRIS counts after 3 weeks of incubation at −20 °C were above background level for ^{35}S-TRIS of the sterilized control assay and the mean rate determined for this 3-week period was 0.06 nmol cm^{-3} day^{-1} (Figure 3A). This very low SRR value probably is due to the time it took the sediment to cool down from 4 °C and freeze. This is supported by a ten-week long, control incubation experiment at −20 °C. The ^{35}S-TRIS counts measured in sediments incubated at −20 °C for ten weeks were at the same level as ^{35}S-TRIS counts determined for the 3-week long incubation. In the subsequent 3-week period at 10 °C, average SRR increased to 38 nmol cm^{-3} day^{-1}, which was higher than the earlier *in-situ* microbial activity,

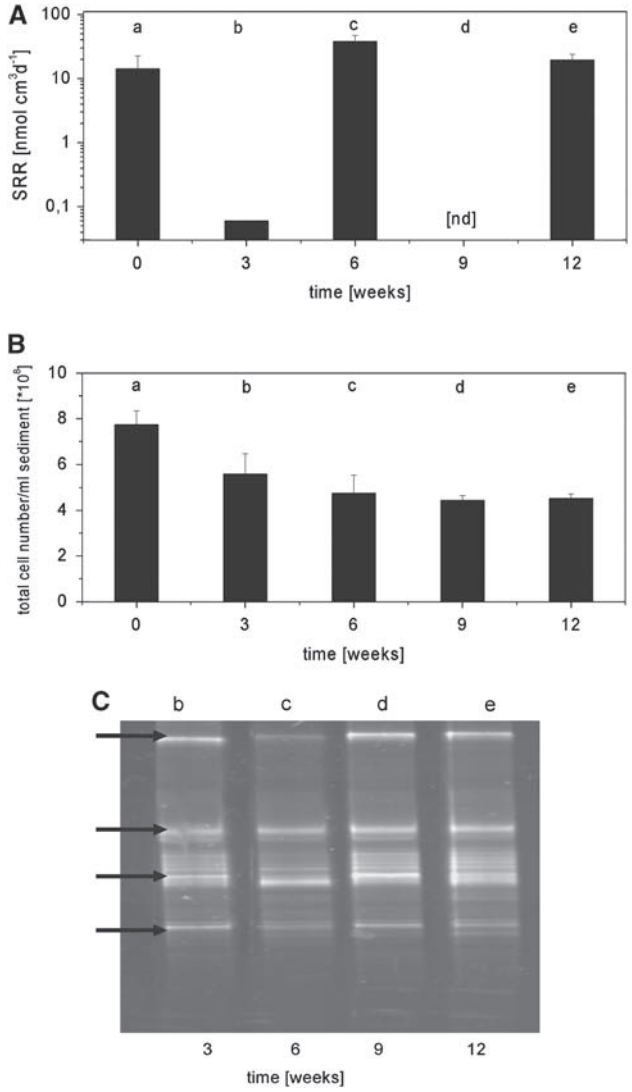

Figure 3 (**A**) The sulfate reduction rates (SRRs) determined at *in situ* temperature (a) followed by alternating freezing (b and d) and thawing (c and e) conditions. During the second frozen phase (weeks 6 to 9) sulfate reduction was not detected (nd). (**B**) Total cell numbers determined for the sediment stored at 0 °C (a) and for the sediment subjected to alternating freezing (b and d) and thawing (c and e) conditions. (**C**) Denaturing gradient gel electrophoresis (DGGE) profiles for 16S rRNA gene fragments obtained from DNA extracted after alternating freezing (b and d) and thawing (c and e) conditions, which was amplified by PCR using primers 338f and 907r. The arrows indicate four dominant bands that were prominent in all samples.

suggesting survival of, at least part of, the sulfate-reducing community during the −20 °C period, and its subsequent reactivation at 10 °C. After the second 3-week freezing period (that is, at week 9 of the experiment), measured ^{35}S-TRIS counts in triplicate tubes were not significantly higher than the ^{35}S-TRIS counts measured at the beginning of the freeze period (that is, at week 6). Thus, because of the high ^{35}S-TRIS background, SRR values could not be determined for the second freeze period. After the second 3-week period at + 10 °C (that is, at week 12), ^{35}S-TRIS counts had increased further. The mean rate for this second thaw period was 19 nmol cm^{-3} day^{-1}, which indicated that sulfate-reducing microorganisms were reactivated after successive freeze–thaw cycles.

Total cell numbers were determined by DAPI staining at the end of each frozen and thawed incubation interval (Figure 3B). The initial cell abundance was 7.7×10^8 per ml wet sediment. After the first freeze interval, the cell numbers dropped to 5.6×10^8 per ml. After the second freeze interval, total cell numbers decreased further to 4.7×10^8 per ml and remained at this level throughout the experiment. The DGGE profiles of PCR-amplified rRNA gene fragments extracted after each freeze and thaw period were similar (Figure 3C). At least, four dominant bands were consistently detected for each freeze and thaw period, suggesting that several major taxa persisted under the freeze–thaw conditions.

Multiple freeze–thaw incubation

In this experiment, the highest SRR value, 33 nmol cm^{-3} day^{-1}, was determined in the first of the eight freeze–thaw intervals (Figure 4a). In the second and third cycles, the SRR value decreased to 27 and 7 nmol cm^{-3} day^{-1}, respectively, and remained low (3–5 nmol cm^{-3} day^{-1}) in all subsequent thawed phases. In a control incubation that did not experience any freezing, the SRR values were higher and gradually decreased from 40 to 17 nmol cm^{-3} day^{-1} over the course of the experiment.

Three VFAs: acetate, lactate and formate were detected during the multiple freeze–thaw incubation. As in the gradual freeze–thaw experiment, no significant trend was apparent for the individual VFA, therefore, summed concentrations are presented in Figure 4b (for three VFAs, the sum analytical error is about 9%). A pronounced increase in VFAs was measured over the course of this experiment. Over the first six freeze–thaw cycles, concentrations of VFAs increased from 23 to 202 μM, and then increased more gradually up to 225 μM over the final two cycles. In the control experiments, the concentration of VFAs dropped from 40 to 19 μM during the first 30 h and remained at this level for the duration of the experiment.

Discussion

Reactivation under freeze–thaw conditions

Continued activity in cyclical freeze–thaw experiments seems to be influenced by the ability of the microbial community to metabolize substrates released by the freeze–thaw treatment (Schimel and Mikan, 2005). Several studies on soil bacteria have demonstrated detrimental effects of freeze–thaw

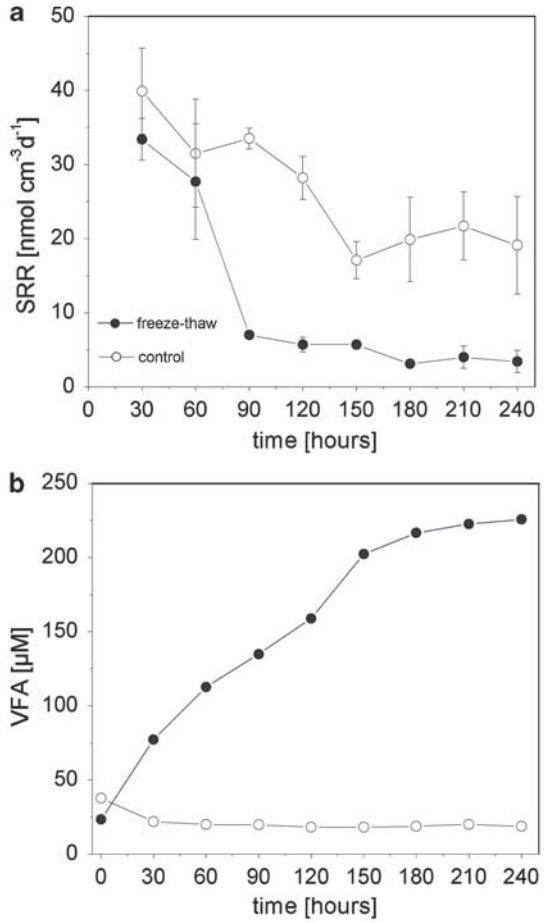

Figure 4 (**a**) The sulfate reduction rates (SRRs) associated with eight successive 30-h freeze-thaw cycles. Closed symbols correspond to SRR values measured during 18-h thaw phases (at 10 °C) that followed 12-h frozen phases (at −20 °C). Open symbols correspond to SRR values measured at the same corresponding time points in a control that was constantly maintained at 10 °C. (**b**) Volatile fatty acids (VFAs) associated with eight successive 30-h freeze–thaw cycles. Closed symbols correspond to concentrations of VFAs measured at the end of each cycle and open symbols correspond to concentrations of VFAs measured at the same times in the control experiment. The analytical error for the summed VFA concentration is about 9%, as described in the text.

event on microbial communities. Such a treatment killed up to 50% of the microbial population in the first freeze–thaw cycle (DeLuca *et al.*, 1992) and irreversibly reduced the soil DNA content by 33% (Pesaro *et al.*, 2003). Multiple freeze–thaw cycles decreased culturable populations in soils by four orders of magnitude and reduced morphological diversity (Walker *et al.*, 2006).

Our results demonstrate that freezing temperatures in freeze–thaw regimes temporarily eliminate bacterial activity, but that sulfate-reducing microorganisms can resume active carbon cycling shortly after thawing of the sediment (Figures 2A and 3A). Arctic soil mesocosm studies showed that microbial respiration remained at a high level in multiple diurnal freeze–thaw cycles although the microbial biomass declined (Larsen *et al.*, 2002). By contrast, microbial biomass in alpine soils subjected to moderate freeze–thaw cycles, a temperature change between 3 °C and −5 °C, was not affected by these temperature fluctuations (Lipson *et al.*, 2000). In our experiments sulfate-reducing bacteria survived in sediment that was gradually frozen and thawed as SRRs changed with each successive temperature shift between 4 °C and −5 °C (Figure 2a). The most distinct temperature response only occurred in the early stages of the experiment, and it is noteworthy that SRRs did not return to initial levels after repeated freeze–thaw cycles (Figure 2a). Lower SRR values after the first freeze–thaw cycle indicate a decreased capacity for sulfate reduction, possibly due to the loss of cells that could not cope with freeze–thaw stress (Figure 3B). Psychrophilic bacteria generally prevail in permanently cold arctic sediments (Helmke and Weyland, 2004). These organisms possess a broad range of cold-adaptive strategies, such as increased membrane fluidity, low-temperature-adapted enzymes, cold-shock and antifreeze proteins and cryoprotectants (D'Amico *et al.*, 2006), which implies that psychrophiles should be dominant community members in Arctic sediments subjected to freeze–thaw conditions. Similar conclusions were drawn by Walker *et al.* (2006), who showed that those soil microorganisms that could withstand multiple freeze–thaw cycles treatment possessed cold-adaptation mechanisms. Psychrophiles are understood to be dominant in permanently cold environments, whereas psychrotolerant bacteria can adapt faster to fluctuating temperature (Robador *et al.*, 2009). It is not clear whether this characteristic of psychrotolerant bacteria extends to the temperature fluctuations imposed by the freeze–thaw cycles used here, leaving it uncertain whether psychrophilic or psychrotolerant bacteria were dominant in our incubated sediments.

Multiple freeze–thaw cycles have been shown to decrease microbial respiration by 50–70% in a soil mesocosm (Larsen *et al.*, 2002). In our multiple freeze–thaw incubations, three successive freeze–thaw cycles resulted in a decrease in SRR value by 80%, however, those sulfate reducers that survived this treatment were able to resume without delay when the sediment was thawed (Figure 4a). Similarly, in the long-term freeze–thaw incubation sulfate reduction, which was inhibited during frozen phase, resumed when the temperature increased again (Figure 3A). The DAPI counts for the long-term experiment showed that the first freeze–thaw cycle decreased cell number irreversibly by 30% (Figure 3B). Our observations are in line with those of other studies that showed a decrease in DNA content due to cell lysis under freeze–thaw conditions, whereas microbial respiration appeared unaffected (Pesaro *et al.*, 2003).

Comparative DGGE analysis of PCR products obtained using universal bacterial 16S rRNA gene primers showed that the microbial community composition in the sediment did not experience major shifts as a result of the freeze–thaw treatments. Our results do not indicate that freeze–thaw treatments selected for or enriched microbial taxa; rather, dominant taxa were maintained throughout the experiment. This finding is in agreement with results of the study by Männistö *et al*. (2009) in Arctic tundra soil, where only minor changes in microbial community structure were observed after repeated freeze–thaw cycles. Our results are also consistent with lipid biomarker studies of high-latitude soil samples that concluded, on the basis of temperature gradient gel electrophoresis images, that neither microbial community structure nor microbial biomass was affected by freeze–thaw stress (Koponen *et al*., 2006). It is very likely that the bright bands in the DGGE gel do not necessarily represent sulfate-reducing bacteria but include other bacterial groups as well. In a study on the sulfate-reducing bacterial community structure in Svalbard marine sediments, Ravenschlag *et al*. (2000) determined that sulfate reducers accounted for only 16% of the total microbial community. Thus, members of the freeze/thaw-resistant community from Ymerbukta sediment, represented by stable intense bands on the DGGE gel, could indicate a broad community of freeze/thaw-resistant bacteria that catalyzed carbon degradation and mineralization in these sediments.

Tolerance of different physiological groups to freeze–thaw cycles

Low concentrations of VFAs during gradual freeze–thaw incubations (Figure 2b) suggest that the coupling between fermentation and sulfate reduction was maintained during this experiment. These incubation conditions, therefore, seemed to recreate the normal situation in arctic sediment, that is, low concentrations of VFAs (for example, Figure 1b and 4b) due to close coupling between fermentation and terminal oxidization processes (Finke and Jørgensen, 2008; Robador *et al*., 2009).

This pattern was not observed during multiple freeze–thaw cycles in which concentrations of VFAs gradually increased during the experiment (Figure 4b). This suggests that detrimental effects of freeze–thaw cycles may have been greater for sulfate reducers than for fermentative microorganisms. These effects suggest major changes in the pathways of carbon processing under repeated freeze–thaw cycles. The long-term fate of VFAs that accumulated after freezing requires further exploration.

