Thermophilic endospores in Aarhus Bay and Svalbard sediments: insight into the dispersal of marine microorganisms

PhD Thesis by
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Front page photos:

Left: view of the city of Aarhus from the harbor (Photo by Laura Lapham)

Center: sediment sampling by Rumohr Lot corer in Aarhus Bay (Photo by Britta Gribsholt)

Right: Smeerenburgfjorden, Svalbard (Photo by Carol Arnosti)

Back photo: sediment sampling by gravity corer in Aarhus Bay (Photo by Nils Risgaard-Petersen)

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THERMOPHILIC ENDOSPORES IN AARHUS BAY AND SVALBARD SEDIMENTS: INSIGHT INTO THE DISPERSAL OF MARINE MICROORGANISMS

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Preface

Over the last three years I have studied the distribution, diversity, physiology and abundance of endospores of thermophilic sulfate-reducing bacteria (SRB) in sediments of Aarhus Bay and Svalbard. The main results are presented in this PhD thesis.

Chapter 1 is an introduction to the research field of marine microbial biogeography followed by a synthesis of my work. The chapter introduces the evolution of this science, including significant studies of distribution patterns of marine microbial populations, the relation of these patterns to environmental factors and the challenges of studying the dispersal of marine microorganisms. I present the opportunity offered by thermophilic endospores in cold sediments for investigating microbial dispersal and summarize my work in the context of other studies focused on this phenomenon. The main findings of my PhD study and perspectives of further research conclude the chapter.

Chapters 2 and 3 comprise most of my PhD work and are presented as manuscripts I have prepared for publication, together with collaborators at Aarhus University, Newcastle University and the University of Vienna. Chapter 2 focuses on our findings regarding the diversity and abundance of endospores of thermophilic SRB in surface and deep sediments of Aarhus Bay, and their significance to the understanding of the dispersal of these microbial populations in the ocean. Chapter 3 describes a new model to estimate the abundance of thermophilic SRB endospores and its application to quantify successive populations that develop in sediments incubated at high temperature.

Chapter 4 presents the quantification of thermophilic SRB endospores in sediments from Svalbard, and the identification of thermophilic SRB and putative thermophilic fermentative bacteria in these sediments. The work described in chapter 4 is an antecedent to the Aarhus Bay studies and is therefore closely connected to the work described in chapters 2 and 3. I have contributed to this work in collaboration with 11 other colleagues.

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Summary

The distribution of microbial populations is a consequence of extinction, speciation and dispersal processes. The individual effects of each of these factors, however, are difficult to distinguish. The presence of thermophilic endospores in cold marine sediments represents an opportunity to study microbial dispersal in the ocean. During my PhD, I have investigated this phenomenon in the temperate sediments of Aarhus Bay and the arctic sediments of Svalbard, aiming to contribute to the understanding of marine microbial biogeography.

In this PhD thesis, I present that clone libraries of two phylogenetic markers, 16S rRNA and dsrAB genes, revealed more than 20 taxa of thermophilic endospore-forming sulfate-reducing bacteria (SRB) in Aarhus Bay, which become active upon sediment incubation at different temperatures. Tracer-enhanced most-probable-number enumerations (T-MPN) showed that the abundance of thermophilic SRB in Aarhus Bay was ca. 10^4 endospores per cm^3 in the upper 10 cm of sediment. It is thus estimated that these sediments have received an influx of ca. 10^7 endospores of thermophilic SRB per m^2 per year for at the least the past 100 years. The abundance of thermophilic SRB decreased exponentially in deeper sediments to ca. 1 endospore per cm^3 at 6.5 m below the seafloor. This indicates that the dispersal of thermophilic endospores has occurred for more than six thousand years, and that buried thermophilic SRB endospores likely die off on that timescale. A half-life on the order of 400 years was calculated for the Aarhus Bay populations.

In addition to the quantification of thermophilic SRB endospores by T-MPN, I present a new model for estimating the abundance of these microbial populations. This model is based on the measurement of exponential increases of sulfate reduction rates upon incubation of sediment at high temperatures. The method includes an approach for estimating cell-specific sulfate reduction rates of the distinct SRB populations that develop in succession during sediment incubation, and provides information regarding their relative composition and physiology. Using a simplified version of this model, we have shown that thermophilic SRB endospores have been supplied to arctic sediments of Svalbard in a flux of ca. 10^8 endospores per m^2 per year for the past 120 years. Clone libraries of 16S rRNA revealed putative thermophilic sulfate-reducing and fermentative bacteria to be present in these sediments. Warm environments such as hydrothermal fields, deep sub-seafloor sediments and oil reservoirs are potential sources of these bacteria. At least one source may be common to Aarhus Bay and Svalbard, based on the close phylogenetic similarity (16S rRNA and dsrAB genes) between particular taxonomic groups found in both locations.

In conclusion, all of these results indicate that the dispersal of thermophilic SRB to these temperate and arctic sediments has occurred over long timescales, of hundreds or thousands of years, as a large flux, providing more than 10^7 and 10^8 endospores per m^2 per year to Aarhus Bay and Svalbard, respectively, and over great oceanic distances of thousands of kilometers. This process of passive dispersal is unlikely to act only on thermophilic SRB endospores and may thus influence the biogeography of various marine microbial populations.

Resumé

Fordelingen af mikrobielle populationer er bestemt af følgende faktorer: artsdannelse, uddøen og spredning. Effekterne af de enkelte faktorer er imidlertid svære at adskille. Tilstedeværelsen af termofile endosporer i kolde havsbundsedimenter er en mulighed for at studere mikrobiel spredning i havet. I løbet af mit ph.d.-studium har jeg undersøgt dette fænomen i tempereret sediment fra Århusbugten og i arktisk sediment fra Svalbard med det mål at bidrage til forståelsen af marin mikrobiel biogeografi.

I denne ph.d.-afhandling præsenterer jeg, at klonbiblioteker af to fylogenetiske markører: 16S rRNA og dsrAB gener, har afsløret over 20 taxa af termofile endosporedannende sulfatreducerende bakterier (SRB) fundet i Århusbugten, der bliver aktive ved sediment inkubering ved forskellige temperaturer. Tracer-enhanced most-probable-number enumerations (T-MPN) viste, at tætheden af termofile SRB i Århusbugten var ca. 10^4 endosporer per cm^3 i de øverste 10 cm sediment. Det anslås derfor, at der i disse sedimenter er sket en indstrømning af 10^7 termofile SRB endosporer per m^2 per år over de sidste 100 år. Sporetætheden af termofile SRB falder eksponentielt med sedimentdybden ned til omkring 1 endospore per cm^3 ved 6,5 meters dybde under havbundens overflade. Dette indikerer, at spredningen af termofile endosporer er sket over en periode på mere end 6000 år, og at begravede termofile SRB endosporer sandsynligvis nedbrydes indenfor dette tidsrum. Halveringstiden for populationen i Århusbugten blev beregnet til 400 år.

Foruden kvantificeringen af termofile SRB endosporer med T-MPN præsenterer jeg en ny model udviklet til at estimere tætheden af disse mikrobielle populationer. Denne model er baseret på målinger af eksponentielt stigende sulfatreduktionsrater ved inkubering af sediment ved høje temperaturer. Metoden inkluderer mulighed for estimering af cellespecifikke sulfatreduktionsrater for de specifikke SRB populationer, der opstår i succession under inkubationer, og den giver information om deres opbygning og fysiologi. Ved at anvende en forsimplet version af denne model har vi vist, at termofile SRB endosporer er blevet tilført arktisk sediment i Svalbard med en rate på ca. 10^8 endosporer per m^2 per år i løbet af de sidste 120 år. Klonbiblioteker på 16S rRNA afslørede tilstedeværelsen af formodede termofile sulfatreducerende- og fermenterende bakterier i sedimentet fra Svalbard. Varme miljøer såsom hydrotermiske felter, dybe havbundssedimenter eller olieholdige sedimenter er potentielle kilder for disse bakterier. Mindst én kilde til SRB endosporer havde Svalbard og Århusbugtens sedimenter til fælles på baggrund af stærke fylogenetiske ligheder (16S rRNA og dsrAB gener) mellem specifikke taksonomiske grupper fundet på begge lokaliteter.

Tilsammen indikerer resultaterne, at spredningen af termofile SRB til disse tempererede og arktiske sedimenter er sket over et langt tidsforløb på hundrede eller tusinde år ved tilførsel af henholdsvis 10^7 og 10^8 endosporer per m^2 per år til Århusbugten og Svalbard. Spredningen er sket over store afstande på flere tusinde kilometer. Det er usandsynligt, at denne passive spredningsproces kun gør sig gældende for termofile SRB endosporer – den kan påvirke biogeografien for en række forskellige marine mikrobielle populationer.

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Chapter 1

Introduction

1. Introduction to microbial biogeography

1.1. The science of microbial biogeography

Biogeography is the study of the geographic distribution of organisms and the mechanisms that regulate their patterns. In simple words, it attempts to answer the questions of *who is where* and *why they are there*. These questions are central to the understanding of ecology, as they relate to how biodiversity is generated and maintained (Green and Bohannan, 2006), and of evolution, as geographical isolation is a crucial mechanism for speciation (Green and Bohannan, 2006; O'Malley, 2007). While for animals and plants these questions have been studied since the early eighteenth century, for microorganisms the study of biogeography started almost three centuries later (O'Malley, 2007).

The geographical range of a species is determined by its ability to disperse and to survive environmental heterogeneity across space and time (Fierer, 2008). Since the early days of microbiology, it has been believed that the geographical range of microbial populations was unlimited. This was succinctly expressed by Lourens Baas Becking (1934) as a memorable tenet, *"everything is everywhere, but the environment selects."*. It suggests that microorganisms are so easily dispersed that they are not affected by geographical barriers or distances, as opposed to animals and plants. Second, it recognizes that there are distribution patterns, yet these patterns are determined by the present characteristics of the environment (O'Malley, 2008). These conclusions derived from the first studies of enrichment cultures, initiated a few years earlier and mainly attributed to Martinus Beijerinck and Sergei Winogradsky. Their studies showed that, providing the appropriate chemical and physical conditions, "the same types of organisms would appear" (van Niel in O'Malley, 2007). This predictability was important to establish microbiology as an academic science in the beginning of the twentieth century. Baas Becking's statement provided a standardized status to microbiology, which continued to develop away from the notion of biogeography (O'Malley, 2007).

In the last decade, the paradigm of unlimited dispersal of microorganisms began to be questioned, especially due to the increasing use and development of molecular techniques. These methods provided the possibility to assess genetic differences in isolated strains, allowing identification and classification that more closely reflected evolutionary traits, rather than physiological characteristics. Moreover, these techniques provided the possibility to increasingly reveal and assess the large diversity of uncultured microorganisms (Amann et al., 1995; Sogin et al., 2006). With the discovery that microbial communities are composed of a large number of mostly unknown taxa in low abundance (the rare biosphere; Sogin et al., 2006) and admitting the detection limits of all techniques used so far, it became questionable whether the statement of "everything is everywhere" can be tested at any level, as the absence of a taxon ("something is *not* somewhere") cannot be assured. The second part of Baas Becking's statement, "but the environment

selects", might not be testable either, as it is virtually impossible to determine all environmental parameters that may influence a microbial population and therefore find two entirely identical environments in different locations. Thus, the classical tenet is now often considered a metaphor, and not a hypothesis (Foissner, 2006). Instead of attempting to confirm or reject it for "all" microbial groups at all taxonomic levels, Baas Becking's statement might be better applied to instigate the question of whether biogeographic patterns of populations found in distinct places are more influenced by dispersal or by environmental characteristics, or at which sampling scale and by which definition of environment particular microbial populations are indistinguishable. It is unlikely that one single rule applies to the biogeography of all microbial taxa, yet there may be a spatial scale, degree of sampling effort and a level of taxonomic resolution at which the biogeography of different microorganisms and macroorganisms are underlined by the same processes and principles. If this is confirmed, it would be another evidence of the unity of life. If it is not confirmed, and it becomes evident that the biogeography of microorganisms is fundamentally different from that of animals and plants, then it is necessary to investigate the particular differences and processes that regulate each of them, improving the understanding of ecology and evolution of all kinds of organisms (Green and Bohannan, 2006; Martiny et al., 2006; Fierer and Lennon, 2011).

1.2. Marine microbial biogeography

It has recently been demonstrated that bacterial communities in natural environments are mainly differentiated between saline and non-saline habitat types. This was concluded by creating an environmentally annotated phylogenetic tree compiling more than 20,000 sequences of bacterial 16S rRNA genes from more than 200 environmental samples. The habitat types included soil, lakes, seawater, sediments, ice, hot springs, acidic springs, hydrothermal vents, among many others (Lozupone and Knight, 2007). After salinity, the second major environmental factor that distinguished microbial community composition was substrate type, i.e., water or sediment. Furthermore the same study showed that sediments, in particular saline sediments, were the most diverse habitat types (Lozupone and Knight, 2007). However, so far marine microbial biogeography has been mostly investigated for communities in the water column.

Oceans are not one single homogeneous environment (e.g. Azam, 1998; Hewson et al., 2006; Teira et al., 2006). The combination of several physical and chemical parameters, such as temperature, light incidence, dissolved gases, nutrients, organic matter content and quality, and the interaction with other micro- and macroorganisms create an immense variety of niches for microbial populations in both space and time. It is a dynamic system, where currents, tides and the global circulation of water masses (for example, surface currents, Figure 1) promote the transport of its biotic and abiotic components (Stommel, 1958; Broecker, 1991; Galand et al., 2010). Potential dispersal barriers, geographic distances and the various environmental characteristics may thus lead to differences in microbial community composition in the ocean,

yet these factors do not affect all microbial populations in the same manner (Fierer, 2008). To understand whether marine microbial populations are more affected by environmental heterogeneity or dispersal limitation, it is first necessary to clearly define the population in question, as demonstrating biogeographic patterns depends on the microbial organism analyzed (bacteria, archaea, unicellular eukaryotes, functional groups, viruses), the taxonomic level (from phyla and classes to species and ecotypes or simply different definitions of operational taxonomic units), the scale of sampling (a few centimeters to thousands of kilometers) and the methodology applied (e.g. microscopy, community fingerprinting, 16S rRNA sequencing, multi-locus sequencing analysis; Horner-Devine et al., 2007).

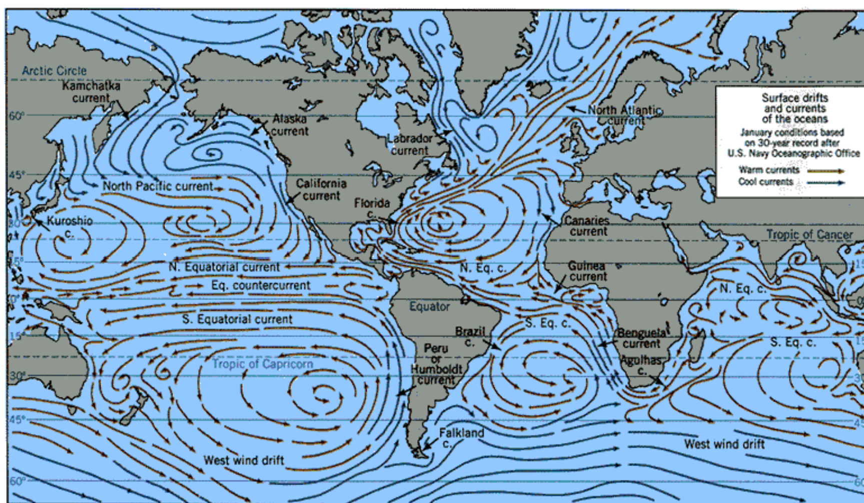


Figure 1. Schematic drawing of the main surface drifts and currents of the oceans, based on a 30-year record of January conditions by the U.S. Navy Oceanographic Office. Red arrows: warm currents. Blue arrows: cool currents. Image from http://www.learner.org**.**

1.2.1. Defining "everything" before asking whether it is "everywhere"

It has been suggested that organisms smaller than 1 mm long are capable of unlimited dispersal (Finlay and Clarke, 1999; Fenchel and Finlay, 2004). For example, a recent study (Cermeño et al., 2010) investigated the distribution of marine phytoplankton, mainly composed of diatoms, dinoflagelates and coccolithophorids (unicellular eukaryotes), along a meridional transect in the Atlantic Ocean from tropical/subtropical regions north and south of the Equator until the sub-Antarctic front. Despite crossing more than 10,000 km, community similarity was only weakly correlated to geographic distance, and strongly correlated to environmental gradients. Furthermore, the phytoplankton communities sampled in different water masses with similar environmental characteristics (North and South Atlantic gyres) were largely similar. These results led to the conclusion that there are no geographical barriers to the dispersal of planktonic microorganisms in the marine environment. However, it is important to note that although the taxonomic level analyzed in that study was the smallest possible (usually species; Cermeño et al., 2010), this was determined by morphological identification. It has been shown that the same morphospecies of

unicellular eukaryotes may be composed of multiple genetically distinct taxa, which present more restricted geographic distribution than indicated by the analysis of the morphospecies (Kooistra et al., 2008). It has also been suggested that the genetic variation observed in these unicellular eukaryotes are not relevant for ecological or biogeographic patterns, as they might be due to neutral intra-species mutation (Fenchel, 2005), yet this is not broadly accepted (Scheckenbach et al., 2006; Chen and Hare, 2008). The discussion regarding species definition in unicellular eukaryotes and their relevance to biogeographic and ecological studies is far from resolved (Caron, 2009; Watts et al., 2011).

Discussions on species definition and biogeographic units are equally important in the prokaryotic domains (Whitaker, 2006). For bacteria and archaea, morphology and physiology alone are no longer the standards for microbial identification. Molecular techniques, in particular targeting the 16S rRNA gene, have become the basis of phylogenetic identification and classification for the last decades (Head et al., 1998). In many cases, however, the 16S rRNA gene might be a phylogenetic marker that is too conserved for detecting ecological differences between closely related microorganisms (e.g. Staley and Gosink, 1999; Ramette and Tiedje, 2007). Therefore, conclusions from microbial biogeography studies are still largely dependent on the technique used and its degree of resolution (Horner-Devine et al., 2007). Ultimately, the comparison of entire genomes reveals a complete picture of the evolutionary distance between strains, yet the degree of dissimilarity at the whole-genome level may be so large (Welch et al., 2002) that it obscures biogeographic patterns in natural microbial assemblages (Ivars-Martínez et al., 2008). As assessing whole genomes of environmental bacteria is currently an expensive, low-throughput and laborious task, the finest phylogenetic resolution so far applied to investigate marine microbial biogeography has been obtained from two methodologies: one is the analysis of concatenated genes (multilocus sequence analysis, MLSA; Maiden et al., 1998) and the other is the analysis of the internal transcribed spacer (ITS) region, by direct sequencing or by fingerprinting (automated ribosomal intergenic spacer analysis, ARISA; Fisher and Triplett, 1999).

MLSA consists of analyzing DNA sequences of multiple phylogenetic marker genes. It has been suggested that at least 20 concatenated genes were necessary to obtain a robust phylogeny for yeast species (Rokas et al., 2003), yet for bacteria and archaea, which normally have a genome size ca. 10 times smaller than yeast, significant phylogeny may be obtained from fewer genes. The concatenation of nine marker genes has already revealed well-supported phylogenies and biogeographic patterns that are unnoticed at 16S rRNA level, as demonstrated for *Sulfolobus* spp. and *Alteromonas macleodii* (Whitaker et al., 2003; Ivars-Martínez et al., 2008). However, the use of MLSA has so far required the isolation of target strains, as all concatenated genes must be undoubtedly assigned to the same strain, which presently cannot be done from bulk DNA extraction of environmental samples. Advances in techniques to sequence genes and whole genomes from single uncultured cells may provide ways to soon overcome this limitation (Ottesen et al., 2006; Stepanauskas and Sieracki, 2007; Ivars-Martínez et al., 2008).

The analysis of the ITS region does not require the isolation of strains, and has been more frequently applied in studies of marine microbial biogeography (e.g. Fuhrman et al., 2006; Hewson et al., 2007; Fuhrman et al., 2008). The ITS region is a highly variable nucleotide sequence between the 16S and the 23S rRNA genes. It contains phylogenetic information that differentiates operational taxonomic units with more than 98% 16S rRNA sequence identity (Brown and Fuhrman, 2005). The phylogenetic information of the ITS region may be assessed not only by direct sequencing, but also by the fluorimetric detection of ITS fragments by ARISA (Fisher and Triplett, 1999). This high-throughput community fingerprinting method appears to be highly reproducible within taxa and to provide a reasonable measure of the relative abundance of populations in a microbial community (Brown et al., 2005; Fuhrman et al., 2006). Moreover, it has been shown to result in similar phylogenetic topographies as MLSA (Ivars-Martínez et al., 2008).

Although MLSA and ITS analyses are currently the state of the art, techniques with lower phylogenetic resolution, based solely on 16S rRNA as a phylogenetic marker gene also provide relevant information regarding the biogeography of marine microorganisms, within their limitations. For example, catalyzed reporter deposition-fluorescence *in situ* hybridization (CARD-FISH), denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP) and clone libraries of the 16S rRNA gene have revealed distribution patterns and differences in the composition of marine microbial populations at various sampling scales. Although cosmopolitan bacterial and archaeal taxa have been reported (e.g. Massana et al., 2000; Pommier et al., 2007; Yan et al., 2009; Wietz et al., 2010), most taxa have a restricted distribution (e.g. Selje et al., 2004; Pommier et al., 2007; Kataoka et al., 2009; Jungblut et al., 2010; Wietz et al., 2010; Rink et al., 2011). The same occurs in viral biogeography, which is largely associated with the biogeography of their bacterial hosts. There are phages with global distribution, others that are more common in low latitudes (Thurber, 2009), others that present considerable genetic differences in kilometer scales (Tucker et al., 2011), and others that are very restrictedly distributed, for example associated to specific stromatolites and thrombolites (Desnues et al., 2008). Therefore it appears that ubiquity in microbial populations is the exception, rather than the rule.

1.2.2. Who is where in the marine environment: examples of distribution patterns of marine microbial populations and their correlation to environmental factors

Studies of bacterioplankton distribution have been conducted at global, regional and local scales, addressing whole community composition (domain level) broadly, and narrowing in taxa until sub-species (ecotype) level. In a global scale, it has been shown by 16S rRNA libraries, ARISA and CARD-FISH that the differences in the distribution patterns of marine bacterioplankton communities are mainly related to latitude (Pommier et al., 2007; Fuhrman et al., 2008; Wietz et al., 2010) and depth (DeLong et al., 2006; Pham et al., 2008). For example, one of the main components of bacterioplankton, *Alphaproteobacteria* from

the *Roseobacter*-clade affiliated cluster (RCA) are common in temperate and polar waters, but not tropic and subtropic regions (Selje et al., 2004); certain cyanobacteria are restricted to high latitudes (Jungblut et al., 2010); ecotypes of *Prochlorococcus* spp. and *Alteromonas macleodii* are differentiated between surface and deep waters (Johnson et al., 2006; Ivars-Martínez et al., 2008). These patterns may be a consequence of the direct influence of several environmental factors on microbial populations, such as temperature, light incidence, chlorophyll a (a proxy for phytoplankton abundance), nutrient concentration, oxygen consumption, viral lysis, grazing and several others, which can vary in horizontal and vertical directions, and also in time. Microbial populations respond differently to these different factors. For example, Herfort et al. (2007) showed that the abundance of pelagic *Crenarchea* in the southern North Sea was positively correlated with ammonia, nitrate, nitrite and phosphorus concentrations and inversely correlated to phytoplankton abundance and composition. Pelagic euryarcheotal abundance had a different result, and was correlated with phytoplankton abundance, but not with its composition, and both crenarchaeal and euryarchaeal populations had a temporal variability (Herfort et al., 2007). Another study in the North Sea showed that free-living and particle-associated bacterioplankton were both influenced by hydrographic and biogeochemical conditions, and mainly differed between offshore and inshore environments, but particle-associated *Alphaproteobacteria* were more correlated to temperature, while free-living *Alphaproteobacteria* were more correlated to salinity (Rink et al., 2011).

Microbial communities in marine sediments also present biogeographic patterns. Most studies have focused on their millimetric zonation with sediment depth, in particular according to gradients of redox potential, organic matter and nutrient concentration (e.g. Llobet-Brossa et al., 1998; Urakawa et al., 2000; Böer et al., 2009). When comparing the distribution of benthic microbial populations at different sites, the sediment type (sand, mud, clay and further variations) is one of the main factors influencing microbial community structure (Meadows and Anderson, 1966; Weise and Rheinheimer, 1977; de Oliveira et al., 2007). Permeable sediments allow an effective coupling of benthic and pelagic environments, due to advective exchange of fluids between porewater and the water column. Less permeable sediments, on the other hand, are dominated by diffusion rather than advection, and are highly stratified. For example, a comparison of bacterial communities in carbonate and silicate coral reef sands in the Red Sea showed that the patterns of microbial assemblages were mainly associated to the type of sand. Season and sediment depth further contributed to the differences in bacterial diversity, although these factors partly reflected the mineralogical differences. The effects of sand type, season and depth were most strongly evident in the distributions of *Gammaproteobacteria*, *Actinobacteria* and *Acidobacteria* populations (Schöttner et al., 2011). Water column depth (Polymenakou et al., 2005; Hewson et al., 2007) and its productivity (Polymenakou et al., 2005; Sapp et al., 2010) are also strongly related to microbial community composition in surface sediments of different locations. Furthermore, as for pelagic communities, the main environmental factors that influence sediment microorganisms may be determined by the spatial scale of sampling. This has

been exemplified for denitrifying bacteria in continental shelf sediments off the coast of New Jersey, which varied at centimeter, meter and kilometer scales and may be respectively related to meio- and macrofaunal abundance, bottom topography and the overall characteristics of the sediment (Scala and Kerkhof, 2000).

These examples of association between microbial populations and environmental factors evidence that microbial distribution patterns are not random (Martiny et al., 2006). However, it is not clear whether the patterns are a result of environmental selection alone or whether there are dispersal barriers, especially at regional and global scales. A closer look into dispersal mechanisms of marine microorganisms is thus necessary.

1.2.3. Dispersal of marine microorganisms

Due to the minute size and often the lack of self-propulsion of microorganisms, passive dispersal plays a more important role in microbial biogeography than active dispersal (Martiny et al., 2006; Jenkins et al., 2007). In the marine environment, passive dispersal of microorganisms can occur by various mechanisms, including atmospheric precipitation (Agogué et al., 2005) and association to marine vertebrates and invertebrates (Ward and Bora, 2006). However, the main mechanism of marine microbial dispersal is expectedly the movement of currents and water masses. Planktonic bacteria are naturally drifting with the water flow, while benthic bacteria in surface sediments can be suspended to the water column due to physical disturbance by near-bed currents and macroorganisms (Hughes and Gage, 2004; Queric and Soltwedel, 2007; Schauer et al., 2010). Furthermore, advective fluid flow may export particles and consequently cells from deep sediment layers to the water column, from where these microorganisms might be carried in currents or hydrothermal plumes (Hubert and Judd, 2010; Orcutt et al., 2011).

Seawater movement may transport cells in a local, regional or global range; however, the separation of water masses in the ocean can be an important dispersal barrier for marine microorganisms. In fact, microbial community composition has been often associated with water masses. For example, Kataoka et al. (2009) reported that bacterioplankton communities in the western North Pacific differed according to temperature, salinity, phytoplankton abundance and nitrate concentration that were characteristic of different water masses and partially of seasons. The correlation of bacterioplankton composition and water mass has also been shown for the deep Arctic Ocean. Once below the euphotic layer, depth was not enough to explain microbial biogeography in this region, which was better explained by a more careful characterization of water masses (Galand et al., 2010). Similarly, water masses appear to be the main drivers of bacterioplankton community composition in the Atlantic Ocean. Agogué et al. (2011) analyzed microbial communities between 100 to 4,500 m depth along a 8,000 km transect in the North Atlantic Ocean, from 60°N to 5°S, and concluded that both the abundant and rare taxa are more similar between locations that are thousands of kilometers apart, but in the same water masses, than between locations less than a few hundred kilometers

apart, but in different water masses. Dissolved organic carbon and oxygen concentration were specific characteristics of the water masses and correlated with the bacterial community composition. Furthermore, the authors observed that bacterial community composition in deep waters was more similar in the northern part than in the southern part of the transect. This may be related to the large-scale formation of deep waters in the northern North Atlantic (sinking of cooled, denser waters), resulting in smaller differences in physico-chemical factors and therefore a more uniform bacterial community (Agogué et al., 2011). The deep water flow to the South is accompanied by an increasing differentiation of abiotic factors and bacterial communities. This may explain why a study in the eastern tropical Atlantic Ocean did not detect a significant correlation between bacterial and archaeal community composition and water masses (Winter et al., 2008). In this case, depth and temperature strongly correlated with bacterial communities, while temperature and frequency of viral infections correlated with archaeal communities. Neither water masses (indicated by potential density) nor horizontal distances of up to 4,600 km correlated significantly with microbial community composition (Winter et al., 2008).