Microbial activity in the frozen state

Various studies have reported that microorganisms are capable of growth below the freezing point of water, down to temperatures of −20 °C (Gilichinsky *et al*., 1993; Rivkina *et al*., 2000; Junge *et al*., 2004). We find no clear evidence that sulfate reduction remained active at −20 °C. In addition, the fact that sulfate reduction was only detected in the first freeze cycle of the gradual freeze–-thaw experiment at −5 °C (Figure 2a) suggests that part of the microbial community, active initially in the first freeze period, was damaged in the subsequent freeze–thaw cycle. Even the fraction of the microbial community that was not damaged during freeze–thaw event was not active when the sediment was frozen, probably because of changes in cell structure at low temperature, for example, membrane lipids stiffen and this decreases the efficiency of embedded transport proteins (Nedwell and Rutter, 1994; Nedwell, 1999; Ponder *et al*., 2005).

At the Antarctic sites, studied by Mountfort *et al*. (2003), it was shown that freezing had a strong impact by uncoupling sulfate reduction from acetate oxidation and shifting the carbon flow towards methanogenesis. Mountfort *et al*. (2003) suggested that freezing provides a physical barrier preventing access of the microbes to sulfate or by reducing the affinity of sulfate reducers for their substrate. Our experiments demonstrate that microbial carbon turnover through bacterial sulfate reduction was not sustained over repeated freeze–thaw cycles in frozen intervals.

Conclusions

Moderate freeze–thaw conditions have little effect on microbially mediated organic carbon degradation in these intertidal Arctic sediments. It is apparent that the *in situ* microbial communities can largely withstand drastic temperature fluctuations and are reactivated without delay. Substantial re-growth of microbial populations to resume carbon mineralization is not required. Although this study considered natural communities from intertidal sediments, the results are relevant for considering other coastal marine environments that are subject to temperature fluctuations. Climate warming studies predict an increase in the amplitude of temperature fluctuations in the Arctic regions (Moritz *et al.,* 2002). Many Arctic coastlines are currently in transition as rising sea level inundates and thaws coastal permafrost (for example, Rachold *et al*., 2000). Thawing coastal permafrost is a significant component of the changing Arctic carbon cycle (Semiletov *et al*., 2007). At present, mineralization rates of organic matter in thawing permafrost are not well quantified but are likely critically dependent on the reactivation and recovery of bacteria.

Conflict of interest

The authors declare no conflict of interest.

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Manuscript II

Temperature characteristics of bacterial sulfate reduction in continental shelf and slope sediments

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Temperature characteristics of bacterial sulfate reduction in continental shelf and slope sediments

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Abstract

The temperature responses of sulfate reduction in continental shelf and slope sediments from the southwest and southeast Atlantic were compared with sediment from Arctic fjords. Sediments were collected from water depths ranging from the intertidal zone to 4327 m where *in situ* temperatures range from 8ºC on the shelf to 1ºC on the lower slope and in the Arctic. Sulfate reduction rates (SRR) were measured using a ^{35}S radiotracer technique in subsampled sediment incubated in a temperature gradient block. An optimum temperature (T_{opt}) of between 27 and 30°C for the south Atlantic shelf sediments and for an intertidal flat sediment from Svalbard is indicative of psychrotolerant or mesophilic sulfate-reducing communities, whereas T_{opt} ≤20°C in south Atlantic slope and Arctic shelf sediments suggests a predominantly psychrophilic community. In South Atlantic slope sediments, SRR at the *in situ* temperature were relatively high (20-50% of that measured at Topt) further supporting the presence of a cold-adapted community. The lower T_{opt} in Arctic shelf and south Atlantic slope sediments reveals how *in situ* temperature may determine the predominant thermal physiologies within sulfate-reducing communities. High metabolic rates at T_{opt} and a broad temperature range for sulfate reduction in several South Atlantic slope sediments indicated a contribution from mesophilic sulfate-reducing bacteria. The presence of these microorganisms may be due to passive dispersal down-slope via sediment movement from the warmer shelf where mesophilic bacteria are more predominant to the slope sediment with lower *in situ* temperature.

Introduction

95% of the seafloor is permanently cold with *in situ* temperatures below 4°C (Levitus and Boyer, 1994). Bacteria carrying out carbon mineralization in the cold sea-bed must be adapted to operate effectively under such low temperatures. Psychrophilic bacteria with a suitable fluidity of the cell membrane and cold adapted enzymes are particularly abundant in the cold deep sea (Margesin and Miteva, 2011). As a result of such microbial adaptation to low temperature the rate and efficiency of organic carbon mineralization in the cold may be comparable to those in temperate and warm habitats (Kostka et al., 1998). However, bacteria that grow effectively in temperate environments and function at temperatures extending into the mesophilic range can also be isolated from the cold deep-sea floor (Rüger et al., 1989; Rüger et al., 1992; Finster and Bak 1992; Chen et al., 2003; Aono et al., 2010).

Based on the temperature response of respiration or growth, different thermal groups of bacteria may be defined with different cardinal temperatures, i.e. temperature minimum (T_{min}) and temperature maximum (T_{max}) limiting bacterial activity and temperature optimum (T_{opt}) indicating highest rate. Psychrophilic bacteria have minimum temperature <0°C, optimum ≤15°C, and maximum ≤20°C. Psychrotolerant bacteria have minimum temperature ≤0°C, optimum ≤25°C, and maximum ≤35°C. Mesophilic bacteria have minimum temperature >0°C, optimum at 25-40°C, and maximum at 35°C-40°C (after Morita, 1975).

Bacterial sulfate reduction is the main anaerobic carbon mineralization pathway in continental shelf and slope sediments (Jørgensen and Kasten, 2006) and is also detected in sediments of the continental rise and the abyssal plains (Ferdelman et al., 1999; D'Hondt et al., 2003; Lee et al., 2008). Sulfate reduction rates can be quantified by incubating marine sediment with ^{35}S-sulfate and measuring the rate of ^{35}S-sulfide formation (Fossing, 1995; Kallmeyer et al., 2004). ^{35}S-sulfate reduction is a method that links a direct quantification of

organic carbon mineralization and the microorganisms responsible for the process (Lee et al., 2008; Leloup et al., 2007). Since carbon cycling in permanently cold shelf sediments contributes significantly to the global carbon cycle, the temperature response of sulfate reducing bacteria and their respiration rate have been studied in high latitude sediments by incubation experiments in a temperature gradient block, by cultivation, and by DNA/RNA based studies (Knoblauch and Jørgensen, 1998; Karr et al., 2005; Bowman and McCuaig, 2003).

Incubation of sediments or of pure cultures along a temperature gradient can be used to determine the temperature dependence and the cardinal temperatures for growth or respiration of microbial communities (Battley 1964). Such incubations have shown that temperate sediments and permanently cold Arctic or Antarctic sediments host bacteria with widely different temperature adaptations (Isaksen and Jørgensen 1996; Knoblauch and Jørgensen 1998; Sahm et al., 1999; Brüchert et al., 2001; Hubert et al., 2009). Rates of metabolism at *in situ* temperatures compared to the rates at T_{opt} are indicative of how well bacteria perform under ambient, low temperatures (Knoblauch and Jørgensen, 1998). The Arrhenius plot can be used for a graphical representation of the temperature dependence of bacterial metabolism (Arrhenius, 1908). Thereby, the logarithm of the rate of bacterial respiration or growth versus the inverse absolute temperature yields a linear relationship in the temperature range where the bacteria are well adapted. Deviations from the linear at the upper or lower extreme express the inability of SRB to maintain a well-controlled metabolic activity and may uncouple electron flow from ATP formation. The slope of the correlation can be used to calculate the apparent activation energy, E_a, where the E_a can be defined as the minimum energy required to initiate a chemical reaction. A reduction of the E_a value will therefore result in an increase of the reaction rate. Apparent E_a values are not activation

energies in the strict chemical sense, however. Sulfate reduction occurs through a series of enzymatic reactions and calculated E_a values therefore measure an ecological response of the whole SRB community to temperature changes rather than the cooperative process between structural elements of an enzyme or a rate-limiting chemical step. From the energy of activation in a given temperature interval we can calculate Q_{10}, i.e. the factor by which the rate of reaction increases by a temperature increase of 10°C (Arrhenius, 1908).

In the present study we analyzed the temperature dependence of sulfate reduction in shelf and slope sediments from the southwest and southeast Atlantic and compared these to permanently cold shelf sediments at Svalbard in the Arctic Ocean. We wanted to assess to which extent the temperature response of the microbial communities reflected the ambient temperature and whether their cardinal temperatures were the result of a narrow adaptation to ambient temperature or rather reflected mixed communities of different temperature groups.

Material and methods

Sediments from the South Atlantic were collected in 2008 and 2009 at six stations located on the shelf and slope off central Namibia and off Uruguay and Argentina, respectively. Sediments from four stations in the Arctic were collected in 2007 in fjords and from an intertidal flat on the west coast of Svalbard. Samples were taken from the zone of highest sulfate reduction, which was typically in the depth range of 3-10 cm (Jørgensen, 1982). Sediments were stored in gas-tight plastic bags at 4°C until further processing in the laboratory. For measurements in whole sediment cores, sediment cores of 26 mm diameter and ca 15 cm long were taken, sealed at both ends with rubber stoppers leaving air in the headspace, and stored at 4ºC. Coordinates for the study sites, *in situ* temperatures, and water depths at which sediments were collected are given in Table 1.

Oceanography and sedimentary setting

Namibian shelf and slope. Two stations were sampled in the Namibian upwelling region, one on the shelf in 130 m water depth and one on the continental slope in 2000 m water depth. Sediments were collected on the RV Meteor cruise M76/1 (MARUM). Sediments accumulate here under the highly productive Benguela upwelling system. Sediment from the shelf and from the slope is characterized by a high total organic carbon (TOC) content of up to 10% dry weight. The Benguela upwelling system is characterized by seaward and downslope particle transport that maintains local high sedimentation rates in a depo-center at 1000-1500 m water depth (Inthorn et al., 2005). The Benguela upwelling system has extremely high primary productivity of 175-240 mmol C m^{-2} d^{-1} and is among the most productive ocean areas. Sulfate reduction rates (SRR) decrease strongly with increasing water depth together with the organic carbon content in the surface sediment (Table 2, Ferdelman et al., 1999).

SW Atlantic margin and basin. Sediments from the SW Atlantic were collected during the RV Meteor cruise M78/3a/b (MARUM) on the continental shelf off Uruguay and on the slope off Argentina. This region is characterized by high sedimentation rates, gravity mass flows due to major turbidities and slides, and strong surface currents (Riedinger et al., 2005). We measured SRR TOC content of the same magnitude as in shelf sediments off Namibia (Table 2). The sediments in the study area are characterized by low carbonate concentrations and high concentrations of organic carbon and iron oxides (Hensen et al., 2003). The TOC content of Argentine slope sediments decreased with water depth from 5% to 1%. The region off Uruguay and Argentina has dynamic oceanographic conditions due to the confluence of two different water masses that cause high primary productivity and high deposition of organic matter (e.g., Behrenfeld and Falkowski, 1997).

West coast of Svalbard. Along the west coast of Svalbard primary productivity is controlled by light availability and ice coverage and is also related to the different water masses. Warm and nutrient rich Atlantic water of the west Spitsbergen current leads to early melting of the ice and stimulates primary production and subsequent sedimentation.

Table 1. Sampling site description.

Station	Coordinates	Water depth (m)	in situ *T*°C
1) Namibian shelf	25°0‘S14°23‘E	130	8
2) Uruguay shelf	36°08’S53°16’W	244	8
3) Namibian slope	25°45'S13°3'E	2000	2
4) Argentine Basin	38°12'S54°56'W	627	4
5) Argentine Basin	37°57’S53°50’W	3400	2
6) Argentine Basin	39°28'S53°42'W	4327	1
7) Arctic intertidial flat	78°16’N14°02’E	0	6
8) Krossfjord	79º08`N.11º39`E	80	0
9) Kongsfjord	79°00′N 11°40′E	110	-1
10) Smeerenburgfjord	79°42’N11°05’E	215	2

Mean annual primary production is 120 g C m^{-2} y^{-1} along the west coast (Sakshaug, 2003). SRR are also relatively high and comparable to rates of many temperate shelf areas. Sediment was collected from four stations along the west coast of the main island, Spitsbergen. Three stations were located centrally in fjords while the fourth was an intertidal mud flat. The fjord sediments were taken in July 1998 and July 1999 with a Haps corer while the intertidal flat was sampled in August 2008 from the shore.

Sulfate reduction rate measurements

Sulfate reduction rates, SRR, were measured in two parallel sediment cores using the whole core incubation method (Jørgensen, 1978). These data are called *in situ* SRR. 5µl of carrier-free $^{35}SO_4^{2-}$ tracer solution in 4% NaCl (~100kBq per injection) was injected at 1 cm intervals to a depth of 16 cm. Incubation time was 8 h at *in situ* temperature. All samples were analyzed using the low-blank cold chromium distillation method described by Kallmeyer et al. (2004). Briefly, centrifuged sediment was diluted with 10 ml dimethylformamide and placed in a distillation flask. Total reduced inorganic sulfide (TRIS) was acid-distilled under nitrogen at room temperature after adding 12ml 6N HCL and 12ml 1M chromium chloride. The TRIS was recovered as zinc sulfide in traps containing 7ml of 5% w/v zinc acetate solution and ^{35}S was counted in a liquid scintillation counter (Packard, Tricarb 2500 TR). The scintillation cocktail was Lumasafe Plus (Lumac BV, Groningen, The Netherlands) mixed 2:1 (v/v) with the ZnS suspension.