The effect of distinct water masses becomes even more evident when the sampling scale is limited to much smaller geographic distances (Figure 2). For example, Pinhassi et al. (2003) analyzed bacterioplankton communities within only 35 km across the Skagerrak-Kattegat front, where deep water from the North Sea meets surface water from the Baltic Sea (Figure 2A; Danielssen et al., 1997). The bacterioplankton community composition was separated according to the water masses, which also differed in salinity, dissolved organic carbon concentration, phytoplankton abundance and composition, bacterial production and limiting nutrient for bacterial growth (Figure 2B; Pinhassi et al., 2003). Furthermore, the effect of distinct water masses over small distances in frontal areas can be observed not only at community level, but also for specific taxonomic groups, which can be illustrated by the presence of different *Roseobacter*-clade-affiliated phylotypes north and south of the polar front, which separates the subantarctic and Antarctic water masses (Figure 2C; Selje et al., 2004).

The repeatedly observed correlation between microbial community composition and water masses may be explained by two models. The first is that different water masses, separated by density or by geological features in the seafloor, act as islands within the ocean, and their hydrographic boundaries are barriers for microbial dispersal. This would favor the divergence of populations through allopatric speciation (i.e. generation of new species through physical separation of populations). The second model is that the different properties of each water mass characterize them as distinct environments, thus creating different ecological niches. This would mean that microbial dispersal may not be limited, but the extinction of certain migrating populations would be caused by environmental heterogeneity (Galand et al., 2010). Testing these two models, however, is a difficult task, as they are not mutually exclusive.

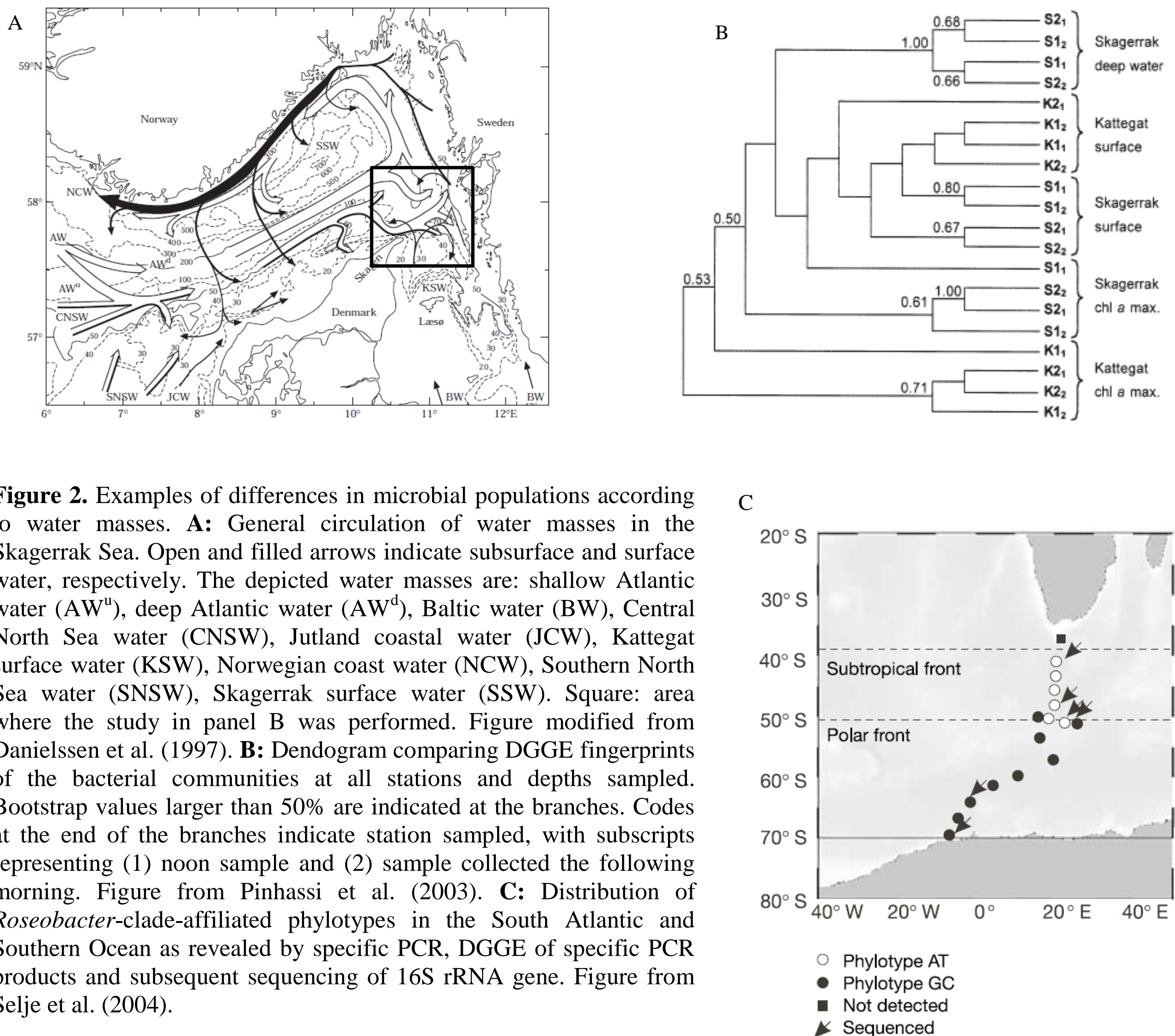


Figure 2. Examples of differences in microbial populations according to water masses. **A:** General circulation of water masses in the Skagerrak Sea. Open and filled arrows indicate subsurface and surface water, respectively. The depicted water masses are: shallow Atlantic water (AW^u), deep Atlantic water (AW^d), Baltic water (BW), Central North Sea water (CNSW), Jutland coastal water (JCW), Kattegat surface water (KSW), Norwegian coast water (NCW), Southern North Sea water (SNSW), Skagerrak surface water (SSW). Square: area where the study in panel B was performed. Figure modified from Danielssen et al. (1997). **B:** Dendogram comparing DGGE fingerprints of the bacterial communities at all stations and depths sampled. Bootstrap values larger than 50% are indicated at the branches. Codes at the end of the branches indicate station sampled, with subscripts representing (1) noon sample and (2) sample collected the following morning. Figure from Pinhassi et al. (2003). **C:** Distribution of *Roseobacter*-clade-affiliated phylotypes in the South Atlantic and Southern Ocean as revealed by specific PCR, DGGE of specific PCR products and subsequent sequencing of 16S rRNA gene. Figure from Selje et al. (2004).

Another complicating factor comes from the cases of evident dispersal across water masses, for example by sinking (e.g. García-Martínez and Rodríguez-Valera, 2000) or lateral migration through several water masses. This has been observed for sediment bacteria in three deep-sea basins in the eastern South Atlantic Ocean, which included a physical barrier (the Walvis Ridge) that separates the Antarctic lower circumpolar deep water and the North Atlantic deep water. While bacterial communities were significantly different in the distinct water masses, thus indicating dispersal limitation, phylotypes of *Gammaproteobacteria*, *Deltaproteobacteria* and *Acidobacteria* were not influenced by the Walvis Ridge nor by the distinct water masses (Schauer et al., 2010). Another compelling example is the detection of almost identical ITS sequences in different oceans, despite the overall clustering according to their geographic origin. This suggests that their dispersal is primarily limited, yet a common ancestor or subpopulation was

effectively dispersed within less than a few hundred generations. This has been observed, for example, for a marine crenarchaeon in the eastern North Pacific, Mediterranean Sea and the Gulf of Biscay (García-Martínez and Rodríguez-Valera, 2000) and for the planctomycete *Candidatus* Scalindua arabica, in the oxygen minimum zones of the Arabian Sea and the Peruvian Margin (Figure 3; Woebken et al., 2008).

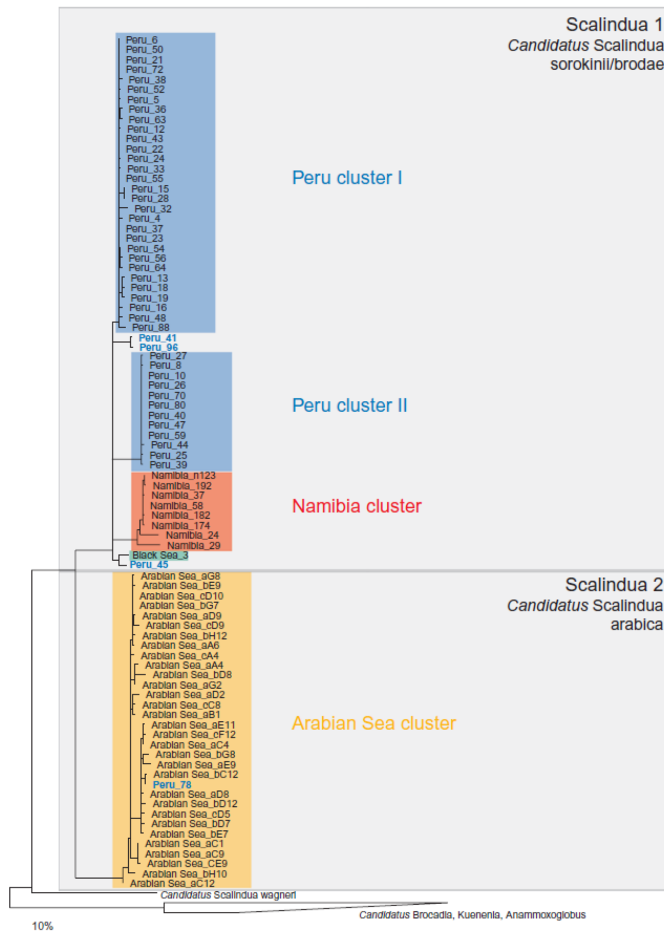


Figure 3. Indication of long-distance dispersal across several water masses. The tree shows the phylogenetic relation of ITS sequences of the planctomycete *Candidatus* Scalindua sorokinii/brodae and arabica originated from the Arabian Sea (yellow), Peruvian (blue) and Namibian (red) oxygen minimum zones and the Black Sea (green). The presence of an ITS sequence from the Peruvian coast that is almost identical to the ones in the Namibian coast indicates that a common ancestor may have been dispersed between these two regions within a few hundred generations (García-Martínez and Rodríguez-Valera, 2000). Figure from Woebken et al. (2008).

All of these examples indicate that the biogeographic patterns of marine microorganisms result from a complex interaction of dispersal history and environmental selection, and it is difficult to distinguish between the effects of each of these factors (Fierer, 2008). One interesting opportunity to investigate dispersal, its rate and extent, while excluding the selective pressure of the environment, is by analyzing populations that are clearly exogenous and remain dormant in the environment. This is the case of thermophilic endospores in cold sediments, the target of the present study.

1.3. Thermophilic endospores as tools for studying microbial dispersal

Ocean currents may carry cells stochastically between various microbial niches, at distances from millimeters to several kilometers. If they encounter favorable conditions, the dispersed microbes may colonize the new habitat, becoming part of the local active community. Active cells are subjected to genetic drift, lateral gene transfer and differential selective pressures in the new environment, which may lead to speciation and conceal a dispersal history (Martiny et al., 2006). However, not all microbial populations are constantly active. The ability to produce dormant life stages has evolved in various forms in all domains of life (Kaprelyants et al., 1993; Lamarre et al., 2008; Schubert et al., 2010). It has been estimated that ca. 40% of microorganisms in the oceans are dormant (Lennon and Jones, 2011), hence this microbial trait likely influences biogeographic processes of a large proportion of marine populations. Dormancy may provide a faster survival response to environmental pressure than speciation and reduce the chances of extinction of a species (Locey, 2010). Moreover, it increases the chances of dispersal over long distances (Fierer, 2008) and of immigration success (Lennon and Jones, 2011), as it improves survival in unfavorable conditions for extended periods of time.

One of the most resistant forms of dormancy is the formation of endospores. This trait has evolved within the bacterial group of *Firmicutes*, providing sporulating species strong resistance to harsh conditions over thousands (Sneath, 1962; Gest and Mandelstam, 1987; Kennedy et al., 1994; Potts, 1994; Nicholson et al., 2000) to millions of years (Cano and Borucki, 1995; Vreeland et al., 2000), although the most extreme claims of longevity are questionable, since modern contamination could not be ruled out (Maughan et al., 2002; Nickle et al., 2002; Willerslev et al., 2004). Endospores are also the only form of dormancy often considered to be metabolically inert (Setlow, 1995), thus even less susceptible to environmental pressure than other forms of dormant cells. However, although endospores are inactive, they are not necessarily exogenous. They can be produced by local endospore-forming bacteria, which might be the main reason why endospores seem to constitute a large part of microbial communities, especially in deep sediments, where they may even outnumber vegetative cells (e.g. Köpke et al., 2005; Batzke et al., 2007). Therefore, a more specific subgroup of endospores must be selected to investigate passive dispersal mechanisms. Studying thermophilic endospores is thus one possibility.

Most of the ocean is cold. Approximately 90% of the ocean water and surface sediments have temperatures below 4°C (Levitus and Boyer, 1994; Robador, 2009). Nevertheless, thermophilic endospores, which are activated at temperatures above 40°C, have been observed in cold marine sediments of various locations (e.g. Bartholomew and Paik, 1966; Isaksen et al., 1994; Vandieken et al., 2006). As there have been no indications of their activity at the low *in situ* temperature of the sediments where they have been found, these populations of thermophilic endospores must originate from elsewhere. The combined features of long-

term resistance, almost complete inactivity, and exogenous origin make thermophilic endospores interesting tools to investigate passive dispersal in the ocean.

A first step into understanding their dispersal is to analyze the diversity, abundance and potential origin of these misplaced organisms. These were the central topics of my PhD studies. In the following section, I will summarize the scientific achievements of my work over the last three years, together with other significant studies investigating the phenomenon of thermophilic endospores in cold sediments. Most of our work has focused on endospores of thermophilic sulfate-reducing bacteria (SRB) in Aarhus Bay and Svalbard sediments, therefore relevant aspects of these sites will be introduced below.

2. Scientific context and synthesis of my work

2.1. Main study sites

2.1.1. Aarhus Bay

The presence of endospores of thermophilic sulfate-reducing bacteria has been previously investigated in the surface sediment of station M1, western Aarhus Bay (Figure 4; Isaksen et al., 1994). During my thesis, I examined the water column at the same station, and sediments from 0 to 650 cm depth at station M5, eastern Aarhus Bay (Figure 4) for the presence, diversity, abundance and activity of thermophilic SRB (Chapter 2).