Temperature dependence of SRR

The temperature dependence of SRR was determined in temperature gradient incubation experiments using a thermostated aluminum block (Isaksen and Jorgensen, 1996). The temperature span in the gradient block was -5°C to +40°C. The temperature increment between each sample was 1.5°C. Sediment slurries were prepared by 1:2 (w/v) dilution with anoxic artificial seawater (Widdel and Bak, 1992). Sediment slurries were made anoxic by bubbling with N_2, and 5 ml of slurry was transferred to each Hungate tube. Hungate tubes were flushed with N_2 (Bryant, 1972) and sealed with butyl rubber stoppers. The Hungate tubes were immediately placed in a temperature gradient block and pre-incubated to allow the sediments to reach thermal equilibrium. Then ^{35}S-labeled carrier-free sulfate (100 kBq final activity) was injected and the slurries were incubated with radiotracer. Incubations were

stopped by transferring the sediment to 50 ml polyethylene centrifuge tubes with 20 ml 20% zinc acetate to stop bacterial activity and to fix sulfides. Samples were kept frozen until further analysis. Subsequent processing followed procedures described above (Kallmeyer et al., 2004). South Atlantic sediments were pre-incubation for 12 hours and then radiotracer was injected for 24 hours i.e., incubations lasted 36 hours. Sediments from three Arctic seafloor stations (Kongsfjorden, Krossfjorden and Smeerenburgfjorden) were preincubated for an hour and incubations with the radiotracer lasted 12 hours for Kongsfjorden and Krossfjorden and 8 hours for Smeerenburgfjorden sediment.

Arrhenius plot and Q_{10}.

Activation energies were calculated from the linear range of Arrhenius curves of the ^{35}S-sulfate reduction rates, *k*, as a function of temperature:

$$\ln(k) = \ln(A) + \left(\frac{-E_a}{R} \cdot \frac{1}{T} \right)$$

where E_a is the activation energy (J mol^{-1}), *k* is the rate of sulfate reduction (nmol cm^{-3} day^{-1}). *A* is a constant, *R* is the gas constant (8.314 J K^{-1} mol^{-1}), and *T* is the absolute temperature (K). Q_{10} values between 0°C and 10°C were calculated according to:

$$Q_{10} = \exp \left[\frac{E_a \cdot 10}{RT(T + 10)} \right]$$

Solid phase analyses

Freeze-dried and homogenized sediment was analyzed for total carbon (TC) and total nitrogen (TN) with a Fisons NA 1500 (Series 2) Elemental Analyzer. Total inorganic carbon (TIC) was measured with a CM 5240 Orbis BV coulometer. Total organic carbon (TOC) was calculated by subtracting TIC from TC.

Sulfate measurements

Pore water sulfate concentrations were determined after centrifugation of sediment at 3500 rpm in capped centrifuge tubes with nitrogen headspace at 4°C for 15 min. Supernatant pore water (1 ml) was preserved with 200 µl 1% (w/v) Zn-acetate solution and stored at -20°C. Sulfate concentrations were measured by suppressed ion chromatography at 1:100 dilution with 18MOhm water on a Metrohm 761 compact IC. Sulfate standards were prepared from Na_2SO_4, with concentrations ranging from 5 to 400 µM using an eight point calibration curve. Quality control samples, treated as unknowns were prepared from calibrated seawater (IAPSO) and analyzed at the start and end of every sample run.

Results

Characterization of study sites

The organic carbon and nitrogen content, expressed as % dry weight and molar C:N ratio are listed in Table 2. The highest organic carbon content was measured in Namibia (4.4 %) and Uruguay (5.0%) shelf sediments and Namibia (6.5%) slope sediment. In the other sediments, TOC ranged from 1.2% to 2.3%. The TN ranged from 0.1% to 0.9%. Except for the Arctic intertidal flat sediments where the molar C:N ratio was 16, C:N ratios calculated for other sediments ranged between 8 and 10. C:N ratios of ca.10 determined for South Atlantic sediments are typical for sediments with high TOC content deposited under highly productive marine systems with associated high organic matter fluxes (Meyers, 1994). C:N ratios of 10 generally characterize labile organic matter easily accessible for microorganisms.

Mean rates of *in situ* SRR in the zone of highest sulfate reduction (top 3 to 9 cm) are presented in Table 2. Highest rates were found in Namibia shelf sediment, 65.8 nmol cm^{-3} d^{-1} and Uruguay shelf sediment, 43.6 nmol cm^{-3} d^{-1}. The lowest SRR, 6 nmol cm^{-3} d^{-1}, were found

in Argentine sediments from 3300 m water depth, in the Arctic Krossfjorden, 4.29 nmol $cm^{-3}d^{-1}$, and in Namibia slope sediment from 2000 m water depth, 3.59 nmol cm^{-3} d^{-1}.

Table 2. Bulk geochemical analysis, carbon and nitrogen concentrations determined for Namibian, Uruguayan, Argentine and Arctic sediments.

Station	Organic carbon (wt %)	Nitrogen (wt %)	C/N	SRR nmol $cm^{-3}d^{-1}$ (SR zone mean)
Namibia 130 m	4.4	0.5	10.5	65.8
Namibia 2000 m	6.5	0.9	10.4	3.59
Uruguay 244 m	5.0	0.6	10.0	43.6
Argentina 627 m	2.3	0.3	10.3	nd
Argentina 3400 m	1.3	0.2	9	nd
Argentina 4327 m	nd	nd	nd	6
Arctic 0 m	1.4	0.1	16	11.
Krossfjord 80 m	nd	nd	nd	4.29
Kongsfjord 110 m	nd	nd	nd	12.6
Smeerenburgfjord 215 m	1.2	0.2	8.8	19.4

Temperature dependence of SRR, South Atlantic

SRR measured in slurried sediments in the temperature gradient block are not representative of *in situ* rates, yet they are clearly related to water depth and availability of organic matter. The temperature curves of sulfate reduction in the different sediments can be characterized in terms of three cardinal temperatures, T_{min}, T_{opt}, and T_{max}, and the SRR at *in situ* temperatures relative to the T_{opt}. Figure 1 shows the temperature dependence of SRR in all sediments studied. The corresponding T_{opt} and other derived parameters are listed in Table 3. The *in situ* temperature of Namibia and Uruguay shelf sediments varies between 7 and 10ºC throughout the year and the temperature response of SRR can be characterized as mesophilic to psychrotolerant (Figure 1 a, b). SRR in both shelf sediments had optimum at 25-30ºC. SRR in Namibia slope sediments were 7 nmol cm^{-3} d^{-1} at the *in situ* temperature and increased to 72 nmol cm^{-3} d^{-1} at T_{opt}. In Uruguay sediment SRR at the *in situ* temperature were 3 nmol cm^{-3} d^{-1} and increased to 16 nmol cm^{-3} d^{-1} at T_{opt}.

In situ temperatures in the slope sediments off Namibia and Argentine range annually between 1 and 4°C. T_{opt} of sulfate reduction was lower than in the shelf sediments and the temperature response of SRR can be classified as psychrotolerant. In the Namibia and Argentine slope sediments highest SRR were found at 22°C and 20°C, respectively (Figure 1 c, d, f), and the T_{max} was near 30°C. A T_{opt} of only 12°C was found in sediment from the Argentine slope in 3400 m water depth indicating that this sediment hosted a psychrophilic community (Figure 1 e). The temperature profile was relatively broad between 0 and 15°C and SRR dropped to near detection above 20°C. SRR were 0.1 to 2 nmol cm^{-3} d^{-1} at *in situ* temperatures in slope sediments (Figure 1 c, d, e, f). Rates at T_{opt} in Namibia slope sediment reached 29 nmol cm^{-3} d^{-1} (Figure 1 c) but were only 0.7 to 4.3 in Argentine slope sediments (Figure 1 d, e, f).

Temperature dependence of SRR, Arctic

In the Arctic sediments the highest rates were measured in Smeerenburgfjorden (Fig 1 j). In this sediment the SRR at *in situ* temperature were at 53 nmol cm^{-3} d^{-1} and increased to 200 nmol cm^{-3} d^{-1} at T_{opt}. In the Arctic intertidal mud flat (Ymerbukta) and in Kongsfjorden sediment SRR were lower by around 40% at T_{opt} (Figure 1 g, i). The rates increased from 4 to 120 nmol cm^{-3} d^{-1} at T_{opt} in the Arctic intertidial flat sediment of Ymerbukta and from 27 to 141 nmol cm^{-3} d^{-1} in Kongsfjorden. In the other Arctic fjord sediments rates increased from 4 to 44 nmol cm^{-3} d^{-1}at T_{opt} (Figure 1 h).

The broad temperature profiles of SRR suggest that Arctic sediments host microbial communities with divergent temperature characteristics (Figure 1 g-j). In the Arctic intertidal mud flat summer temperatures can be as high as 6°C, but drop to -20°C during winter. SRR increased in the temperature range from -4°C to 30°C and dropped to near-zero at a T_{max} of 35°C (Figure 1 g). The other Arctic sediments (Kongsfjorden, Krossfjorden,

Smeerenburgsfjorden) have very constant temperatures throughout the year ranging from -1°C to +2°C.

The temperature profile of SRR in Smeerenburgfjorden sediment was broad and increased from -4°C to 27°C while activity was still detected at 40°C. In Krossfjorden SRR increased from below 0° and T_{opt} was in the psychrotolerant range of 23°C while activity was not detected above 34°C (Figure 1h). Also in Kongsfjorden SRR showed a psychrotolerant response as activity was detected at -4°C, reached maximum at 18°C, and was barely detectable above 34°C (Figure 1 i)

Arrhenius plots and Q_{10}

The metabolic rates at in situ temperatures compared to Topt were between 9 and 50% for all the stations (Table 3, Figure 2). The activation energies calculated from the Arrhenius plots ranged from from 25 to 55 kJ mol^{-1} while the Q_{10} factors were in the range of 2 to 3 (Table 3). In Namibia sediments activation energies ranged from 33 to 55 kJ mol^{-1} and in Argentine sediments from 26 to 55 kJ mol^{-1}.

Table 3. Sulfate reduction activities and Arrhenius parameters determined in the temperature-gradient incubation experiments for Namibian, Uruguayan, Argentine and Arctic sediments.

Station	T_{opt} (ºC)	SRR nmol cm^{-1} d^{-1}		%SRR	E_a $kJmol^{-1}$	Q10
		at in situ *T*	at T_{opt}			
Namibia 130 m	25	7	72	9	55	2.8
Namibia 2000 m	22	2	29	7	38	2
Uruguay 244 m	27	3	16	20	32	2.9
Argentina 627 m	20	0.15	0.7	21	30	2.8
Argentina 3400 m	12	0.1	0.2	50	52	2.7
Argentina 4327 m	20	1.2	4.3	28	26	2.7
Arctic 0 m	30	8	120	15	25	2.7
Krossfjord 80 m	25	9.1	44	20	27	2.3
Kongsfjord 110 m	18	27	141	19	31	2.1
Smeerenburgsfjord 215 m	27	41	213	19	25	2

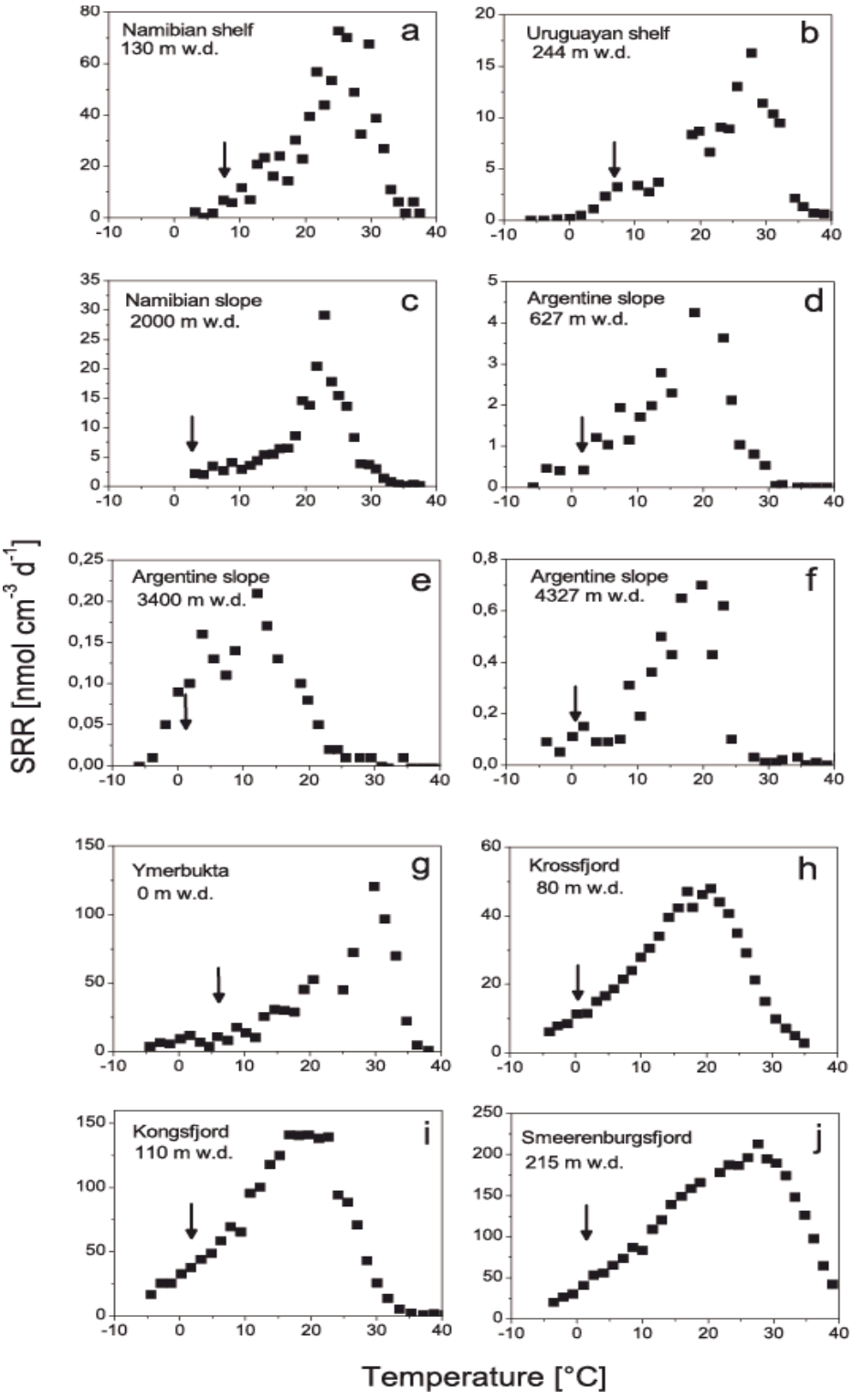

Figure 1. SRR measured in temperature-gradient incubation experiments of sediment slurries from the different sampling sites.

Discussion

Temperature response of SRR depends on the *in situ* T°C and on the water depth

Our results demonstrate how the ambient temperature regime selects for microbial populations with different temperature physiology. The temperature response of SRR from permanently cold sediments was in the psychrophilic to psychrotolerant range while in temperate sediments it was in the mesophilic range (cf. Isaksen et al., 1994; Sagemann et al., 1998; Isaksen and Jørgensen, 1996). The sediment temperatures in the Uruguayan and Namibia shelf were determined by ocean currents of different thermohaline characteristics that maintained sediment temperatures of 7-10°C (e.g., Lass and Mohrholz, 2005; Hansen et al., 2003; Ortega et al., 2007). Similar temperatures were recorded during the Arctic summer for an intertidal mud flat of Svalbard where the air temperature during low tide may heat the surface sediments up to 9°C (Nørdli 2005). Accordingly, the temperature response of SRR in these sediments was in the psychrotolerant to mesophilic range, the T_{opt} was 25-30ºC, and the activity declined above 35ºC (Figure 1 a, b, j). The T_{opt} for respiration and growth of sulfate reducing communities is generally found to be well above the *in situ* temperature (Sagemann et al., 1998; Isaksen and Jørgensen, 1996). In cold environments the T_{opt} for anaerobic respiration is up to 10°C higher than the T_{opt} for growth (Sagemann et al., 1998; Knoblauch and Jørgensen, 1999). Broad temperature profiles with relatively high T_{opt} and T_{max} values have been reported for Arctic sediments (Arnosti et al., 1998; Sagemann et al., 1998). Thus, in Smeerenburgfjorden sediments, which are around 2°C year round, T_{opt} values of 30°C have been demonstrated several times (Sagemann et al., 1998; Robador et al., 2009; Brüchert et al., 2001). The *in situ* temperature of the south Atlantic sediments from greater water depths was lower than on the shelf and the T_{opt} and T_{max} decreased with increasing water depth (Figure 1 b, c, d, f). Temperature-activity profiles from those deep stations indicate a predominance of

psychrotolerant and even psychrophilic bacteria. In Argentine sediment from 3000 m depth we measured a T_{opt} of 12°C after 36 hour incubation (Figure 1 e). A similarly low T_{opt} of 12.5°C for sulfate reduction had been observed in sediment from Antarctica from Kap Norvegia in the Weddell Sea (Isaksen and Jørgensen, 1996). This may be the lowest published temperature optimum for an anaerobic microbial process in nature. The authors reasoned that the temperature profile resembled the response of psychrophilic isolates and therefore likely reflected also the growth rate optimum of a predominantly psychrophilic community (Isaksen and Jørgensen, 1996).

A relationship between the sulfate-reducing populations T_{opt} and water depth was not observed in the Arctic fjord sediments as the bottom water of the Svalbard fjords is permanently near 0°C at all water depths. In our study with Smeerenburgfjorden sediment a broad temperature profile was observed with T_{opt} at 27°C after 8 hour incubation (Figure 1 j).

In an earlier study of Smeerenburgfjorden sediment the T_{opt} was observed to be lower if the incubation period was extended, e.g., 21°C after 4.5 day incubation (Arnosti et al., 1998). A similar shift in temperature response was found by Finke and Jørgensen (2008) in Arctic sediment where the T_{opt} dropped from 27°C after 0.3 days to 18°C after 8 days of incubation. It is apparent that sulfate-reducing microorganisms from these cold sediments maintain high activity at the highest temperatures only for a limited time (Finke and Jørgensen, 2008). It is interesting that we found a low T_{opt} of 18°C in Svalbard sediment from 100 m water depth after only 12 hour incubation (Fig 1 i). The T_{opt} of 18°C for sulfate reduction in Kongsfjorden sediment is comparable to the optima reported for growth of pure cultures of sulfate reducing bacteria isolated from cold environments (Knoblauch and Jørgensen, 1998; Isaksen and Jørgensen, 1996).

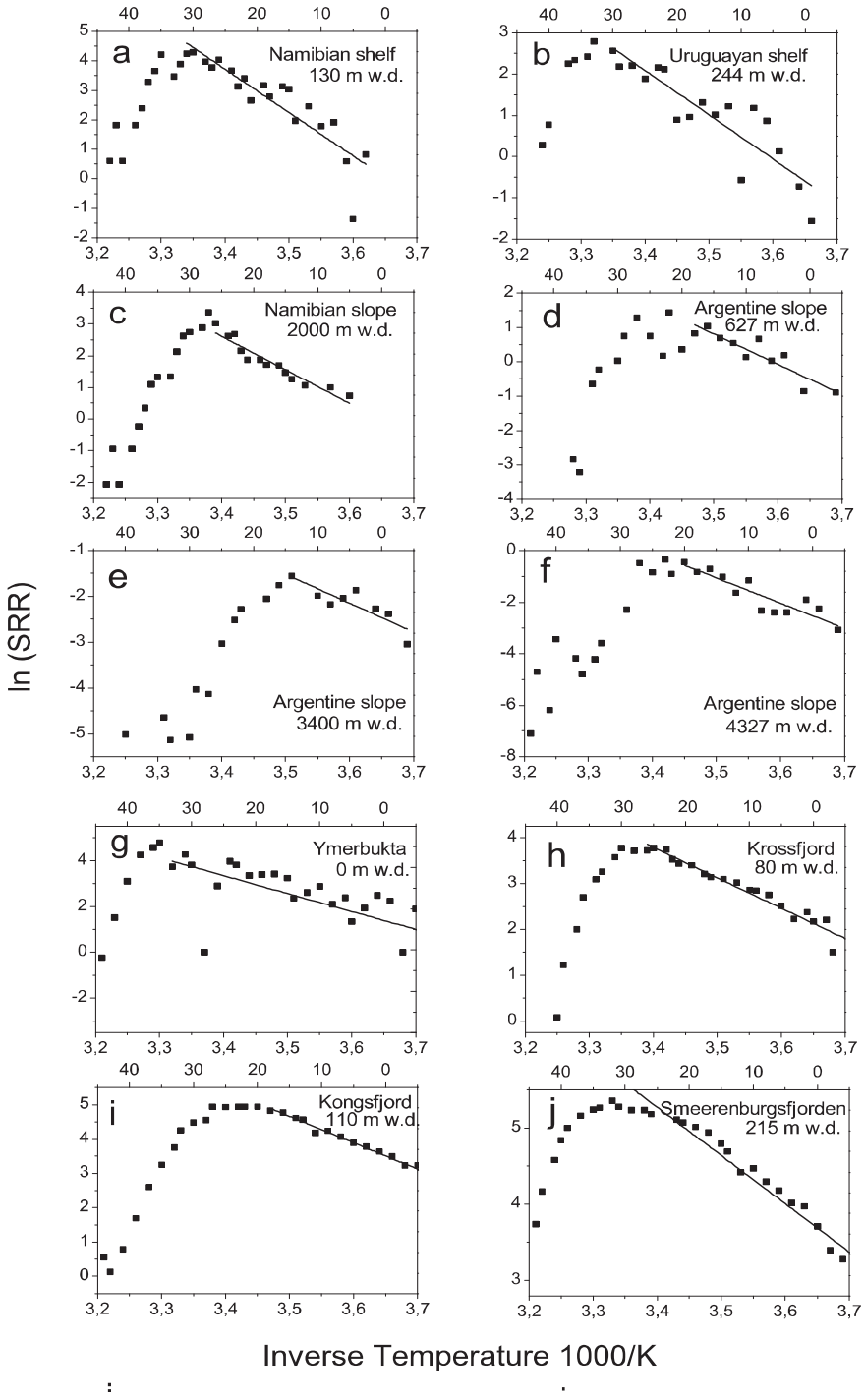

Figure 2 Arrhenius plots of data in Figure 1.

SRR in shelf and slope sediments fall into three temperature groups

The temperature characteristics of SRR imply the presence of mixed SRB communities composed of mesophilic, psychrotolerant and psychrophilic members in south Atlantic and Arctic sediments (Arnosti et al., 1998; Rysgaard et al., 2004; Sahm et al., 1999). In south Atlantic sediments the spatial distribution of distinct thermal groups was related to the *in situ* temperature of the sediment and consequently to the water depth. Thus, high T_{opt} of SRR on the shelf indicates a predominance of mesophilic and/or psychrotolerant SRB, whereas in the deeper sediments T_{opt} was lower, implying the presence of SRB adapted to permanently cold conditions typical for psychrotolerant and psychrophilic microorganisms. The predominant temperature responses of SRR in south Atlantic incubations were psychrotolerant, which is consistent with earlier reports that psychrophiles do not prevail in these permanently cold sediments (Nedwell and Rutter ,1989).

The adaptation of SRB populations to the *in situ* temperatures was described from experimentally measured temperature curves by comparing SRR at *in situ* temperature with SRR at T_{opt} and by calculating the activation energy, E_a (Knoblauch and Jørgensen, 1999). The *in situ* SRR in the Argentine and Arctic sediments were high compared to the rates at T_{opt} falling in the 20% to 50% range (Table 3). These values are comparable to those reported for cold-adapted Arctic SRB (Robador *et al*. 2009) and are consistent with similar relative growth rates of 24% to 41% determined for psychrophilic strains isolated from Arctic sediments (Knoblauch and Jørgensen, 1999). In Namibian shelf and slope sediments the in situ rates relative T_{opt} were <10% (Table 3), but E_a determined for Namibian sediments suggests that SRB are well adapted to the ambient temperature. The E_a were similar to those measured for sulfate reduction, denitrification and anammox in Arctic sediments (Figure 2, Table 3) (Rysgaard et al., 1998; Gihring et al., 2010). Q_{10} values for SRR in south Atlantic sediments

were around 2, which is a typical for many marine environments. This low Q_{10} value was found repeatedly for metabolic processes in Arctic and Antarctic sediments and implies a microbial community well adapted to ambient temperature. Many biological reactions have a Q_{10} of 2 which is roughly equivalent to an activation energy of 50 kJ mol^{-1} at 20°C (Kirchman et al., 2009). E_a values reported for permanently cold sediments vary in the range of 30-50 kJ mol^{-1}, depending on whether the community is more psychrotolerant or psychrophilic.

Sediment transport effects on experimentally-determined temperature-activity relationships

Deposited labile organic matter from the Benguela upwelling system over the Namibia shelf undergoes suspension and re-deposition leading to a net down-slope transport. The shelf material accumulates in depo-centers at 1000-1500 m water depth where the sediment is rich in organic matter (Inthorn et al., 2005; Inthorn et al., 2006). This down-slope transport of sediment material from the warm shelf may explain the relatively high SRR and a temperature-activity response indicative of the presence of mesophilic SRB in the cold slope sediments (Figure 1 a, c). Although psychrotolerant or psychrophilic SRB may be better adapted to live in the slope sediments at the prevailing temperature of 3°C, the down-slope dispersal of SRB from warmer sediments enables mesophilic community to be maintained. A mesophilic signature was observed also in the temperature response of SRR from upper slope sediments off Argentina (Figure 1d) whereas sediments from greater water depths had rather a psychrotolerant to psychrophilic signature (Figure 1e, f). Also the slope sediments off Uruguay and Argentina are characterized by dynamic depositional conditions with generally high sedimentation rates, gravity mass flows due to turbidities and slides, and strong surface

currents (Riedinger et al., 2005). Thus, also here some mesophilic SRB may be transported down from the shelf to the slope.

Conclusions

T_{min}, T_{max}, and T_{opt} temperatures for SRR in the continental margin shelf and slope sediments indicate that all three thermal groups are present. T_{opt} of SRR determined from short-term temperature experiments depended on the depth of the water column and was lower in the deep-sea sediments and in the cold Arctic than T_{opt} in warmer sediments. Mesophilic sulfate reducing bacteria in south Atlantic slope sediments are likely transported with sediment from the adjacent shelf where such mesophilic bacteria dominate.

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Manuscript III

Adaptation of sulfate-reducing bacteria to ambient sediment temperatures in polar, temperate, and tropical marine environments

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Adaptation of sulfate-reducing bacteria to ambient sediment temperatures in polar, temperate, and tropical marine environments

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Keywords: Sulfate reduction, temperature adaptation, marine sediments

Summary

Temperature is an important factor regulating the rate of biological processes and, therefore, is likely to exert a selective pressure in the environment. The temperature response of carbon mineralization via bacterial sulfate reduction of polar, temperate and tropical marine sediments was studied in temperature-gradient incubations experiments by measuring sulfate reduction rates (SRR) using ^{35}S-sulfate. Sediment slurries were incubated in a thermal gradient between -10°C and +50°C to cover the physiological temperature range of the active sulfate-reducing bacteria (SRB) and the resulting temperature response profiles were used to characterize the competitiveness of SRB, in terms of relative SRR at in situ temperatures, the temperature dependence for energy metabolism of SRB and the correlation of cardinal temperatures of sulfate reduction and sediment temperatures. In polar regions, only temperatures close to the freezing point of the sediment limit the rates of sulfate reduction. In these environments SRB exhibited high metabolic rates of ca 10-17% of maximal potential rates at the in-situ temperature of 0°C. Similar relative SRR in temperate and tropical sediments were only observed at temperatures around 15-20°C. These observations imply psychrophilic adaptation of polar SRB and the predominance of mesophilic SRB in warmer latitudes. Further examination of the temperature dependency for sulfate reduction using Arrhenius plots in temperate sediments revealed that tropical sediments exhibited a more limited metabolic regulation at temperatures below 8-18°C and optimal temperature conditions for sulfate reduction closer to their ambient temperatures. Together, the inspection of the temperature responses for metabolic activity of SRB in marine sediments showed that temperature adaptations of SRB form a continuum with respect to their environmental latitude, which implies the potential of environmental temperatures for the selection of adaptive physiologies and for evolutionary divergence of microbiota in different latitudes.