Aarhus Bay is a semi-enclosed embayment adjacent to the Kattegat, in the transition area between the North Sea and the Baltic Sea (Figure 4). It covers an area of 320 km^2 and the water depth varies between ca. 10 m and 30 m from the western to the eastern part, where it connects to the southern Kattegat. Kattegat water is composed of surficial, less saline Baltic Sea water and deeper, more saline North Sea and Atlantic Ocean water (Skyum and Lund-Hansen, 1992; Jørgensen, 1996). Surface water temperature may reach up to 21°C in summer, while bottom water and sediment temperatures do not exceed 15°C (Jørgensen, 1996; Thamdrup et al., 1998). The water column in the Kattegat is stratified during 70% to 80% of the year, especially in spring and summer. This stratification is less strong in autumn, as frequent storms promote mixing of the water layers, and in winter the stratification is weak or absent (Skyum and Lund-Hansen, 1992; Jørgensen, 1996). Due to the freshwater surplus of 15,000 m^3 s^{-1} from the Baltic Sea, the net current in the Kattegat is northbound, towards the North Sea. However, the instantaneous flow frequently alternates from northbound to southbound due to wind and atmospheric forcing (Møller, 1996). The flow of water masses in and out of Aarhus Bay, mainly through a deep trough in the northeastern part, follows these hydrographic

events in the Kattegat. The calculated residence time of the water in the bay is on average only 12 days (Jørgensen, 1996).

A B

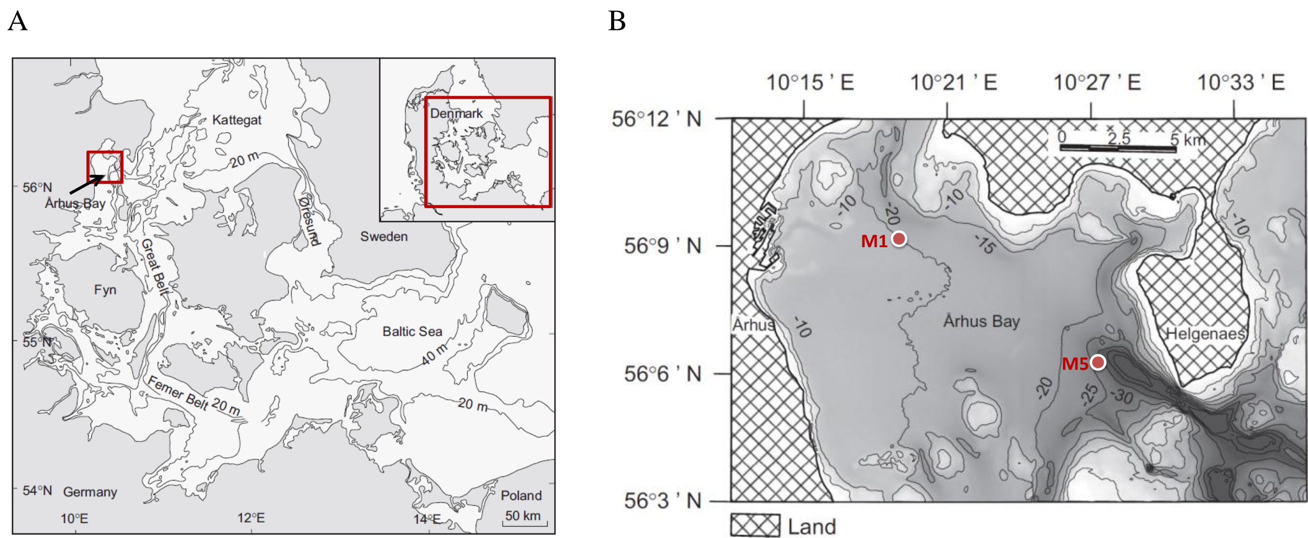


Figure 4. Location of Aarhus Bay. **A:** Map of the south-western Baltic Sea and the Danish straits. Aarhus Bay is framed on the left. **B:** Bathymetric map of Aarhus Bay and location of stations M1 (56˚09'10''N, 10˚19'20''E) and M5 (56˚06'20"N, 10˚27'48"E). Figure modified from Jensen and Bennike (2009).

Currents and waves frequently cause sediment resuspension in the relatively shallow Aarhus Bay. For example, during a summer which had a well-developed pycnocline and no storms, gross deposition was 30-fold higher than the net deposition of ca. 3 g m^{-2} d^{-1} in the western part of the bay (station M1; Jørgensen, 1996; Pejrup et al., 1996). Despite its high frequency, resuspension decreases exponentially with height above sediment, and less than 10% of resuspended material reaches 4 m above the bottom. Furthermore, the resuspended particles have a short residence time in the water column, with mean settling velocity of 0.5-2 m h^{-1}. Consequently, the lateral transport distance of resuspended material is only a small fraction of the water transport distance, and sediment particles are mostly retained within the bay, despite the open exchange of waters with the Kattegat (Jørgensen, 1996).

In a larger scale, Aarhus Bay has been a sedimentation basin for the past nearly ten thousand years. After the last glaciation, Aarhus Bay transitioned from dry land with bogs and shallow lakes (ca. 12-11 ky before present) to fully marine conditions at around 8,000 years before present. However, nowadays the incoming Kattegat water circulation promotes a differential sedimentation in the bay. The eastern part, which includes station M5, presents recent and continuous net sedimentation, while the western part, which includes station M1, has had little or no net deposition for the past 3,000 years. The sedimentation rate at station M5 is ca. 1 mm y^{-1} (Jensen and Bennike, 2009).

2.1.2. Svalbard

The presence of thermophilic endospores has been investigated in surface sediment of several sites around Svalbard, in particular Smeerenburgfjorden, in the northwest region (Vandieken et al., 2006; Christensen, 2009; Hubert et al., 2009 (Chapter 4); Hubert et al., 2010; Müller et al., unpublished). The archipelago of Svalbard is located between the Arctic Ocean, Barents Sea, Greenland Sea and Norwegian Sea (Figure 5). The water mass circulation is different in the East and West of Spitsbergen, the main island of Svalbard. The eastern fjords of Spitsbergen and the other islands of the archipelago receive cold polar and Arctic water, whereas the western fjords of Spitsbergen mostly receive relatively warm Atlantic water from the South via the West Spitsbergen Current, and to a smaller extent the polar and Arctic masses from the North (Figure 5; Hald et al., 2001). The West Spitsbergen current is the main branch of the Norwegian Current, which flows northwards along the coast of Norway and divides into two branches at ca. 72˚N (Ebbesen et al., 2007). The warm surface water from the Atlantic Ocean is the major source of heat to the high northern latitudes and contributes to the generation of deep water in the Nordic seas, sinking below the cold polar surface waters after it passes the western Svalbard shelf and slope. Atlantic water has been continuously present on the southwestern Svalbard fjords over the last 20,000 years (Rasmussen et al., 2007).

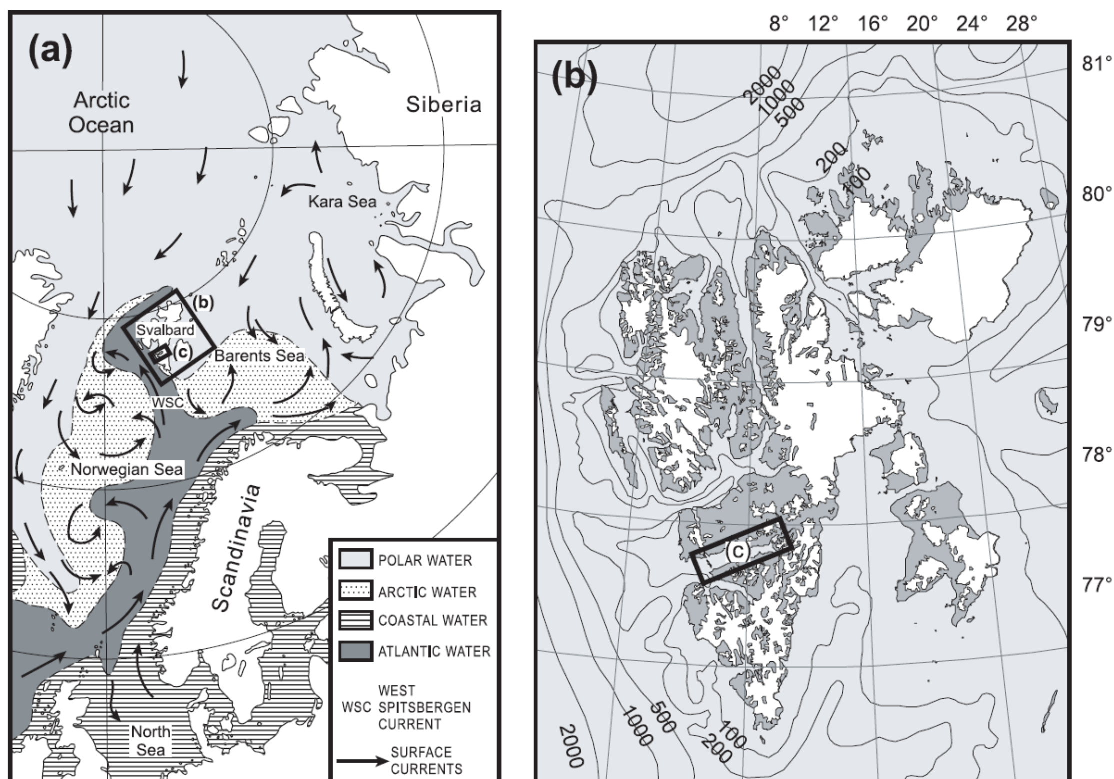


Figure 5. Location map showing: (a) major surface water masses of the Norwegian-Greenland Sea and adjacent seas; (b) the Svalbard Archipelago. The area marked as (c) is Van Mijenfjorden, the second largest fjord in Svalbard. Figure modified from Hald et al. (2001).

Fjords are in general a unique kind of estuary that acts as an efficient sediment trap. In subpolar fjords, such as Svalbard and West Greenland, sediment accumulation rates are on the order of several millimeters per year. In the central basins of Svalbard fjords, these rates range from 0.8 (Billefjorden) to 18.7 mm y^{-1} (Adventfjorden; Szczuciński, 2009). In Smeerenburgfjorden, the sediment accumulation rate for the last century has been ca. 1.9 mm y^{-1} (Hubert et al., 2009 (Chapter 4)). The sediment temperature in Svalbard fjords varies between -2 and +4°C during the year (Knoblauch and Jørgensen, 1999).

2.2. Diversity of thermophilic endospore-forming bacteria in cold sediments

Until 1994, only aerobic thermophilic bacteria had been detected in permanently cold marine sediment. This was reported from sediments in the Kara Sea, Arctic Ocean, as early as 1938 (Egorova, 1938), and later from several stations in deep ocean basins off the coast of Mexico and California, where isolated strains were identified as endospore-forming *Bacillus* species (Bartholomew and Rittenberg, 1949; Bartholomew and Paik, 1966). The first detection of anaerobic thermophiles was from Aarhus Bay sediment. Isaksen et al. (1994) isolated a sulfate-reducing strain able to grow between 52 and 69°C, with optimum growth at 63°C. The strain was able to use several carbon sources (formate, acetate, propionate, butyrate, caprylate, methanol, ethanol, lactate, pyruvate and fumarate) with sulfate as electron acceptor, and to ferment pyruvate and fumarate. Based solely on its physiological characteristics, this thermophilic strain was identified as *Desulfotomaculum kuznetsovii.* The *Desulfotomaculum* genus is a phylogenetically broad group (Stackebrandt et al., 1997) of endospore-forming sulfate-reducing bacteria. Another *Desulfotomaculum* strain, moderately thermophilic, was later isolated from arctic sediment of Nordfjorden, off the west coast of Svalbard. *D. arcticum* strain 15^T sustained growth between 26 and 46.5°C (optimum at 44°C), still far above the *in situ* temperatures of 0 to 4°C (Vandieken et al., 2006).

In the following years, the diversity of anaerobic thermophiles in cold sediments began to be investigated in more detail and beyond isolated strains. Sulfate reduction was measured between 41°C and 62°C in sediment from Smeerenburgfjorden, northwest Svalbard, and further experiments indicated that a complex community of thermophilic endospores is present in those arctic sediments (Hubert et al., 2009 (Chapter 4); 2010). These thermophilic endospore-forming bacteria were inactive at *in situ* temperatures, as expected, but capable of extensive mineralization of complex organic matter upon sediment incubation at 50°C. Extracellular enzymatic hydrolysis of polyssacharides and production of volatile fatty acids were detected at this temperature, indicating the presence of diverse thermophilic bacteria. Furthermore, sulfate reduction appears to be performed by different populations of thermophilic SRB associated to the consumption of distinct substrates (Hubert et al., 2010). Clone libraries of 16S rRNA gene revealed three putative thermophilic fermentative *Firmicutes* and five thermophilic *Desulfotomaculum* phylotypes which

were distinct from *D. arcticum* (Hubert et al., 2009 (Chapter 4); 2010; Figure 6). Representatives of two of these sulfate-reducing phylotypes have been isolated and characterized (Christensen, 2009).

In light of the findings in Svalbard sediments, the diversity of thermophilic endospores in Aarhus Bay was revisited as part of this thesis research (Chapter 2). In brief, clone libraries of 16S rRNA revealed more than 20 *Desulfotomaculum* spp. phylotypes that are activated at distinct temperatures, from 46 to 69°C. Furthermore, three of the *Desulfotomaculum* spp. phylotypes discovered in Svalbard sediments were also present in Aarhus Bay. The similarity of phylotypes found in both locations was supported by clone libraries of dsrAB genes. These genes encode the two major subunits of the dissimilatory (bi-)sulfite reductase, a key enzyme of sulfate reduction and phylogenetic marker gene for SRB. They showed cases of more than 99.9% full sequence identity between phylotypes in these two locations, separated by more than 3,000 km (Chapter 2). Diverse thermophilic bacteria other than SRB were also detected in Aarhus Bay sediment and were activated at temperatures of up to 75°C (see Section 3.3).

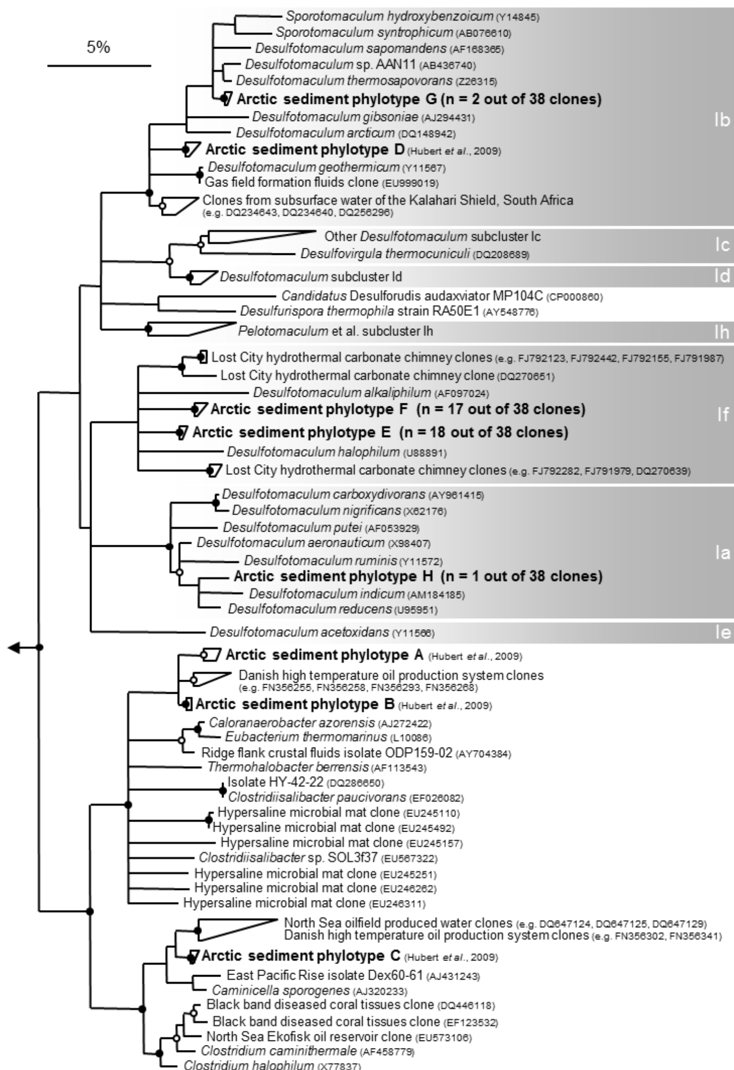


Figure 6. 16S rRNA consensus tree of *Firmicutes* with putative thermophiles in arctic marine sediment highlighted in bold. Arctic sediment phylotypes A-D were detected in a previous study (Hubert et al. 2009 (Chapter 4)) and include putative hydrolytic and fermentative thermophiles (phylotypes A-C) and putative thermophilic sulfate-reducing *Desulfotomaculum* spp. (phylotype D). Arctic sediment phylotypes E-H represent sequences retrieved from heated sediment using PCR primers that specifically target the *Desulfotomaculum* lineage. Relative abundances of phylotypes E-H in the *Desulfotomaculum* clone libraries are indicated in parentheses. Figure from Hubert et al. (2010).

2.3. Abundance of thermophilic endospores in cold sediments

There are several challenges regarding the quantification of thermophilic endospores in cold sediments, as problems arise from any cultivation-based and cultivation-independent methods currently available. For vegetative communities, it is well established that cultivation-based methods tend to underestimate their abundance by at least one order of magnitude, since only populations that are able to grow in the specific cultivation conditions are enumerated (Sahm et al., 1999). Therefore cultivation-independent methods have been increasingly used for the quantification of vegetative cells. However, enumeration methods used for vegetative cells are generally inadequate for the enumeration of endospores (Fichtel et al., 2007; Chapter 3). This is mainly due to the peculiar structure of endospores. For example, fluorescence *in situ* hybridization (FISH) and quantitative polymerase chain reaction (qPCR) are widely used for the quantification of vegetative populations and are both based on access to nucleic acids. However, in endospores this is prevented by the numerous, exclusive and resistant layers of endospore walls (Figure 7). There has been recent advance in overcoming these methodological limitations by improving nucleic acid extraction from bacterial endospores (Marentis et al., 2005; Warner et al., 2009; Geissler et al., 2011), required for qPCR, and permeabilization of endospore walls, required for FISH (Filion et al., 2009). However these improvements have yet to be tested beyond pure cultures of *Bacillus* species. Therefore, currently these methodologies cannot be directly applied to the quantification of thermophilic endospore populations. Further adaptations or strategies must be developed and tested.

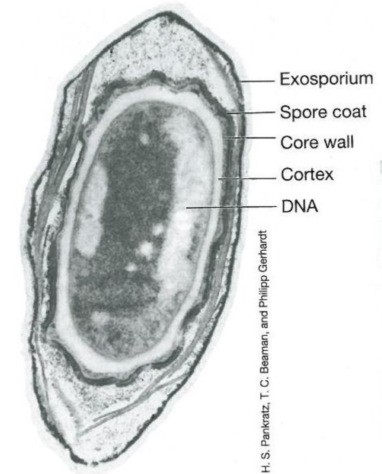


Figure 7. Example of a bacterial endospore. Transmission electron micrograph of a thin section through an endospore of *Bacillus megaterium*, showing the various endospore layers. The exosporium is not present in all endospores (Setlow, 2007). Figure from Madigan et al. (2009).

Other quantification methods that are exclusive for endospores have been developed and applied in environmental samples, including sediments. One successful example is based on indirect fluorescence detection of dipicolinic acid (DPA), a specific and universal component of bacterial endospores (Powell, 1953; Fichtel et al., 2007; 2008). DPA extraction from sediment samples has allowed the quantification of total numbers of endospores in North Sea tidal flats (10^5 to 10^7 endospores per gram of sediment; Fichtel et al., 2008) and recently also in Aarhus Bay (ca. 10^7 endospores per gram of sediment; Langerhuus, Lever, Inagaki, Røy, Jørgensen and Lomstein, in prep.). However, due to the universal presence of DPA in bacterial endospores, the method cannot be directly applied for the specific quantification of thermophilic endospores, which constitute only a subpopulation among all other endospores. Another method based on the detection of DPA from endospores has been developed for genus-specific single-cell quantification of viable endospores

and applied to ice cores and desert soils. However this microscopy-based endospore viability assay is limited to the quantification of *Clostridium* spp., as it relies on the stimulation of germination by addition of specific germinants for members of this genus (Yang and Ponce, 2011). At the current stage of knowledge on the diversity of thermophilic endospores in cold sediments and especially the factors that promote their germination, this method is not suitable for their quantification.

Given these methodological obstacles, our studies so far have focused on the quantification of thermophilic SRB endospores, using two distinct methods based on sulfate reduction measurement. The first method is most probable number enumeration with autoclaved sediment as medium (T-MPN, Vester and Ingvorsen, 1998; Chapter 2). The second method is based on monitoring exponential increases of sulfate reduction rates (SRR) following endospore germination in sediment incubation experiments. Measured SRR are fit to a function that allows the calculation of initial SRR and cell-specific SRR, thus leading to the estimation of SRB abundances at any given time in an exponential growth phase. This novel method is described in Chapter 3. The two methods above are cultivation-based and may underestimate the actual number of thermophilic SRB endospores in the sediment. However, they provide a minimum reliable estimate of the abundance of thermophilic SRB endospores and thus of thermophilic endospores in general.

In this thesis research, the SRR monitoring method was developed and applied to quantify thermophilic SRB endospores in Smeerenburgfjorden (Svalbard), where it indicated a stable abundance of ca. 10^5 thermophilic SRB endospores per cm^3 of sediment between 0 and 23 cm depth. Several other fjords on the west coast of Svalbard also appear to harbor more than 10^4 thermophilic SRB endospores per cm^3 of sediment. These results suggest that a significant flux of thermophilic endospores have been supplied to this Arctic location for the past 120 years (Hubert et al., 2009 (Chapter 4)).

For Aarhus Bay surface sediments, both methods have been used and independently indicated a similar abundance, between 10^3 to 10^5 thermophilic SRB endospores per cm^3 of surface sediment (Chapters 2 and 3). As in Svalbard, this estimate refers to thermophilic SRB that were active at 50°C. According to 16S rRNA clone libraries, the diversity of thermophilic SRB in Aarhus Bay sediment that develops at 50°C is different from the diversity that develops at 60°C (Chapter 2), yet a similar result, of 2.8×10^4 cells per gram of surface sediment was obtained in MPN enumerations conducted at 60°C (Isaksen et al., 1994), indicating that both populations are similarly abundant. Thermophilic SRB endospores were also quantified for deeper sediments in Aarhus Bay. The abundance of thermophilic SRB decreased exponentially from ca. 10^4 endospores per cm^3 at the surface to ca. 1 endospore per cm^3 at 650 cm depth. This result indicates that the influx of thermophilic SRB into Aarhus Bay sediments has occurred for more than 6,000 years. Furthermore, it suggests that this flux has either increased over a thousand-year timescale or that buried thermophilic SRB endospores die off on that timescale (Chapter 2). Therefore, not only the identity and diversity of thermophilic endospores in various cold sediments, but also their high numbers in surface sediments and detection through long time scales may hint to potential sources, as discussed below.

2.4. Potential sources of thermophilic endospores to cold sediments

The presence of diverse thermophilic endospores in Aarhus Bay and Svalbard sediments, including phylotypes that are activated at different temperature intervals, suggest that they might originate from multiple warm, anaerobic habitats. These source environments provide a significant efflux of cells, resulting in the deposition of at least 10^7 and 10^8 thermophilic SRB endospores per m^2 per year in Aarhus Bay and Svalbard, respectively, for the past hundred years (Chapter 2; Hubert et al., 2009 (Chapter 4)). The temperature range at which these thermophiles have been activated in laboratory experiments (40-70°C) is common in deeply buried sediment, and these are known to host diverse thermophilic microorganisms (Parkes et al., 2007). Advective flow of deep pore fluids and light hydrocarbons at pockmarks, mud volcanoes and other cold seep formations can connect these sub-seafloor environments to the water column and transport particles and cells to abyssal water masses (Judd and Hovland, 2007). The basaltic rock basement below deep sediment layers is also a potential source environment, as large volumes of fluids circulate through the ocean crust, influenced by seawater recharge and discharge zones at exposed fractures, in particular at mid-oceanic ridges (Cowen, 2004; Judd and Hovland, 2007). Hydrothermal plumes and diffuse fluid flow through warm shallow sediments around hydrothermal vents may also carry microorganisms from these habitats into the water column. This may happen along oceanic ridge axes or in serpentinization-driven, off-axis hydrothermal systems. The Lost City hydrothermal field, 15 km away from an active spreading zone in the Atlantic Ocean, is the only non-magma driven hydrothermal system known so far, yet it is predicted that similar environments exist around the globe (Orcutt et al., 2011). The venting temperatures at the Lost City field are typically between 40 and 90°C, while venting temperatures at the on-axis, black smoker systems may vary between <100°C and (more commonly) >300°C (Kelley, 2005). Finally, oil and gas reservoirs and offshore production facilities may also host and spread thermophilic endospores (Judd and Hovland, 2007; Hubert and Judd, 2010). These various potential sources are illustrated in Figure 8.

The closest relatives of the thermophilic phylotypes found in Aarhus Bay and Svalbard are *Firmicutes* detected in or isolated from these various warm environments, which supports that these environments may be potential sources of thermophilic endospores (Hubert et al., 2009 (Chapter 4); 2010; Chapter 2). Furthermore, the presence of identical 16S rRNA and dsrAB gene sequences in clone libraries of both Aarhus Bay and Svalbard indicate that these locations may have at least one common source of thermophilic SRB endospores (Chapter 2). This could be related to the irrigation of both regions by Atlantic water masses, which may contain and transport thermophilic endospores towards both locations. Several potential sources, including hydrothermal fields and oil and gas reservoirs are present in the path of this current (Figure 9).