Introduction

Mineralization rates of organic matter in coastal marine sediments frequently show a strong variability associated with seasonal changes at ambient temperatures (e.g. Middelburg et al., 1996; Arnosti et al., 1998; Rysgaard et al., 1998; Thamdrup and Fleischer, 1998). Among the different processes implicated in the anaerobic benthic degradation of organic carbon, bacterial sulfate reduction has the quantitatively dominant role (Jørgensen, 1982) and the measurement of respiratory reduction of sulfate to sulfide is one of the few available methods that targets a physiological defined group of bacteria. Consequently, most work on the environmental temperature dependence of sedimentary metabolism has involved seasonal studies of sulfate reduction in coastal sediments (e.g. Jørgensen, 1977; Aller and Yingst, 1980; Moeslund et al, 1994; Kristensen et al., 2000). Coastal environments, however, are highly variable and dynamic systems and the interpretation of the response of sulfate reduction rates to ambient temperatures is not straightforward.

Sulfate reduction in marine sediments is constrained by factors such as the concentration of organic matter and its degradability which is commonly interpreted as the overriding limiting factor controlling the response of SRR to temperature. Westrich and Berner (1988) observed that the temperature dependence of SRR is more pronounced in sediments with lower benthic mineralization rates, and suggested that the temperature response of sulfate reduction may depend on the reactivity of accessible organic carbon. In fact, although rates of sulfate reduction are generally considered to be limited by the quantity and quality of available organic substrates (Westrich and Berner, 1984), the consequence of temperature variations is not only overall changes in turn-over rates but also qualitative, as well as quantitative, shifts in species composition and community structure (Robador et al. 2009). When similar permanently cold and temperate sediments were compared over long-

term temperature incubation experiments, Robador et al. (2009) observed that the response of bacterial sulfate reduction to warming was closely related to the physiological characteristics of the active SRB community. The microbial SRB community in Arctic sediments, in contrast to their temperate counterparts, exhibited a high sensitivity to increasing temperatures and a rapid decline of specific groups of SRB.

The examination of the temperature characteristics for sulfate reduction of bacteria isolated from marine sediments has shown the coexistence of SRB populations with different temperature adaptations. Isaksen and Teske (1996) isolated a moderately psychrophilic sulfate-reducing bacterium from temperate sediments able to grow and sustain a higher catalytic activity at lower temperatures when compared to a mesophilic strain isolated from the same sediments (Isaksen and Jørgensen, 1996). Laboratory studies on the temperature dependence of sulfate reduction in Arctic and Antarctic marine sediments (i.e. Sagemann et al., 1998; Isaksen and Jørgensen, 1996) showed that the bacterial community is predominantly psychrophilic, while in temperate sediments the SRB were mostly mesophilic (Isaksen et al., 1994). Together, these observations suggest the strong influence of environmental temperatures on microbial selection. In competition, the physiological adaptations of the active SRB to ambient temperatures may have an important role in the temperature response of the benthic mineralization of organic matter.

The aim of the present study is to investigate the physiological adaptation to environmental sediment temperatures, in terms of sulfate reduction rates (SRR), as an important mechanism controlling competition and other microbial interactions and, ultimately, the efficiency of carbon cycling. In order to understand the effect that ambient temperatures may have on the microbial carbon cycling in marine sediments, we compared the temperature

dependence of the SRB community in sediments from different latitudes using temperature gradient incubation experiments.

Material and methods

Study sites

Study sites for the present work were selected extending from polar regions to temperate and tropical latitudes in order to obtain a representative range of sediments exposed to different environmental temperatures. A detailed description of the study sites is provided in Table 1. Samples were obtained from the upper 10 cm depth of sediment from each site, which generally corresponds to the depth range where bacterial sulfate reduction peaks in 95% of all sediment studies published to date.

Index properties

Wet-bulk density and porosity were calculated from one sediment sample, taken at each sampling site, from measurements of the wet and dry masses of the sample and from the volume using calibrated glass cylinders.

Elemental analysis

The elemental analyses, at each sampling site, were performed on triplicate samples of about 20-100 mg of freeze-dried ground sediment.

Total carbon (TC) and nitrogen (TN) content of sediment samples were determined using a Fisons NA 1500 (Series 2) elemental analyzer. Freeze-dried material with vanadium pentoxide catalyst added is converted into elemental simple gases by combustion at 900-1000°C in a stream of oxygen, moved through a separation column and, subsequently, detected by a thermal conductivity detector.

Inorganic carbon was measured in order to determine the amount of total organic carbon (TOC) in the sediments. Total inorganic carbon (TIC) was determined using a CM5240 TIC auto-acidification sampler module attached to a coulometer CM5014 CO_2 analyzer (UIC, Inc.) which measures automatically the absolute mass amount of CO_2 evolved from sample acidification. The TOC contents of the sediment samples were determined by calculating the difference between TIC and the TC value.

Temperature-gradient experiments

The temperature response of microbial energy metabolism was evaluated in temperature-gradient incubation experiments (Battley,1964). Sediment slurries were incubated in Hungate tubes in an aluminum temperature-gradient block heated electrically at one end and cooled at the other end with a refrigerated and thermostated water bath. The temperature span was from 0° to +50°C to cover a large potential physiological temperature range of the active organisms, which was well above the optimal conditions for growth in some of the studied sediments. Additionally, the incubation temperature gradient for sites 3, 4 adn 5 (Table 1) was extended to -10°C in order to explore the physiological limits of microorganisms at temperatures below the freezing point. Sediments slurries were prepared by dilution 1:1 with anoxic artificial seawater. Anoxic artificial seawater was prepared as described by Widdel and Bak (1992). Sediment slurries were prepared under N_2 and 5 ml of slurry were transferred into each Hungate tube. Hungate tubes were flushed with N_2 according to the Hungate technique (Bryant, 1972) and sealed with butyl rubber stoppers. The Hungate tubes were immediately placed in a temperature-gradient block and preincubated for at least 5 hours to allow them to reach thermal equilibrium. Measurements of bacterial sulfate reduction were performed using ^{35}S-sulfate according to Kallmeyer et al. (2004). In order to minimize bacterial growth during the experiment, the incubation time with the radiotracer was only 24

hours. Triplicate samples were incubated in parallel wells (at the same temperature) at several points along the temperature gradient block in order to investigate reproducibility of SRR. All data (including replicate measurements) are shown in Fig. 1.

Temperature dependence

Activation energy and Q_{10} values were calculated from the slope of the linear range in Arrhenius plots to characterize the temperature dependence of metabolic activity. The Arrhenius curves were obtained from temperature-gradient incubations and represent the variation of metabolic rate as a function of temperature as follows:

$$\ln(k) = \ln(A) + \left(\frac{-E_a}{R} \cdot \frac{1}{T} \right)$$

where E_a is the activation energy (J mol^{-1}), k is the rate of sulfate reduction (nmol cm^{-3} day^{-1}), A is the Arrhenius constant, R is the gas constant (8.314 J K^{-1} mol^{-1}), and T is the absolute temperature (K).

Q_{10} values between 20°C and 30°C were calculated according to the following equation:

$$Q_{10} = \exp \left[\frac{E_a \cdot 10}{RT(T + 10)} \right]$$

Results and discussion

Rates of sulfate reduction and competitiveness of SRB in marine sediments

Studies on the temperature response of initial and terminal steps of organic carbon turnover in marine sediments suggest that absolute rates of sulfate reduction are mainly controlled by the availability of suitable electron donors rather than by temperature (e.g. Arnosti et al., 1998). SRR in marine sediments increase following the amendment with organic carbon compounds which has been interpreted as substrate limitation under in-situ

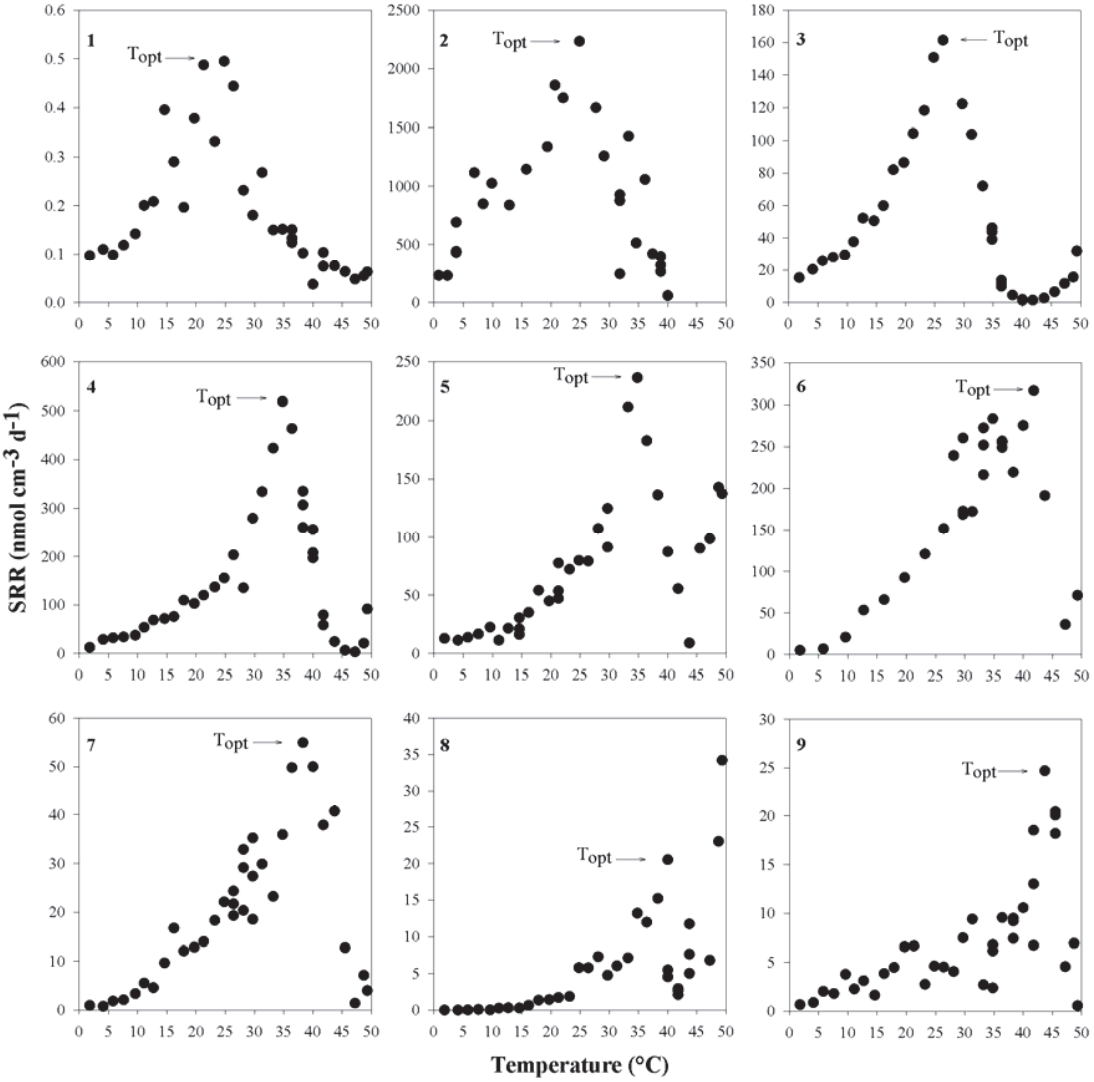

Figure 1. SRR measured in temperature-gradient incubation experiments of sediment slurries from all sampling sites. 1) Southern Ocean (Weddell Sea), permanently cold sediment; 2) Arctic Ocean (Ymerbukta, Svalbard), seasonally freezing-thawing sediment; 3) Arctic Ocean (Smeerenburgfjord, Svalbard), permanently cold sediment; 4)Wadden Sea (German Bight, North Sea), estuary system subjected to strong seasonal temperature changes; 5) Baltic Sea (Arkona Basin), sediment subjected to mild seasonal temperature changes; 6) Andaman Sea (Phuket Island, Thailand), permanently warm tide-dominated mangrove forest sediment; 7) Arabian Sea (off the coast of Goa, India), permanently warm sediment from an upwelling system; 8) Arabian Sea (Sadeyat island, United Arab Emirates), permanently warm hypersaline sediment; 9) South China Sea (Hainan Island, China), permanently warm sediment.

conditions (Sagemann et al., 1998; Arnosti et al., 2005). Therefore, absolute SRR measured in the present study (Figure 1) likely reflect variations in the content and quality of organic matter in the different sediments studied here.

In fact, the lowest SRR were measured in an oligotrophic environment, (Figure 1.1 and Table 1) while the highest rates were observed in a habitat characterized by the high content of organic matter derived from decomposing macrophytes (Figure 1.2 and Table 1). The examination of the competitiveness of SRB, understood in terms of relative metabolic rates at in situ temperatures to maximum rates, may be more useful to understand the overall metabolic capacity of SRB.

The SRR measured in the Arctic and Antarctic study sites at 0 °C relative to the maximum rates measured at the T_{opt}, 10-17 % (Figure 1.1, 1.2 and 1.3 and Table 2), are in the range previously described for psychrophilic SRB communities in similar polar marine sediments (Isaksen and Jørgensen, 1996; Robador et al., 2009). A relatively high metabolic rate at low temperatures represents one of the main physiological adaptations of microorganisms to cold habitats (Harder and Veldkamp, 1968). Among the three polar environments, the Antarctic sediment exhibited the strongest psychrophilic response, with the lowest T_{opt} for sulfate reduction and the highest relative rates at low temperatures. Arctic sediments collected from Smeerenburgfjorden and Isfjorden on the west coast of Svalbard are influenced by slightly warmer water currents than the Weddell Sea, which may be the reason for the broader temperature range of the active SRB community. In permanently, but moderately cold sediments from temperate regions sulfate reduction showed rather a mesophilic temperature response which was comparable to that of other temperate environments (Isaksen and Jørgensen, 1996). These observations together suggest that

ambient temperature is an important factor influencing the metabolic efficiency of SRB in a given environment.