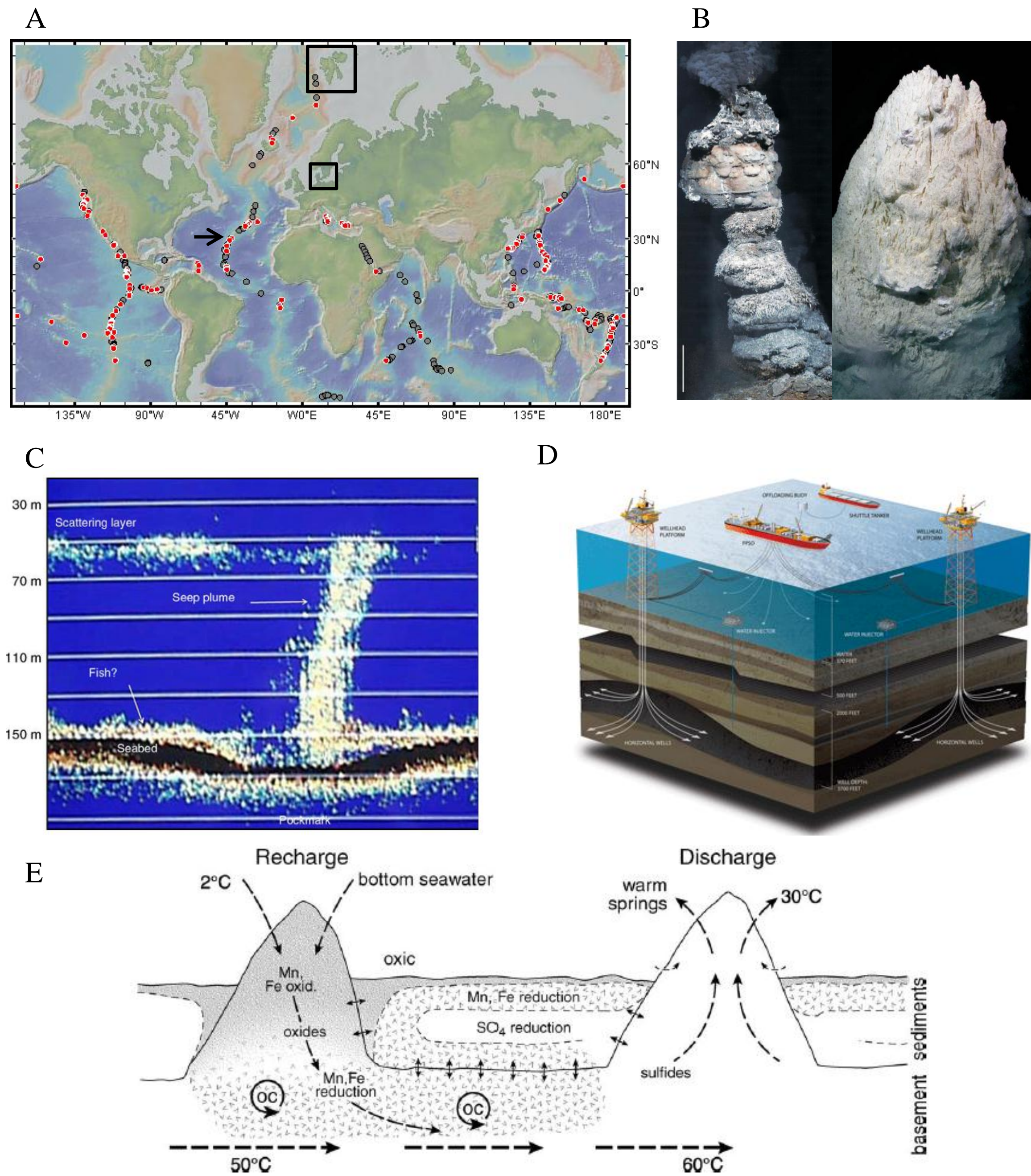


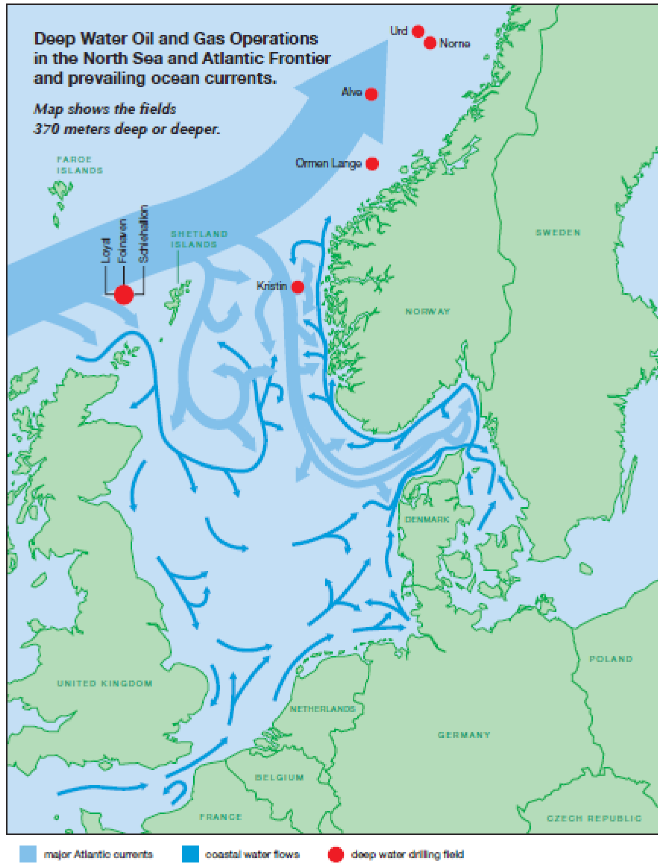
Figure 8. Illustrative examples of potential sources of thermophilic endospores to cold sediments. **A:** Global distribution of hydrothermal fields. Red circles correspond to active venting and gray circles to inactive or unknown. Svalbard and Aarhus Bay are marked in squares. The Lost City Hydrothermal Field, which is the only non-volcanic hydrothermal field, is marked by an arrow. **B:** Examples of hydrothermal vents: on the left, a 350°C black smoker from the Juan de Fuca Ridge, Northeast Pacific Ocean, ca. 5 m tall; on the right, one of the four pinnacles at the summit of the 60-m tall Poseidon edifice in the Lost City Hydrothermal Field, which vent fluids at up to 75°C. **C:** Echo-sounder image of a seep plume rising from a pockmark in the North Sea. The plume rises from the seafloor at 170 m water depth to the thermocline at ca. 50 m depth, where the scattering layer likely indicates particles transported by the fluid flow. **D:** Schematic representation of an offshore oil production platform. **E:** Schematic representation of potential processes during fluid circulation in the buried ocean crust, associated to recharge and discharge zones of exposed outcrops. Dashed arrows represent advective fluid flow and small solid arrows indicate diffusive flow. Figure sources: A: GeoMapApp, using the InterRidge Hydrothermal Vents Database layer. B: modified from Kelley (2005). C: Hubert and Judd (2010). D: http://www.offshore.no/international/article/Bentley_set_for_2012_start_. E: Cowen (2004).

Some of the sources may be unique for each location. For example, the seafloor of western Svalbard exhibits relevant geological features such as pockmarks and active cold seeps. Pockmarks are seafloor craters from less than one to more than a hundred meters in diameter, formed where localized eruption and seepage of gas and pore fluids occurs in soft, fine-grained sediment. Passive seepage of gas and fluid has been detected in the Barents Sea and off western Spitsbergen (Knies et al., 2004; Vanneste et al., 2005; Hustoft et al., 2009). Geological anomalies along these conduits might be explained by active petroleum source rocks, gas hydrates and natural seabed gas seeps in that area, especially at Vestnesa Ridge in the eastern Fram Strait (79˚N, 6˚E; Vogt et al., 1994; Knies et al., 2004; Damm et al., 2005).

Pockmarks with active seepage are also widespread in the North Sea, Skagerrak and Kattegat Sea (Judd and Hovland, 2007). These natural seeps, many of which occur in areas that sustain oil production facilities, especially in the North Sea (Figure 9), may be local sources of thermophilic endospores to Aarhus Bay. *Desulfotomaculum* 16S rRNA gene sequences from the Dan and Halfdan oil fields, located 200 km west of Denmark and characterized by temperatures from 40 to 80°C, were the closest relatives to two of the main phylotypes of thermophilic SRB in Aarhus Bay (Chapter 2). Besides potential marine sources, terrestrial industrial environments may add to the flux of thermophilic endospores into Aarhus Bay sediments, such as local wastewater treatment plants and a nearby coal-fired power plant (Isaksen et al., 1994). These potential anthropogenic sources may have contributed in the recent decades, yet they cannot be the only sources of thermophilic endospores to this location, since these endospores are present in sediment layers which date back thousands of years before industrialization (Chapter 2).

Cold marine sediments may also harbor thermophilic endospores that originate from other terrestrial environments. However, in the case of thermophilic SRB endospores, this appears to be unlikely. Various attempts have failed to detect thermophilic SRB in cold terrestrial environments, including soil near Aarhus Bay (Isaksen et al., 1994), terrestrial layers in deep sediment cores in the tidal flat of Janssand, southern North Sea (De Rezende, 2008) and a terrestrial warm spring in northern Svalbard (Albert Müller et al., unpublished). Furthermore, thermophilic SRB have also been detected in the water column of Aarhus Bay, which confirms that, regardless of their origin, these thermophilic endospores are presently being dispersed by seawater currents and are thus relevant indicators of passive dispersal in the marine environment (Chapter 2).

A



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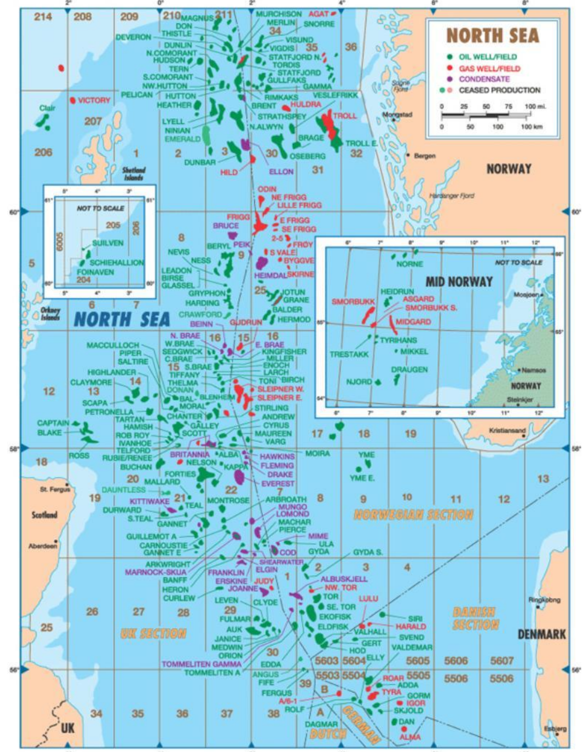

C

Figure 9. Oil and gas reservoirs in the North Sea. **A:** Deep water oil and gas operations in the North Sea and Atlantic Frontier and prevailing ocean currents. More detailed map of oil and gas fields in the northern North Sea (**B**) and the southern North Sea (**C**). Figures from: www.foodandwaterwatch.org /pressreleases/bp-north-atlantic/ (A) and www.acorn-ps.com/web/page/oilgas/nsfields.htm (B and C).

2.5. Conclusions

The main conclusions of my thesis are:

1. Thermophilic SRB endospores are present throughout a 650-cm sediment core in Aarhus Bay. This indicates that passive dispersal of thermophilic endospores to this location has occurred for at least 6,000 years. In Svalbard, only sediments down to 23 cm have been studied, and the constant abundance of thermophilic SRB endospores through this depth indicates that dispersal to this location has occurred as a constant flux for at least 120 years.

2. The detection of thermophilic SRB in the water column of Aarhus Bay confirms that this passive dispersal is an ongoing process involving seawater circulation.

3. The presence of identical 16S rRNA and *dsrAB* sequences in Aarhus Bay and Svalbard suggests that some of these thermophilic SRB are dispersed over great distances, possibly originating from a common source.

4. The diversity of thermophilic endospores in Aarhus Bay, activated at different temperatures, indicates that these populations may originate from multiple sources. There may be sources of thermophilic endospores that are not common to Aarhus Bay and Svalbard.

5. The high abundance of thermophilic SRB, consisting of at least 10^4 endospores per cm^3 of surface sediment in both Aarhus Bay and Svalbard, indicates a large-scale dispersal of these populations.

6. The abundance of distinct populations of thermophilic SRB endospores in cold sediments that develop in succession upon sediment incubation can be estimated by monitoring sulfate reduction rates and calculating average cell-specific rates for each population.

Our studies have focused on the biogeography of thermophilic endospores, in particular those of sulfate-reducing bacteria. However, the mechanisms and effects of dispersal are likely not exclusive for these populations. Subsurface fluid flow and ocean currents may pass through various microbial habitats and similarly act in transporting diverse cells and other endospores in local, regional or global scale, thus influencing the biogeography of diverse marine microbial populations. Furthermore, incoming populations may contribute to microbial ecology and diversity of the colonized habitat, either as active members or as part of a seed bank, which may become active when conditions change. In the particular case of thermophilic endospores, their long-term survival may allow these organisms to reach deep warm sediments via

sedimentation. This is unlikely to occur in Aarhus Bay, yet it may be possible in other locations where sedimentation rates are higher or temperature gradients with depth are steeper. If this occurs, thermophilic endospores once in cold sediments may become active members of the deep biosphere.

3. Other work and future research directions

3.1. Development of qPCR assays for quantifying thermophilic endospore-forming SRB

As discussed in section 2.3., there is no standard method for quantifying thermophilic endospores in sediments. Nevertheless, investigating their abundance provides insight regarding the flux and potential sources of these cells to cold marine sediments, and their survival during sedimentation. This has been demonstrated by T-MPN for thermophilic SRB endospores in sediments as deep as 650 cm in Aarhus Bay (Chapter 2). Furthermore, we have developed a method to quantify thermophilic SRB by monitoring exponential increases in sulfate reduction rates upon incubation at high temperature with radioactive sulfate tracer. This method has been applied to surface sediments in Aarhus Bay and Svalbard (Chapter 3; Hubert et al., 2009 (Chapter 4)) and revealed similar abundances in both sites, which were supported by T-MPN results (Chapter 2). Both methods provide a reliable minimum estimate of the abundance of thermophilic SRB in cold sediments. However, they are both laborious and time-consuming, which hinders their application to a large number of samples (e.g. different sites and depths). Therefore, further methodological development is of interest for this research, and may provide means to address further questions regarding the dispersal and biogeography of thermophilic endospores.

One promising technique for this purpose is the development of quantitative PCR assays for endospores that germinate at high temperatures. A unique advantage of qPCR in relation to the previous methods is that it allows the quantification of selected groups at various taxonomic levels, by the use of specific primers. Additionally, for quantifying thermophilic SRB, the incubation time might be reduced to hours instead of days (SRR-based estimation) or months (T-MPN); processing (DNA extraction, qPCR reaction) can be done in parallel with several samples at once; and fewer or no SRR measurements will be required, thus reducing the need of radioactive incubations.

The primary goal would be to quantify thermophilic endospores by qPCR as soon as germination occurs, when endospores turn into vegetative cells and DNA becomes accessible for extraction, but before the population size changes due to exponential growth. As most of the knowledge so far is on the diversity and abundance of thermophilic SRB, these would be the first target group. Primers can be designed for both 16S rRNA and dsrAB genes, based on the existing clone libraries with more than 300 and 150 sequences, respectively (Chapter 2). Preliminary work on the design of 16S rRNA gene-targeted primers which are

specific to lineages of interest has been performed by collaborators at the University of Vienna (Dr. Alexander Loy and Cecilia Wentrup) and the Max Planck Institute for Marine Microbiology (Dr. Casey Hubert; presently at Newcastle University). Primers targeting phylotypes E and F have been optimized and initial data showed abundances on the order of 10^5 amplicons cm^{-3} in Smeerenburgfjorden sediment slurries after ca. 20 h of incubation at 50°C. Primers targeting phylotype G have been designed but not yet tested. Primers for phylotypes D and R are also of interest and can be designed based on Aarhus Bay clone libraries (see Table 1 in Chapter 2 for a list of thermophilic SRB phylotypes in Aarhus Bay and Svalbard).

Future work could start as follows: first, test the specificity of the designed primers and the detection limit of the qPCR assays on plasmids from the existing clone libraries which are representative of target and non-target lineages for the primer pairs. This can be supported by testing primers on two thermophilic SRB isolated from Smeerenburgfjorden, *"Desulfotomaculum thermohydrogenovorans"* strain Lac-2 and *"Desulfotomaculum thermethanolovorans"* strain Eth-2 (Christensen, 2009). These strains are representative of phylotypes F and G, respectively. Second, apply the qPCR assay on sediment slurry incubated at 50°C and subsampled hourly in the first 20 h for SRR measurement and DNA extraction (Hubert, unpublished). This will allow the characterization of the initial moments of the exponential phase for each population of thermophilic SRB and will provide a comparison between the detection limit of thermophilic SRB by qPCR assay and by SRR measurements.

Applying qPCR in the first hours of sediment incubation experiments may provide a better estimation of the germination time of these thermophilic SRB than currently obtained from SRR measurements. It is known that in both Aarhus Bay and Svalbard sediments two successive populations of thermophilic SRB develop within five days of sediment incubation at 50°C (Chapter 3; Hubert et al., 2010; Figure 10). However, it is not known whether the first and the second exponential phases initiate at the same time, i.e. whether both the faster- and the slower-growing populations germinate simultaneously. Germination time is a relevant parameter for the SRR-based estimation of the abundance of thermophilic SRB endospores. Improved determination of the germination time may narrow in one order of magnitude the uncertainty of the estimated abundance of each of the populations that develop in high-temperature incubations (Chapter 3).