SRR measured at 0°C in polar sediments relative to maximal rates at the T_{opt} were comparable to those of temperate sediments at an approximately 15°C higher temperature, close to their respective in situ temperatures (Figure 1.4, 1.5 and Table 2). This difference provides further indication for the adaptation of psychrophilic SRB to the permanently cold sediment temperatures in polar regions. Temperate sediments, however, are only seasonally exposed to low temperatures during winter and thus, the relative SRR, at 0 °C were only 2-5 % of the rates at T_{opt} (Figure 1.4 and 1.5 and Table 2). This is in agreement with previously published data on the temperature characteristics of SRB communities in similar temperate sediments (Isaksen et al., 1994; Robador et al., 2009) and suggests a suboptimal adaptation to low temperatures in comparison to polar regions.

SRR were only 0.1-3 % of maximal rates at 0°C for the tropical sediments (Figure 1.6, 1.7, 1.8 and 1.9 and Table 2). The permanently warm conditions in these environments may exert a strong pressure on SRB with low temperature adaptations and instead select for a community best adapted to permanently warm temperatures. In fact, sediments in the intertidal zone of the Arabian Sea can occasionally be exposed to temperatures close to 50°C (Al-Najjar, personal communication). At in situ temperatures at the time of collection, approximately 30 °C, SRR were 23-64 % (Figure 1.6, 1.7, 1.8 and 1.9) of maximal rates which suggests that permanently warm sediments are dominated by a mesophilic SRB community with an optimum temperature for metabolism close to the ambient range.

Interestingly, several of the studied sediments, irrespective of their latitudinal position, showed a rapid increase in SRR above the T_{opt} (Figure 1.1, 1.3, 1.4, 1.5, 1.6, 1.7 and 1.8). These rates indicated a thermophilic temperature response. In the present work, none of the

study sites were situated in a region supporting in situ growth at these high temperatures. Only the Antarctic site was located in the vicinity of a methane-venting seep, where thermophilic sulfate reducers could have originated from the deep subsurface through seepage transport to the seawater. Incubation experiments with sediments from temperate zones (Isaksen et al., 1994) also reported SRR in the thermophilic range which were attributed to the germination of SRB endospores. Thermophilic spore-forming bacteria have also been detected in the high Arctic (Hubert et al., 2009 and 2010), but their activity was only induced after incubations for more than 12-16 hours at 50°C.

Temperature dependency for metabolic activity of SRB in marine sediments

Arrhenius plots are commonly used to examine the temperature dependence of thermally-induced process or reactions. The Arrhenius equation (Arrhenius, 1908) is generally applied to model the temperature dependency of the rate of a chemical reaction. The slope of the linear range obtained from plotting the natural logarithm of the reaction rate against the reciprocal of the absolute temperature is proportional to the E_a of the reaction. The E_a can be defined as the minimum energy required to start a chemical reaction and small E_a values will therefore result in an increase of the reaction rate. In the context of temperature adaptations, for instance the catalysis of a chemical reaction by an efficient enzyme will yield a low E_a (Marx et al., 2007; D'Amico, 2002).

In the present study, chemical reaction rates have been substituted for SRR to examine the temperature response of sulfate reduction. The Arrhenius plots (Figure 2) presented here derive from the ^{35}S-determinations of sulfate reduction in sediments (Figure 1) and are characterized by a range of linearity, mostly extending below and above the environmental temperature range. E_a estimated from the slope of the linear temperature range are only

apparent activation energies and reflect the temperature response of the rate-limiting step in a biochemical processes, i.e. membrane transport or enzymatic catalytic conversion. Furthermore, calculated E_a are not necessarily activation energies by a single sulfate-reducing population only, but reflect rather the response of a complex mixture of microbial communities. Despite these limitations, Knoblauch and Jørgensen (1999) found that calculated E_a values for pure cultures of SRB were similar to those estimated for whole sulfate-reducing communities in marine sediments. Coincident apparent E_a indicate that the response of sulfate reduction to increasing temperatures in pure cultures and natural sediments is comparable, and consequently, E_a may be a useful parameter to describe and evaluate the temperature sensitivity of SRB communities between sediments from different climate regimes.

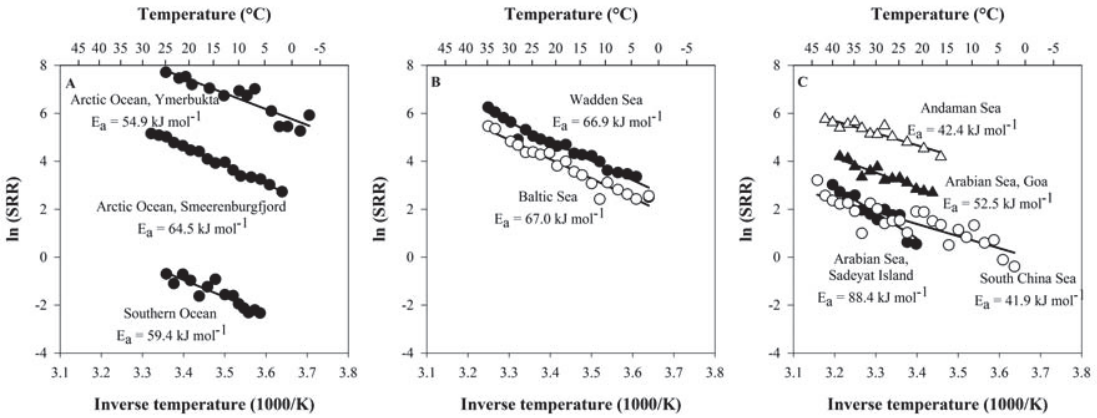

Figure 2. Arrhenius plots of data in Figure 1.

Apparent E_a ranged between 41.9 kJ mol^{-1} and 88.4 kJ mol^{-1} (Q_{10}, 1.8-3.3; Table 2, Figure 2). These values are within the range of apparent E_a estimated in seasonal studies of coastal marine sediments, 36-132 kJ mol^{-1} (Westrich and Berner, 1988). Westrich and Berner (1988) observed that deeper buried sediments with lower SRR exhibited a more pronounced temperature dependency, i.e. higher E_a values, and attributed this effect to variations in the

quantity of organic matter readily amenable to fuel sulfate reduction. However, similar experiments but with a higher temperature resolution showed that apparent E_a in substrate-limited sediments, 40-75 kJ mol^{-1}, did not change significantly after the addition of organic substrates (Sagemann et al., 1998). It is therefore possible that the variability of apparent E_a previously observed in marine sediments is not due to the reactivity of the organic matter but due to the different physiological adaptations of coexisting SRB populations.

The temperature dependency of sulfate reduction in polar and temperate samples was constant down to approximately 0°C (Figure 2) and, when further examined (Figure 3), SRR were measured along temperatures between -3.4°C and -6.4°C, which are at or below the freezing point of the sediment slurries. Below these temperatures SRR decreased abruptly reflecting the physico-chemical constrains (i.e. low water activity, low nutrient content, high salinity) imposed by the freezing process of the sediment. These results clearly indicate that the polar and temperate sulfate-reducing communities are well adapted to tolerate temperatures down or beyond the freezing point of seawater, which may permit survival and recovery even after temporary freeze conditions. There is evidence, from experimental studies with arctic sediments, that sulfate reduction decreases sharply during freezing however, SRB may exhibit relatively high metabolic rates upon thawing or even after repeated freeze-thaw events (Sawicka et al., 2010). In addition to psychrophily, cryotolerance may be an important characteristic of SRB for survival in polar environments with constant low temperatures or even in temperate habitats exposed to extreme seasonal low temperatures.

By contrast, in sediments from tropical latitudes, E_a remained constant over a linear range that extended from the T_{opt} down to an apparent transition temperature between +8°C and +18°C (Figure 2C). Below these temperatures the slope changed sharply demonstrating higher E_a values. Apparent E_a values increased to 124.6-184.6 kJ mol^{-1} (Q_{10}, 5.3-11.8), which

suggests that sulfate-reducing communities in permanently warm climates have a higher temperature dependence than communities in seasonally changing and permanently cold habitats.

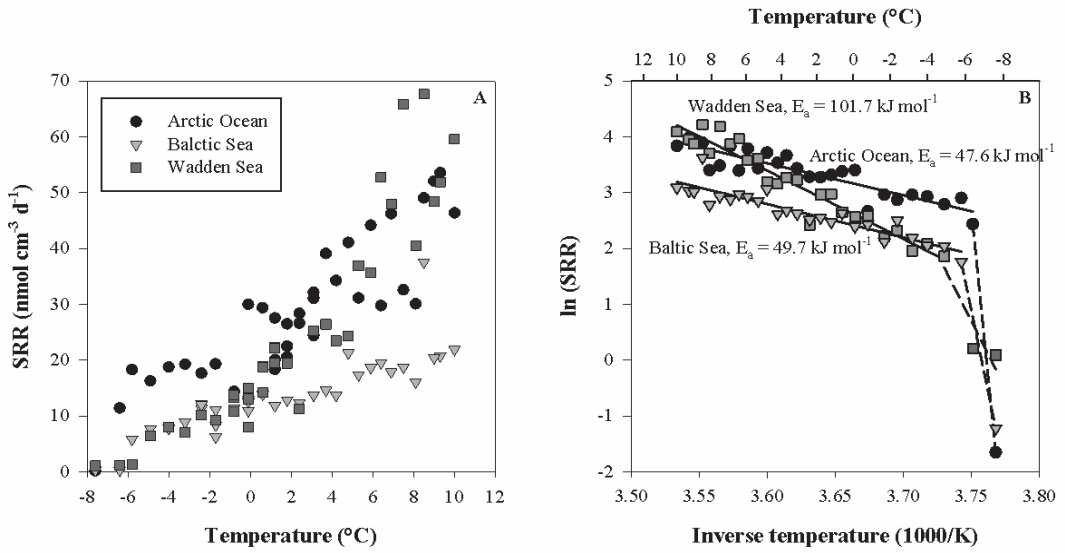

Figure 3. (A) SRR measured in temperature-gradient incubation experiments down to -10°C of sediment slurries from sampling sites 3, 4 and 5. (B) Arrhenius plots of data in panel A.

The temperature at the intersection of the two slopes is defined as the 'critical temperature', T_{crit} (Lamanna et al., 1973) and the existence of T_{crit} has been described for psychrotolerant, mesophilic and thermophilic microorganisms (Harder and Veldkamp, 1968; Mohr and Krawiec, 1980; Reichardt and Morita, 1982). Reports for psychrophilic microorganisms are lacking, probably because growth rates of psychrophiles have not been examined systematically at sufficiently low temperatures (Bakermans and Nealson, 2004). T_{crit} has been described for sulfate reduction in SRB isolates (Tarpgaard et al., 2005), although there are no reports in marine sediments. This is probably because most of the studies have investigated the psychrophilic response of SRB in low-temperature environments at minimum temperatures of -3.5°C (e.g. Robador et al., 2009). Based on experimental data of bacterial cell protein concentration, the T_{crit} has been previously explained as the transition temperature

between optimal and sub-optimal domains for bacterial growth (Guillou and Guespin-Michel, 1996). Although a cellular basis for T_{crit} remains uncertain, this temperature is likely the result of the uncoupling of cellular energy metabolism at low temperatures. In addition to E_a, T_{crit} may be a functional parameter important to explain the physiological temperature dependency of microorganisms.

Conclusions - Correlation between environmental temperatures and cardinal temperatures of sulfate reduction

Our results suggest a direct relationship between the ambient environmental temperature and sedimentary bacterial energy metabolism reflected in the T_{opt} (Figure 4).

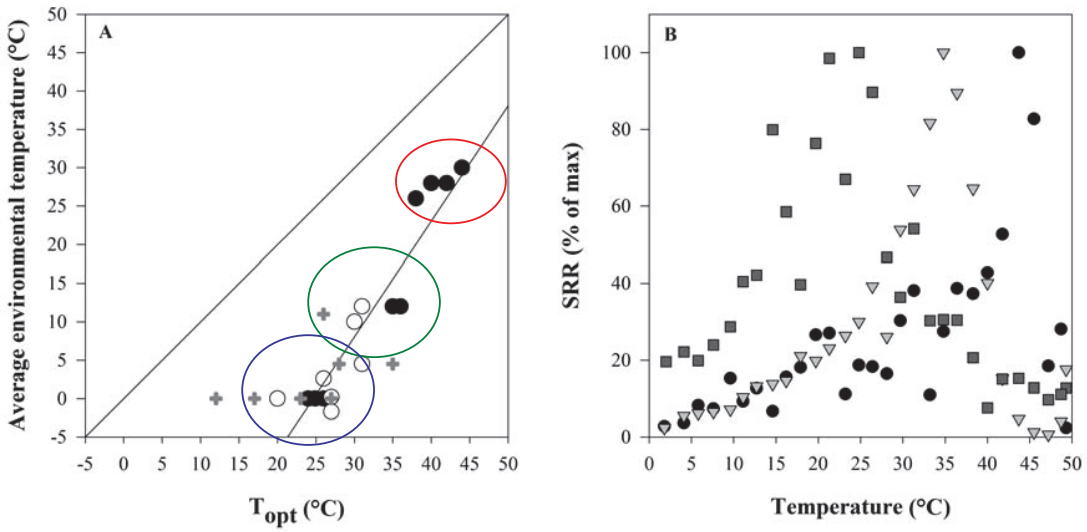

Figure 4. (A) Relations between average environmental temperatures and T_{opt} for sulfate reduction in marine sediments grouped according to sampling latitude: Polar regions, blue line; Temperate regions, green line; Tropical regions, red line. The plot is based on: data presented in this study, full circles; data compiled from Isaksen et al. (1994), Isaksen and Jørgensen (1996), Arnosti et al. (1998) and Sagemann et al. (1998), open circles; and data from seven SRB strains presented in figure 4, plus symbols. The straight line passing through the origin is the theoretical curve if environmental temperatures and T_{opt} for SRR were the same. The regression line indicates the empirical relation between environmental temperatures and T_{opt} for SRR. (B) SRR expressed as percentage of maximum rates, corresponding to data in panels 3, 4 and 9 of fig. 1. Profiles were selected to represent the characteristic temperature responses of each group in panel A

Although T_{opt} generally exceeds the in situ temperatures experienced by the microbial communities, the proportional increase with the mean ambient temperatures (Figure 4A) implies diverse temperature sensitivities of the dominant microbial community in the studied environments. Increasing T_{opt} for sulfate reduction reflect the diverse nature of the SRB involved and their temperature adaptations as shown in figure 4B. Although the composition of temperature response for the dominant SRB in the sediments is unknown, sediment SRR can be interpreted on the basis of mixed communities with different temperature response curves.