A limitation of the qPCR method is that it provides the gene abundance, and this may not be directly converted to cell numbers when targeting the 16S rRNA gene, due to multiple copies of the gene in the same organism (Tourova et al., 2001). Nevertheless, one alternative is to calibrate qPCR results by using both qPCR and fluorescence *in situ* hybridization to quantify the target groups at a later time in the exponential phase and test whether the numbers obtained are in good agreement. If SRR are measured for the same time point, this test will also provide an experimental determination of an average cell-specific SRR value for the analyzed population, which would improve the accuracy of the SRR-based quantification.

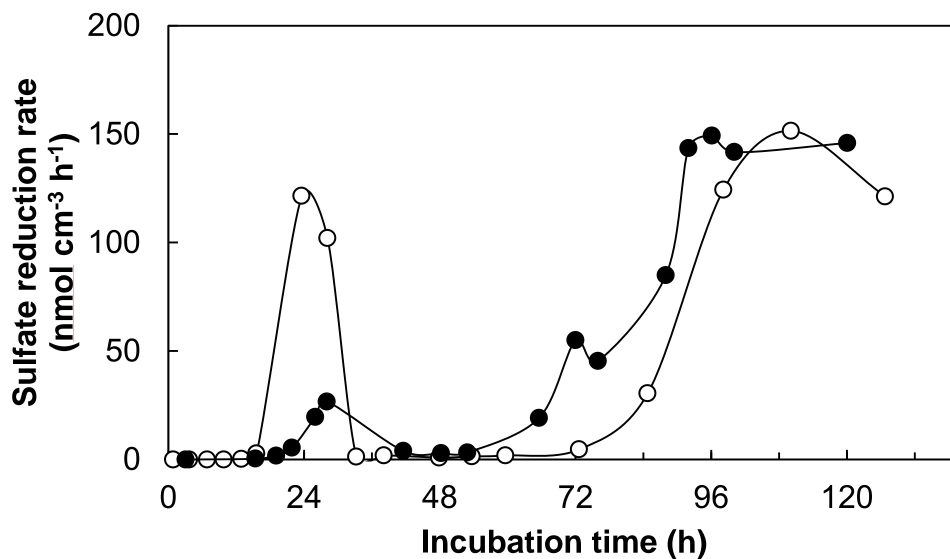

Figure 10. Sulfate reduction rates of pasteurized sediment slurries incubated at 50°C with amendment of seven organic substrates: formate, lactate, acetate, succinate, propionate, butyrate and ethanol (1 mM each). Closed circles: Aarhus Bay surface sediment (data from Chapter 3). Open circles: Smeerenburgfjorden surface sediment (Hubert et al., unpublished).

3.2. Potential applications of qPCR assays for thermophilic endospore-forming SRB

The development of qPCR assays for different phylotypes would open the possibility of quantitatively tracking their distribution, and may reveal a dispersal rate of these specific populations in the ocean. This has never been achieved for any microbial population. In fact, Martiny et al. (2006) hypothesized that microorganisms present a similar frequency distribution of dispersal distances as macroorganisms, for which most passively dispersed propagules reach only very short distances and a small proportion can disperse over great distances. Furthermore, the distance of passive dispersal may be correlated to the abundance of the population at the source (Figure 11). However, these hypotheses have not been systematically tested so far.

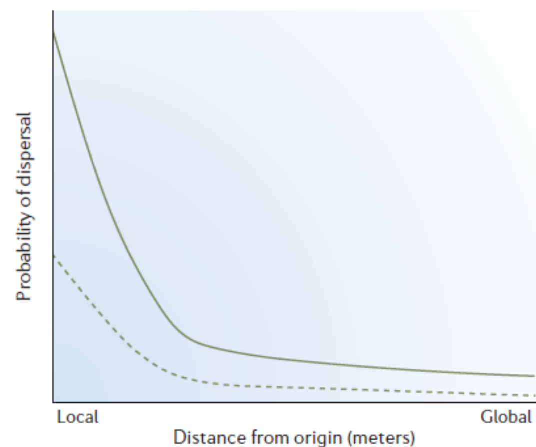

Figure 11. Hypothetical dispersal distribution of a passively dispersed macroorganism. Populations with higher densities present higher chances that an individual will disperse over very long distances (solid line). For taxa with lower population densities (dashed line), passive dispersal might be effectively restricted, as the chances of long-distance dispersal are very low. Figure from Martiny et al. (2006).

One interesting site for starting such

investigations could be the Guaymas Basin. This hydrothermal field is a potential source of thermophilic endospore-forming SRB to cold seawater and sediments. The upward flow of hydrothermal fluid creates high heterogeneity among sites, particularly observed in extreme temperature variations in limited lateral and vertical orientations. The bottom water, usually around 2.8 to 4°C, can be in direct contact with fluids of up to 350°C being expelled from mounds and chimneys. At the same time, surrounding sediments may have temperature variation from 3°C on top to more than 130°C at 30 cm depth at some locations, and more moderate temperature gradients at others, where thick bacterial mats cover the sediment (Weber and Jørgensen, 2002). Thermophilic and hyperthermophilic sulfate reducers, which may include *Desulfotomaculum* spp. (Dhillon et al., 2003) thrive in these environments and might achieve sulfate reduction rates of more than 2000 nmol cm^{-3} d^{-1}. These are among the highest rates measured in the marine environment (Weber and Jørgensen, 2002). The heterogeneity of this site, with major and minor temperature gradients, and the cold deep-sea sediments surrounding the hydrothermal field are then a promising combination for analyzing variations in abundance, activity and distribution of thermophilic SRB endospores.

During my PhD studies, I had the privilege to participate in the research cruise AT15-40 (December 2008), aboard the research vessel *Atlantis* and assisted by the submersible human-occupied vehicle *Alvin*, and collected various sediment and water samples for incubation experiments. These included a transect from within the hydrothermal field into the cold surroundings. Sediment cores were collected from 0, 100, 200 and 400 m away from a large *Beggiatoa* mat ("Megamat"), on the edge of the hydrothermal field, and in cold sediment areas within the hydrothermal field. I have worked in collaboration with Albert Müller, PhD student at the University of Vienna, on preliminary experiments with these sediments, which revealed the development of sulfate reduction upon incubation of pasteurized sediment at 50°C, suggesting the presence of thermophilic SRB endospores. Interestingly, sulfate reduction of pasteurized sediment was highest in cold sediment within the hydrothermal field and decreased in the sediment 400 m away from it, yet it was still more than three times higher than in the warm sediment within the hydrothermal field, where the *in situ* temperature was close to 50°C (Table 1). This may be an indication that thermophilic SRB in the warm sediments are more represented by vegetative cells, while endospores are more abundant in the cold areas within the hydrothermal field and their dispersal extends for at least 400 m. However, it is also possible that the differences in sulfate reduction are not related to the abundance of these populations, but to environmental factors. Identifying and quantifying the populations of thermophilic SRB along this transect may thus clarify this issue. The identification of the thermophilic community developed in these incubation experiments is currently being done by Albert Müller, as part of a promising project analyzing the phylogeography of thermophilic endospore-forming bacteria in a global scale. Knowledge about the diversity of thermophilic SRB in these sediments will support the use of qPCR for the quantification of representative phylotypes and may thus provide a dispersal rate of these populations in the Guaymas Basin.

Table 1. Sulfate reduction in surface sediment slurries (0-2 cm depth) incubated at 50°C after pasteurization. These results were obtained in collaboration with Albert Müller (University of Vienna).

Push core no.	Sulfate reduced (µM)			Originated from	In situ T (°C)
	0 h	72 h	120 h		
4488-6	Below background	90	407	Sediment below "Megamat"	43
4491-1	Below background	1534	5014	Cold sediment within the hydrothermal field	<25
4491-31	Below background	754	1496	Cold sediment 400 m away from the hydrothermal field	4

The idea of quantifying populations of thermophilic SRB endospores in a lateral transect may be used for more than calculating dispersal rates. This could potentially be used for tracking the unknown sources of these populations in cold environments, based on the theoretical correlation between population abundance and dispersal distance (Figure 11). If the chances of dispersal decrease with distance and sedimentation rates are accounted for, then larger deposition of a specific population may indicate proximity of its source. This could be applied, for example, to investigate whether the thermophilic endospores in Aarhus Bay originate from the North Sea or the Baltic Sea, by quantifying these populations at different sites in both directions. In principle, this analysis could be done both with sediments and with water column samples, as thermophilic SRB endospores have been detected at depths that correspond to both water masses in Aarhus Bay (1 m depth corresponded to Baltic Sea water and 14 m depth to North Sea water). However, a study tracking thermophilic endospores in water masses must test whether the presence and abundance of thermophilic endospores in each layer is a result of local sediment resuspension. Furthermore, it is likely that the abundance of these thermophiles in seawater is much lower than in sediments, where they accumulate by sedimentation. In fact, despite the success in detecting thermophilic SRB at 1 m and 14 m in the water column of Aarhus Bay, similar attempts performed by Dr. Casey Hubert and myself with samples from Svalbard and the Guaymas Basin have been unsuccessful. Sampling of very large volumes of seawater in order to concentrate sufficient biomass may be necessary.

Quantitatively tracking thermophilic endospore populations could be interesting not only for biogeography studies, but also for applied research. The sources of thermophilic endospores are often related to oil and gas deposits, thus tracking the abundance of thermophilic endospores may lead to the discovery of seepage from reservoirs unknown so far, as proposed by Hubert and Judd (2010).

3.3. Expanding the investigation of thermophilic endospores in cold sediments from SRB to other metabolic groups

Another interesting future research direction is to analyze the diversity of other groups of thermophilic endospores in cold surface and deep sediments. In Aarhus Bay, denaturing gradient gel electrophoresis (DGGE) showed the presence of diverse thermophilic bacteria activated at temperatures of up to 75°C, an incubation temperature at which no SRB were detected (Figure 12). Investigating the diversity of thermophilic endospores other than SRB may be interesting not only because of the diversity *per se*, but also because they could provide unique hints about potential sources. Furthermore, the time-course experiments conducted in both Aarhus Bay and Svalbard indicate that sediment incubated at a high temperature presents a complex community interaction and succession. In Smeerenburgfjorden surface sediment, it appears that exoenzymatic hydrolysis of polyssacharides is followed by the production of volatile fatty acids, and these are differently consumed by successive populations of sulfate-reducing bacteria. It is intriguing how these different groups depend on each other and compete, and it would be interesting to evaluate how the diversity and community dynamics develop at different incubation temperatures and conditions, sites or depths.

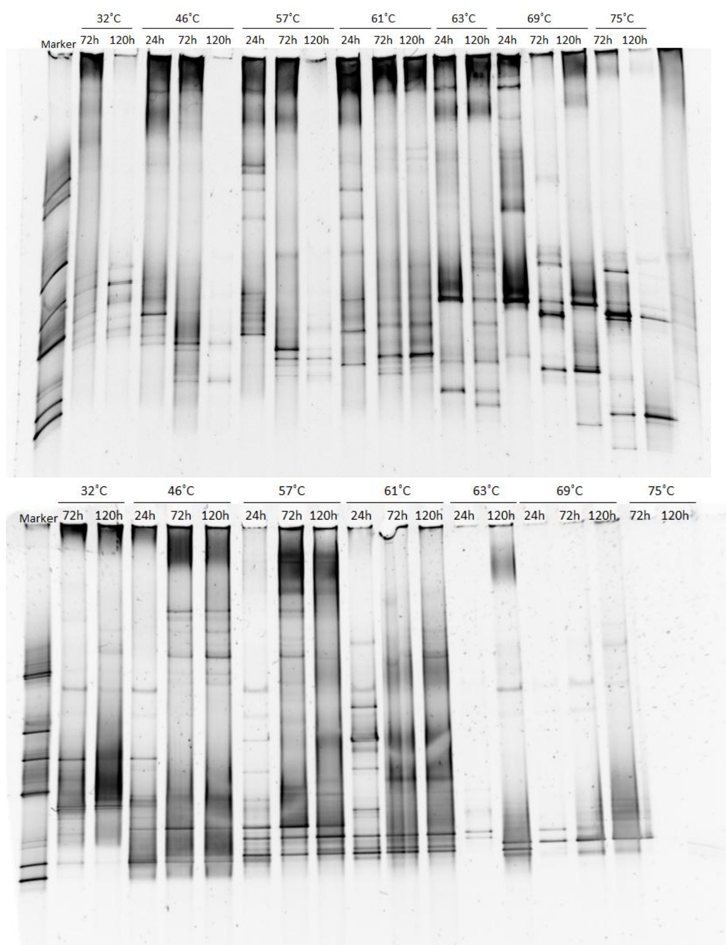

Figure 12. DGGE profiles of cDNA fragments obtained after RT-PCR with general primers 8F/1492R, targeting bacterial 16S rRNA gene (**A**) or specific primers DEM 116F/1164R, targeting *Desulfotomaculum* sp. 16S rRNA gene (**B**; data from Chapter 2). In both cases, RT-PCR was followed by PCR with the general bacterial primers 341F-GC and 907R.

A potential short project could be developed based on the following observation. I conducted a preliminary experiment with sediment samples along a transect in van Mijenfjorden, southwestern Svalbard, which showed distinct patterns of the development of thermophilic sulfate reduction at each station (Figure 13). Although the availability of substrates was homogenized among samples by the addition of 2 mM of seven distinct volatile fatty acids, sulfate reduction developed at different times and reached different levels. As shown in Figure 13, station GH, at the mouth of Van Mijenfjorden, showed the highest sulfate reduction with a lag phase between the two major increases (ca. 24 to 44 h and ca. 72 to 120 h of incubation), which is similar to the pattern observed in Smeerenburgfjorden and Aarhus Bay (Figure 10). Station BJ, in the middle of the fjord, showed sulfate reduction at levels similar to GH between 24 and 72 h, but no further increase until the end of the incubation, at 120 h. Station AG, the next station eastwards, showed relatively constant increase of cumulative sulfate reduction (i.e. constant rate) from ca. 24 h until the end, at 120 h of incubation. Station GC at 0-10 cm depth showed relatively low and late sulfate reduction. Deeper sediment at the same station (100-110 cm depth), however, did not show sulfate reduction above background. Station GB, next to the coal mining plant of Sveagruva, started showing activity only after ca. 96 h, and reached 32 μM of sulfate reduced after 120 h. Finally, station AE, on the southern end of the fjord towards Paulabreen, showed rapidly increasing activity after 64 h.