Arctic and Antarctic sediments exhibited T_{opt} for sulfate reduction of 24-26 °C (Figure 1.1, 1.2, 1.3 and Figure 4B), similar to those previously reported for some psychrophilic SRB isolates (Knoblauch et al., 1999). The T_{opt} observed in warmer temperate and tropical sediments, however, are in the range of those reported for nominal mesophiles (Isaksen and Jørgensen, 1996). Sediments from temperate latitudes showed broader thermal ranges than polar sediments and sulfate reduction could be measured from temperatures below 0°C up to the T_{opt} at 35°C (Figure 1.4, 1.5 and Figure 4B). Tropical sediments exhibited a shift of the thermal range for sulfate reduction towards higher T_{opt}, 38-44°C (Figure 1.6, 1.7, 1.8, 1.9 and Figure 4B).

Figure 5 illustrates how the combination of SRR resulting from different pure SRB cultures (Fig. 5A) generates the type of mixed community response observed in this study for polar sediments (Fig. 5C). The quantitatively dominance of psychrophilic SRB strains translates into the relatively high activity at low temperatures (Figure 5C). Moreover, SRR of individual strains have characteristic temperature ranges (Figure 5B) that, when combined, result in a relatively broader response (Figure 5D).

The difference between environmental temperatures and T_{opt} of bacterial sulfate reduction, however, varied between the sediments. At in-situ temperatures of 0°C in corresponding polar regions the difference was approximately 27°C, while at in situ temperatures of 30°C in tropical habitats this difference was reduced to 15°C (Figure 4A). The explanation for the larger difference in cardinal temperatures is likely due to composition of the active SRB in the sediment. Figure 4A shows how the dominance of SRB with lower T_{opt} could, potentially, influence the overall response of sulfate reduction in the environment.

In conclusion, the physiological responses described in this study demonstrate that psychrophilic and mesophilic SRB in polar and tropical environments, respectively, have evolved to adapt their energy metabolism to the stenothermal environmental conditions. Ambient temperatures outside the upper or lower limits of their thermal range likely result in functional constraints. In eurythermal habitats with strong seasonal temperature fluctuations, the overall rates of organic carbon mineralization are likely determined by the combined metabolic response of coexisting populations to the wide range of temperatures that characterize these environments. The heterogeneous temperature adaptations of the coexisting SRB in these habitats can explain the broad temperature response described above. However, a higher competitiveness of mesophilic SRB compared to their psychrophilic counterparts at their respective in situ temperatures may explain the predominance of mesophilc microorganisms in these habitats.

The potential significance of environmental temperatures and habitat temperature variability has generally not been taken into account in the study of the temperature response of carbon mineralization in marine environment (Wohlers et al., 2009). Bacterial temperature response is generally assumed to be well described by Q_{10} values between 2 and 3 (Pomeroy and Wiebe, 2001). The present study shows that biogeographic variability, selection of

adaptive physiologies, and evolutionary divergence of microbiota in different latitudes need also be considered for an improved quantification of respiration effects in response to ocean warming.

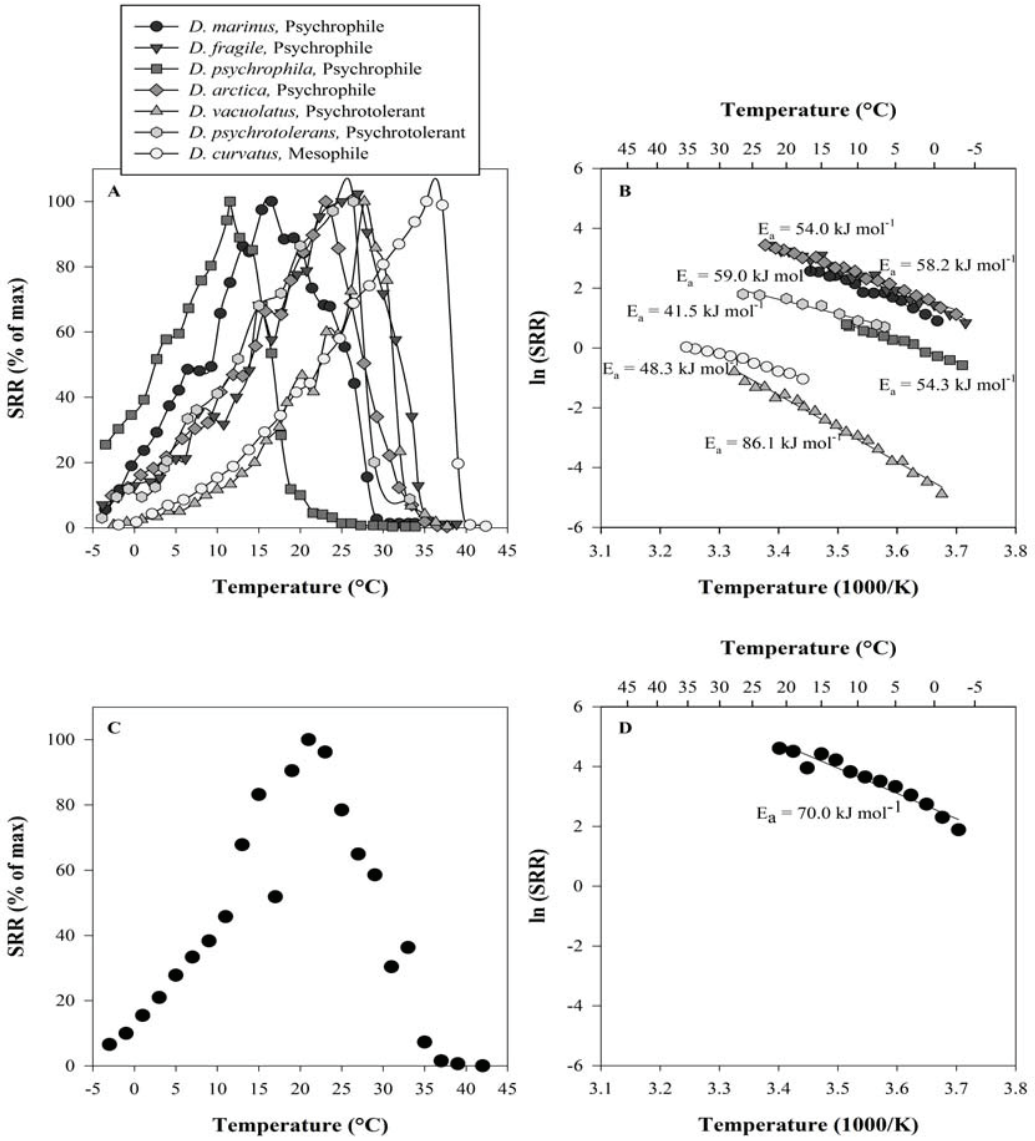

Figure 5. (A) SRR measure in temperature-gradient incubation experiments of seven sulfate-reducing bacteria strains. Data modified from; Isaksen and Jørgensen (1996), Knoblauch and Jørgensen (1999), Tarpgaard et al. (2005). (B) Arrhenius plots of data in panel A. (C) Sum of SRR of the seven strains from panel A at 2°C temperature intervals. (D) Arrhenius plot of data in panel C.

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TABLES

Table 1. Sampling site description

Table 2. Summary from temperature gradient experiments of data in figure 1

Table 1. Sampling site description

Study sites	Study sites	Coordinates		Sampling date	Sampling device	Water depth (m)	Average environmental temperature (°C)	Salinity (‰)	Wet Density (g/cm^3)	Porosity	C/N	TOC (%)	Sediment description
1	Southern Ocean (Weddell Sea)	65° 26´ S	61° 26´ W	Sep-07	Multi core	850	0[a]	34[a]	1.5	0.7	7	0.3	Permanently cold sediment situated in the proximity of a methane-venting cold seep, consisting of light-grey colored stiff clay
2	Arctic Ocean (Ymerbukta, Svalbard)	78° 16´ N	14° 02´ E	Jul-05	Push core	Subtidal	0[a]	27-30[a]	1.5	0.6	13	2.9	Seasonnally freezing-thawing sediment located at the tidal-dominated fringe of a glaciar moraine and consisting of black colored coarse-grained sand
3	Arctic Ocean (Smeerenburgfjord, Svalbard)	79° 42´ N	11° 05´ E	Aug-07	HAPS core	215	0[a]	33-34[a]	1.7	0.6	11	1.6	Permanently cold sediment with abundant worm burrows, soft brown colored in surface grading to clayey mottled dark grey-black over depth

4	Wadden Sea (German Bight, North Sea)	53° 27′ N	08° 07′ E	May-07	Push core	Intertidal	12[b]	22-30[b]	1.3	0.7	13	3.0	Estuary system subjected to strong seasonnal temperature changes with abundant meio- and macrofauna, sediment consisting of light-brown sandy mud changing to black mud over depth
5	Baltic Sea (Arkona Basin)	54° 46′ N	13° 48′ E	Jun-07	Multi core	9	12[b]	8-9[b]	1.2	0.7	9	6.1	Sediment subjected to mild seasonnal temperature changes consisting of dark-brown and black colored mud
6	Andaman Sea (Phuket Island, Thailand)	08° 03′ N	98° 25′ E	Aug-07	Push core	Intertidal	28[a]	28-34[a]	1.3	0.6	30	3.6	Permanently warm tide-dominated mangrove forest, sediment consisting of brown colored coarse-grained sand

7	Arabian Sea (off the coast of Goa, India)	15° 6´ N	73° 24´ E	Apr-07	Multi core	60	26[a]	34-35[a]	1.2	0.8	14	3.0	Permanently warm sediment from an upwelling system consisting of green colored soft fine-grained and watery mud
8	Arabian Sea (Sadeyat island, United Arab Emirates)	24° 31´ N	54° 26´ E	Sep-07	Push core	Intertidal	30[a]	200[a]	1.4	0.7	106	1.4	Permanently warm hypersaline sediment covered by a 0.5 cm-thick microbial mat and consisting of yellow with grey-black streaks fine-grained sand
9	South China Sea (Hainan Island, China)	19° 35´ N	110° 48´ E	Sep-07	Push core	Intertidal	30[a]	15-25[a]	1.8	0.4	10	0.2	Permanently warm sediment with abundant worm burrows, consisting of dark-brown colored dry sand
[a] *In-situ* measurements													
[b] Measurements from closest automated monitoring station													

Table 2. Summary from temperature gradient experiments of data in figure 1

	Sulfate reduction		Sulfate reduction rates (nmol cm^{-3} day^{-1})				
Study sites	T_{opt} (°C)	T_{crit} (°C)	At 0°C	At T_{opt}	% SRR[a]	Range of linearity[b] (°C)	Activation energy (kJ mol^{-1})	Q_{10}
1	21	N/A	0.1	0.5	20	0, +21	51.2	2.0
2	25	N/A	232	2233	10	0, +25	54.9	2.1
3	26	N/A	15	161	9	0, +26	64.5	2.4
4	35	N/A	12	518	2	0, +35	63.7	2.4
5	35	N/A	13	236	5	0, +35	67.0	2.5
6	42	13	5	316	2	+13, +42	44.6	1.8
7	38	11	1	55	2	+11, +38	55.7	2.1
8	40	18	0.02	21	0.1	+18, +40	97.4	3.7
9	44	6	1	25	3	+8, +44	36.0	1.6

a % of SRR at 0°C of max SRR at T_{opt}

b The term "Range of linearity" refers to the linear part of the Arrhenius plot

Appendix

Temperature effects on the microbial community composition in Arctic and temperate marine sediments

Abstract

Understanding the impact of increasing temperature on microbial community in Arctic Ocean may help us to asses and predict the response of carbon cycling to warming in this sensitive region. We have used denaturing gradient gel electrophoresis (DGGE) as a fingerprint technique to screen sedimentary microbial community composition in Arctic and temperate sediments exposed to elevated temperature over a year. Changed DGGE banding pattern in both sediments suggest that long term exposure to increased temperature changes sedimentary microbial community composition.

Introduction

The Arctic Ocean is experiencing currently changes due to anthropogenic and natural factors that include warming, sea ice loss and ecosystem structure changes (Vincent, 2010). Since Arctic Ocean plays an important role in the global carbon cycle the response of microbial mediated carbon cycling has been currently studied in the water column and at the seafloor both at the *in situ* conditions and in the laboratory settings (Robador et al., 2009 and 2010, Kirchman et al*.,* 2005; Wohlers et al*.,* 2009). To test whether microorganisms will be affected by increased temperature perturbation experiments are being performed in which experimental warming is applied. However, these short experiments might fail to incorporate the possibility of adaptations of extant communities over the long term or of shifts in community composition. Long term incubations allow for shifts in community in response to the manipulated environmental parameter.

Long term, 2-year incubation, performed on the permanently cold and temperate sediments has demonstrated that warming can have differential effect on sedimentary microbial communities (Robador et al., 2010). Studies on sulfate reducing bacteria (SRB) - key terminal oxidizers of organic matter in shelf sediments revealed the decline of specific groups of SRB and confirmed that microbial community composition of arctic sediments is particularly sensitive to elevated temperature, while these effect was not observed in a temperate sediment (Robador et al., 2009). In addition functional microbial groups in studied sediments reacted differently to long term warming (Robador et al., 2010). The accumulation of DOC was observed suggesting that the activities of organisms and enzymes responsible for the solubilization/hydrolysis of POC to DOC outpaced DOC consumption by sulfate reducing bacteria (Robador et al., 2010). Low concentration of volatile fatty acids and temperature related decline in sulfate reduction rates demonstrate close coupling between sulfate reduction and fermentation of volatile fatty acids. It was hypothesized that the net accumulation of DOC in warming marine sediments could be related to a change in the composition of the microbial community in response to permanent temperature increases.

Our study provides insight into the response of sedimentary microbial community composition to increased temperature scenario. Using DGGE technique we screened for changes in microbial community composition in permanently cold Arctic sediment samples and temperate sediment samples, incubated at increased temperatures for a year. We hypothesized that increased temperature would affect microbial community composition in Arctic sediment, but would have no effect on the community composition in temperate sediment.

Material and Methods

Sampling site

The sediments were collected, from a permanently cold region (Svalbard, Arctic Ocean, 79°420N, 11°050E; sediment temperature typically around 0 °C) in 2008. The study site was in the central part of Smeerenburgfjord, on the west coast of Svalbard, Arctic Ocean (Station J; 79°42′N, 11°05′E; water depth 215 m). At the times of sampling the temperature was 1.6°C. Sediment was brown-coloured in the upper 2 cm, and contained numerous worm burrows and occasional drop stones and brittle stars. Below ca. 3 cm depth, the sediment was clayey and changed to a mottled dark grey-black. Sediments were collected with a haps corer. Sediments were also collected from a temperate region and the site was located in Aarhus Bay in Denmark. Samples were collected with a box corer in January 2009. The water depth is 15 m and the sediment is silty clay (organic carbon: ca. 3% dry weight). *In situ* temperatures vary between 4 and 15° annually (Rasmussen and Jørgensen 1992). Aarhus Bay is located in the North Sea Baltic Sea transition, and salinity of bottom waters varies between 23 and 33 ‰ (Arnosti et al. 1998).

Sediments from all sampling sites were transferred into 2 l gas-tight plastic bags (Hansen et al., 2000) without airspace and stored at in situ temperatures until further processing. These bags allowed the long-term incubation of anoxic sediment for the study of microbial and geochemical processes over time. Homogenization was performed by simple kneading, thus avoiding continuous stirring, introduction of a gaseous headspace or dilution with seawater.

Sediments from Smeerenburgfjorden collected in 2008 and from Aarhus Bay collected in 2009 were incubated at 4°C, 10°C and 20°C after collection and also subsampled periodically. To maintain anoxic conditions, sediment was sub-sampled under nitrogen gas using an inflatable polyethylene glove bag (Two-hand Atmos- Bag, Aldrich). In order to

avoid the depletion of the electron acceptor for sulfate reduction, prior to every sub-sampling of sediment, incubation bags were homogenized for 10 min by manual kneading and sulfate concentrations in pore water were measured as previously described. Experimental bags contained sediment of a known volume and porosity. In order to avoid sulfate limitation of carbon remineralization during the 24-month incubation, sulfate was added to the bags to reconstitute in situ concentrations whenever concentrations decreased to 3–5 mM. Experimental bags were not replenished with any organic substrates as continuous amendments may result in the enrichment of particular microbial populations over the course of the experiment.

Sediments from Smeerenburgfjorden collected in 2008 and from Aarhus Bay 2009 were sampled periodically after 2 and 12 months and subsamples were taken for DNA extractions and further fingerprinting-DGGE analysis.

DNA extraction and PCR amplification

DNA was extracted, from the sediment using the Power Soil Kit (MolBio#12888-50). The 16S rRNA gene was amplified as described by Muyzer et al. (1997) with the universal primer 907R and the bacterial primer GM5F with a GC clamp (Muyzer et al., 1997). A PCR protocol was used as described by Muyzer et al. (1997) except that “touchdown” PCR was used to increase the specificity of the amplification and to reduce the formation of by-products, i.e., the annealing temperature was set 10°C above the expected annealing temperature and decreased by 1°C every two cycles until an annealing temperature of 55°C was reached at which nine additional cycles were performed. The program started with a hot start at 94°C for five minutes (20 cycles in total) (Muyzer et al., 1997).

Denaturing gradient gel electrophoresis

Denaturing Gradient Gel Electrophoresis (DGGE) was performed using a Bio-Rad DeCode system (BioRad, Munich, Germany). Polyacrylamide gel gradients (20-80%) were

poured with a gradient pump (Econo Gradient Pump, Bio-Rad, Munich, Germany). The gel was polymerized by adding 10% ammonium persulfate (APS) and Temed (BioRad, Munich, Germany) before pouring the gel. 80 µl of each PCR product was applied onto the gel and the DGGE was then performed at 60°C and a constant voltage of 200 V for 3.5 hours. After electrophoresis the gel was incubated for 30 min in an aqueous ethidium bromide solution (0.5 µg/L) and visualized on a UV transilluminator (LTF-Labortechnik, Wasserburg, Germany). The DGGE bands were then excised with a sterile scalpel and eluted in 30 µl sterile water for two days at 4°C. These bands were PCR reamplified using 5 µl of the eluted bands as PCR template. PCR product was amplified as described by Muyzer et al. (1997). A PCR program used was as follows: 95°C for 5 min, 94°C for 1 min, 46°C for 2 min, 72°C for 1min. After purifying the PCR products with the QIA quick PCR purification kit, the products were sequenced.

Results

Smeerenburgfjorden

DGGE analysis of bacterial 16S rRNA gene fragments showed for the Arctic samples revealed changes in community composition as a result of treatment. All manually scored bands from the DGGE image are marked with circles and bands for further analysis are numbered. A total of 4 bands were scored for original Arctic sediment. The number of bands has changed in the course of the experiment. Only two bands (1 and 3) were constant throughout the experiment. The band number 2 was detected in the sediment incubated at 4ºC and 10ºC after two months experiment. After 12 months of incubation band was visible only in the sediment incubated at 4ºC; marked as band number 5. In the sediments incubated at higher temperatures this band was not detected suggesting that temperature affected presence of a species. Band number 4 was detected only in the original Arctic sediment sample it was

not present in the other sediments subject to treatment. Three new bands have appeared as a result of treatment in the Arctic sediments. Bands number 6 and 8 were detected in the sediment incubated for 12 months at 20ºC. Band number 7 appeared already after two months of incubation in sediments at all temperatures. The band was faint after two months of treatment, but it has become more visible after 12 month incubation at 20ºC.

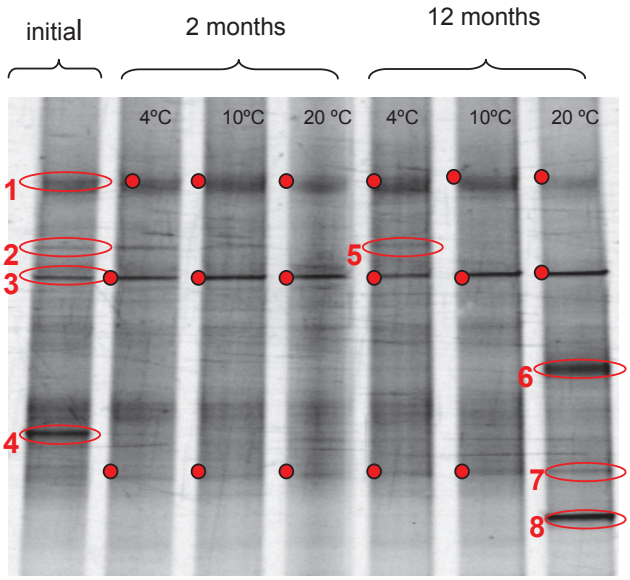

Figure 1. DGGE profiles for 16S rRNA gene fragments obtained from DNA extracted from Arctic sediment samples incubated for different times at increased temperatures.

Temperate sediment Aarhus Bay

In the Aarhus Bay sediment samples 16S rRNA DGGE profile was not resolved properly, however it is visible that DGGE profile varied throughout the experiment as a result of treatment. Only one band, numbered 1 was constant in the course of the experiment. The band number 2 has appeared in all sediments incubated at different temperatures after 2 months of experiment.

Discussion/Outlook

The increasing temperature may cause changes in microbial community composition and reshape microbial carbon cycling in the Arctic Ocean. Our study shows shifts in microbial community composition as a result of increased temperature treatment, both in permanently cold and temperate sediments. The disappearance of some species in the Arctic sediments and appearance of new ones after year of incubation at elevated temperature suggest that richness might change due to perturbations.

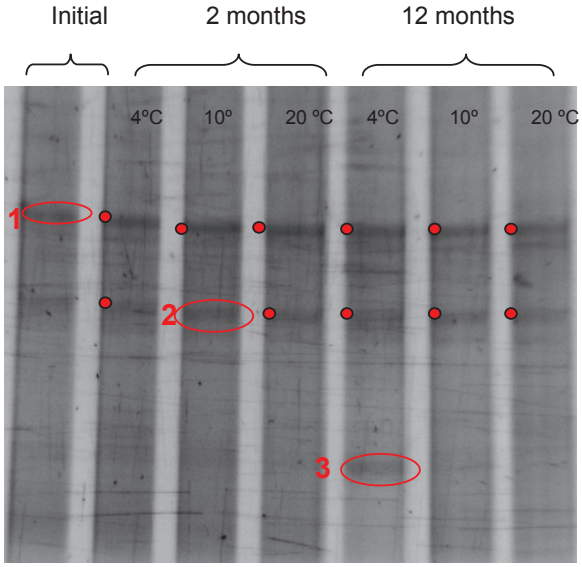

Figure 2. DGGE profiles for 16S rRNA gene fragments obtained from DNA extracted from temperate sediment samples incubated for different times at increased temperatures.

Previous studies demonstrated the steady decrease of the microbial cells and the relative contribution of Bacteria and specific groups of SRB to the total microbial numbers with increasing incubation time and temperature in the Arctic sediment. It implies that a large fraction of the community was negatively affected by the 10°C and 20°C long-term incubation temperatures. In contrast such change was not observed in the temperate sediment sample (Robador et al. 2009).

It is unknown whether compositional shifts will affect ecosystem processes and whether the disturbed community will be functionally similar to the original community (Reed and Martiny, 2007; Allison and Martiny, 2008). If an ecosystem lost an entire functional group, their absence would clearly impact the functioning of an ecosystem. On the other hand some species in a microbial community can be functionally redundant, thus the functioning of ecosystem might not be affected by their disappearance (Reed and Martiny, 2007; Allison and Martiny, 2008).

To help predict carbon cycling under changing environmental conditions long term studies on the microbial community composition are needed. It is also important to measure the rates of organic matter degradation to make the link between the community composition and sedimentary carbon cycling before and after a disturbance manipulation but before microbial composition changes. These measurements give some idea about the direct effect of the disturbance on process rates independent of community composition.

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PART IV Concluding Remarks

Concluding remarks

Psychrophilic and mesophilic SRB have adapted their energy metabolism to environmental temperatures. Thus, thermal groups of microorganisms exhibit globally a biogeographic pattern. It is predicted that increasing temperatures in permanently cold environments (i.e. arctic) will alter biogeographic pattern of thermal groups of microorganisms in polar regions. Invasive microbial species from warmer environments (i.e., warmer latitudes / water bodies) may be generalists having broader thermal tolerances, and are expected to flourish at the expense of psychrophilic species (Vincent, 2010). This expectation is corroborated by the present study of the highly dynamic Namibian and Argentinean slope systems identifying the transport of allochthonous mesophilic bacteria from warmer to colder environments by a change in the overall temperature response profile of a microbial community.

Accepting this scenario, two major questions arise; 1) Are the psychrophilic and psychrotolerant species displaced (i.e. do they become extinct) or maintained (i.e., less active community members as less active community members), and 2) Is this an environmentally significant question?

The present study provides insight into these two issues and provides approaches on how they can be addressed. The study of the Namibian and Argentinean slope systems implies that the transport of mesophilic SRB to the slope sediments does not displace psychrophilic bacteria present at the deep sea floor. Temperature response depth profiles of sediment cores from these environments could give information on the temperature response of SRB, and thus elucidate whether allochthonous mesophiles from the surface sediment survive burial, or if psychrophiles prevail. Such a study, would not only reveal if the subsurface SRB community in deep water sediments is preferentially psychrophilic, but also explore

competitive advantages psychrophilic and psychrotolerant SRB may have over mesophilic SRB. These could include the ability to survive under low energy conditions, or to access non-competitive substrates.

The finding that mesophilic SRB to the slope sediments do not displace psychrophilic bacteria at the deep sea floor does not disprove the possibility that the transport of warm adapted microorganisms will impact the present distribution of psychrophiles, psychrotolerants and mesophiles and will have a permanent effect on the distribution of thermal groups of microorganisms in permanently cold environments. This leads us to the second question, if such a study would have an environmental significance.

It is not known whether the community affected by a temperature increase will be functionally similar to the original community (Reed and Martiny, 2007, Allison and Martiny, 2008). It is very well possible that some species in a microbial community can be functionally redundant, thus the local biogeochemistry might not be affected by their disappearance (Reed and Martiny, 2007; Allison and Martiny, 2008). With regard to this topic, we therefore, need to anticipate what functionalities between psychrophiles and mesophiles may not be fully redundant. Experiments on freeze-thaw conditions presented here, together with the awareness that rapid climate change causes not only temperature increases, but also more variable weather conditions provide a starting point: this work demonstrates that Arctic tidal flat microbial communities can withstand moderate freeze–thaw conditions, which thus have little effect on microbially mediated organic carbon degradation. Drastic freeze-thaw conditions impacted the sulfate reducing community. A mesophile-dominated SRB community may be much more impacted by both moderate and drastic freeze-thaw conditions.

To address these questions in detail longer-term studies on the microbial community composition are required. These should monitor both shifts in community composition (e.g. by tracking disappearing and emergent species of different functional groups by DNA/RNA

based methods) as well as biogeochemical process rates in response to the manipulated environmental parameters. Such experiments are best carried out under continuously maintained conditions where accumulation of metabolites can be avoided. In the rapidly changing Arctic, close attention will need to be paid not only to the temperature response of microorganisms, but also to the structure and functioning of microbial communities.

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Erklärung

Gemäß §6 (5) Nr. 1-3 Promotionsordnung erkläre ich hiermit, dass ich

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I ohne unerlaubte fremde Hilfe angefertigt habe,

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