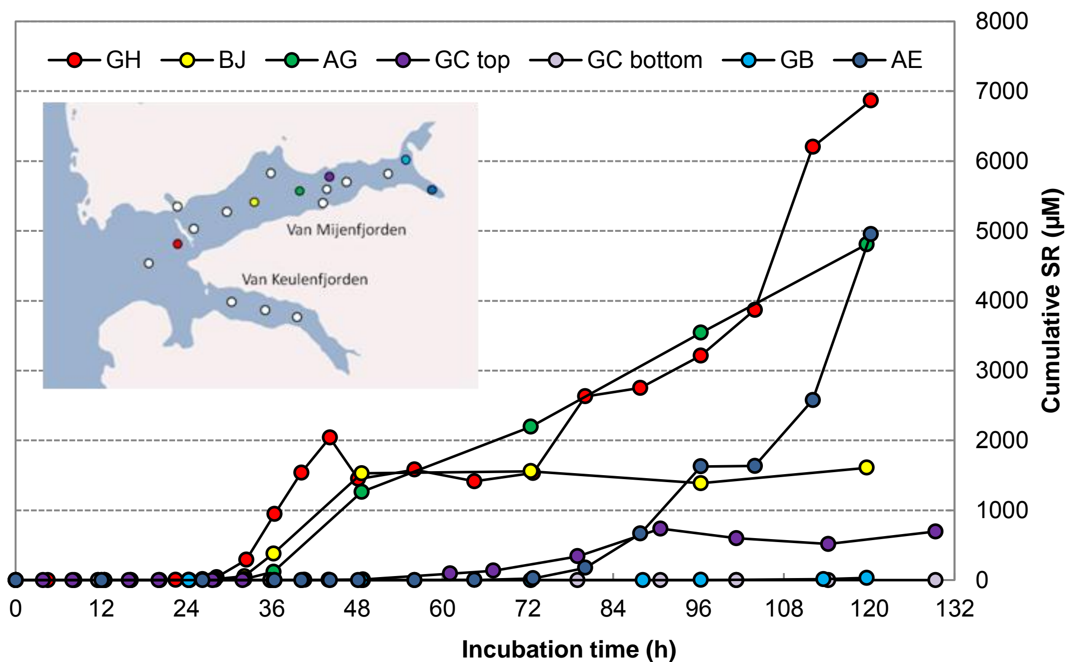

Figure 13. Pasteurized Van Mijenfjorden sediment slurries incubated at 50˚C with 2 mM formate, lactate, acetate, succinate, propionate, butyrate and ethanol. Cumulative SR refers to the total amount of sulfate reduced from the incubation start until the sampling event. The approximate location of sampled (circles) and tested (colored circles) stations is pictured in the inset. GC top: 0-10 cm depth; GC bottom: 100-110 cm depth. All other stations: 0-10 cm depth.

These different patterns could be a result of distinct populations of thermophilic SRB or of other thermophilic bacteria. This could be investigated by DGGE and sequencing of relevant phylotypes, as samples for DNA extraction are available for all of the time points when SRR were measured. Variations in abundance, rather than presence and absence, of these populations may also be an explanation, which would represent yet another application for qPCR assays. Furthermore, a general trend of longer lag phase before detection of SRR was observed towards inner stations. If this trend is directly related to endospore abundances, it may confirm the hypothesis that the thermophilic SRB endospores in Van Mijenfjorden are supplied from the open ocean and are increasingly diluted with freshwater input from land.

3.4. Concluding remark

Little is known regarding microbial dispersal and the diversity and physiology of thermophilic endospore-forming bacteria, and even adequate qualitative and quantitative methods are lacking for addressing these subjects. My PhD work provided valuable contributions to these understudied aspects of microbial biogeography and ecology. The questions raised during my PhD, following experiments, puzzling results and especially the inspiring discussions along the way, indicate that this research is still in its infancy, and motivates further work.

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CHAPTER 2

MANUSCRIPT I

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Dispersal of thermophilic *Desulfotomaculum* endospores to Baltic Sea sediments over thousands of years

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Abstract

Patterns of microbial biogeography result from a combination of dispersal, speciation and extinction, yet the contributions that each of these mechanisms exerts on microbial populations are difficult to distinguish. The influx of endospores of strictly thermophilic microorganisms to cold marine sediments offers a natural model to investigate passive dispersal in the ocean. We investigated the activity, diversity and abundance of thermophilic sulfate-reducing bacteria (SRB) in Aarhus Bay by incubating sediment samples between 28 and 85°C, and by subsequent molecular diversity analyses of *Desulfotomaculum* 16S rRNA and dsrAB genes. The thermophilic *Desulfotomaculum* community in Aarhus Bay sediments consisted of at least 23 species-level 16S rRNA phylotypes. Three of these are also present in arctic sediments of northwest Svalbard, over 3,000 km away. For one phylotype, identical 16S rRNA and *dsrAB* sequences strongly suggest that the same species is present at both cold sediments. Radiotracer-enhanced most probable number analysis revealed that the abundance of endospores of thermophilic SRB in Aarhus Bay sediment was ca. 10^4 endospores cm^{-3} at the surface and decreased exponentially to 1 endospore cm^{-3} at 6.5 m depth. Endospores of thermophilic SRB were also detected in the overlying water column. A *Desulfotomaculum* phylotype shared with Svalbard sediment was abundant in Aarhus Bay surface sediment and was also detected in the deepest sediment tested, suggesting it is widely distributed in the ocean and that its supply into Aarhus Bay sediments dates back at least ca. 6,000 years. The sources of these thermophiles remain enigmatic, but at least one source may be common to both Aarhus Bay and arctic sediments.

Introduction

Mounting evidence indicates that microbial populations exhibit complex biogeographical patterns (e.g. Martiny et al., 2006; Falcón et al., 2008; Ionescu et al., 2010; Schauer et al., 2010; Nemergut et al., 2011). Passive dispersal of cells seems to play an important role in shaping these patterns (Green and Bohannan, 2006; Fierer, 2008), but little is known about the extent of dispersal, due to the difficulty of tracking a given genotype in space and time and unlink dispersal and evolutionary history of free-living microorganisms

(Ramette and Tiedje, 2007). The presence of endospores of strict thermophiles in cold marine sediments appears to be the result of passive dispersal in the ocean, since these bacteria must originate from warm environments distant from the sediments where they have been discovered (Bartholomew and Paik, 1966; Isaksen et al., 1994; Hubert et al., 2009). Given that these exogenous cells are inactive and thereby less prone to selective pressure (speciation and extinction), these misplaced microbes offer a natural model for investigating dispersal processes in the marine environment.

Investigations of the diversity and abundance of these thermophiles have focused on endospores of sulfate-reducing bacteria (SRB) in cold marine sediments of Aarhus Bay and Svalbard. We have estimated that these bacteria have been supplied to arctic sediments of western Svalbard at a rate of 10^8 endospores per square meter per year for at least the past 120 years (Hubert et al, 2009). This influx includes a diverse group of dormant endospores capable of catalyzing anaerobic hydrolysis, fermentation and sulfate reduction upon germination (Hubert et al., 2010). Six different *Desulfotomaculum* spp. – a phylogenetically broad group of endospore-forming SRB (Stackebrandt et al., 1997) – have been enriched in arctic sediments and identified by 16S rRNA gene sequencing. Pure cultures of some of these SRB were isolated by incubating sediment at 28 to 50°C, which is far above the year-round *in situ* temperatures of -2 to 4°C (Vandieken et al., 2006; Hubert et al, 2009). The Svalbard findings coincide with earlier results of experiments conducted with sediment from Aarhus Bay, on the east coast of Denmark. These showed that the germination of endospores of aerobic (Thamdrup et al., 1998), fermentative and sulfate-reducing thermophiles (Isaksen et al, 1994) can be induced in sediment incubated at up to 70°C. A sulfate-reducing thermophile (strain P60) was isolated and classified as *Desulfotomaculum kuznetsovii* based on its physiology (Isaksen et al, 1994). In the present study, we expand the study of thermophilic SRB in Aarhus Bay in light of the new observations at Svalbard. By using sediment incubations with radioactive sulfate tracer, community fingerprinting, and clone libraries of 16S rRNA and dsrAB genes, we provide evidence of large-scale and long-term passive dispersal of SRB endospores in the marine environment.

Materials and Methods

Sampling

Marine sediment was sampled in January 2009 and May 2010 from station M5 (56°06'20"N, 10°27'48"E, 28 m water depth) in Aarhus Bay, located on the transition between the Baltic Sea and the North Sea. Surface sediment (0-10 cm) was collected with a box corer (Hessler and Jumars, 1974) and stored at 4°C in gas-tight plastic bags (Hansen et al., 2000). Sediment from 0 to 650 cm depth was collected using a gravity corer (the top 10 cm were discarded). Cores were sectioned and sediment was stored as described above.

Seawater was sampled in August 2010 at the nearby station M1 (56°09'10"N, 10°19'20"E) in Aarhus Bay, from 1 m and 14 m water depth (25 liters at either depth). The total water depth at this site is 16 m. Seawater was sequentially filtered both through glass fiber (GF) filters (GF-75, Advantec) and through 0.45 µm (HVLP, Millipore) and 0.2 µm polycarbonate (Cyclopore PC, Whatman) filters to collect suspended material. A total of seven GF, three 0.45 and three 0.2 µm filters were used for incubation experiments. In all cases, material collected on each filter was derived from 1 l of seawater. All filters were stored in anaerobic jars at 4°C for three months prior to experimental incubation.

Sediment slurry preparation

Surface sediment slurries were prepared by homogenizing sediment with sterile anoxic synthetic seawater medium at a 1:2 (w/w) ratio under a constant flow of N_2 gas. Synthetic seawater was prepared according to Widdel and Bak (1992) with the following modifications (g/l): NaCl 15.0, $MgCl_2 \cdot 6H_2O$ 2.0, $CaCl_2 \cdot 2H_2O$ 0.2, Na_2SO_4 2.8. The sulfate concentration in the medium was 20 mM, similar to the *in situ* concentration in Aarhus Bay. The sediment slurry was divided into two separate master slurries. One slurry was left unamended and the other slurry was amended with organic substrates: formate, lactate, acetate, succinate, propionate, butyrate and ethanol (each to a final concentration of 1 mM).

Slurry incubations in a temperature gradient

Aliquots of 11 ml from substrate-amended and unamended master slurries were transferred to Hungate tubes under a constant flow of N_2 and stored overnight at 4°C. Replicate tubes were pasteurized at 85°C for 30 min and after pasteurization immediately incubated between 28°C and 85°C in a temperature gradient block (TGB). Replicate substrate-amended and unamended sub-slurries (15 each) all received 200 kBq ^{35}S-labeled carrier-free sulfate tracer and were incubated in alternating slots of the TGB for subsequent measurements of sulfate reduction. Additional replicate sub-slurries without radioactive tracer were incubated in adjacent TGB slots for subsequent RNA extraction. Slurries were sub-sampled after 24, 72 and 120 h. Three ml aliquots were removed from the radioactive (^{35}S-sulfate) slurries, mixed with 6 ml 20% zinc acetate solution and frozen at -20°C until distillation by the single-step cold chromium method (Kallmeyer et al., 2004). At the same time, 2 ml aliquots were removed from the non-radioactive slurries and centrifuged. The resulting pellet was immediately frozen in liquid N_2 and stored at -80°C until RNA extraction.

Radiotracer MPN enumeration of thermophilic SRB endospores

Radiotracer most probable number enumerations (T-MPN, Vester and Ingvorsen, 1998) were performed to estimate the abundance of thermophilic endospore-forming SRB in Aarhus Bay sediment samples. Sediment from seven depths (0-10, 110-120, 210-220, 310-320, 410-420, 510-520 and 640-650 cm) were inoculated into medium prepared according to Vester and Ingvorsen (1998) with the following modifications. Aarhus Bay station M5 surface sediment was diluted with synthetic seawater medium (1:1, w/w; composition described above), homogenized and successively passed through 2.0-, 1.0- and 0.5-mm mesh sieves. This sediment slurry medium was autoclaved for 1 h at 121°C and distributed into Hungate tubes (9 ml aliquots) under a constant flow of N_2, incubated at 50°C for three days, autoclaved a second time for 1 h, incubated again at 50°C for one day and finally autoclaved a third time for 1 h. Prior to use, sodium dithionite (200 µM) was injected as a reducing agent and the sterile sediment medium was amended with organic substrates (formate, lactate, acetate, succinate, propionate, butyrate and ethanol, each at a final concentration of 1 mM).

For each sediment depth, triplicate ten-fold serial dilutions were prepared from undiluted to 10^{-7}. The first tube of each series was inoculated with 1 ml of pristine sediment and pasteurized at 85°C for 30 min prior to the subsequent dilution transfers. Each tube was homogenized by vigorous shaking after inoculation, which was facilitated by the presence of 0.5 cm-wide glass beads in the tubes. Finally each tube received 400 kBq ^{35}S-labeled carrier-free sulfate tracer and was incubated at 50°C. In addition, 35 non-inoculated tubes (i.e. only the autoclaved sediment medium and radiotracer) were incubated as negative controls. After 30 days of incubation, 3 ml aliquots were withdrawn from each test tube and preserved by addition of 6 ml 20% (w/v) zinc acetate and freezing. Following cold chromium distillation of the total reduced inorganic sulfur (TRIS; Kallmeyer et al, 2004), tubes were scored positive for SRB if ^{35}S-calculated sulfate reduction was above the threshold of TRIS% = 0.1% (Vester and Ingvorsen, 1998). TRIS% is calculated as $[a/(a+A)]$x100, where a is the total radioactivity of reduced inorganic ^{35}S-labeled sulfur produced (TRIS) per volume and A is the total radioactivity of ^{35}S-labeled sulfate per volume after incubation (Vester and Ingvorsen, 1998). For sediment samples from 0-10 cm and 640-650 cm depth, tracer-free dilution series were prepared and incubated in parallel to the series that received radiolabeled tracer. From these series, DNA was extracted for further population studies (2 ml aliquots were removed after 30 days and preserved as described above).

Filter incubations

Filters that collected planktonic biomass from 1 m and 14 m water depth were incubated at 50°C in autoclaved sediment medium (described above) that was amended with organic substrates and 400 kBq ^{35}S-labeled sulfate tracer in 10 ml Hungate tubes. Five glass fiber filter incubations were unpasteurized and two were pasteurized at 85°C for 30 min immediately before incubation for both 1 m and 14 m water depth. The 0.45 and 0.2 µm filters were incubated without pasteurization. In addition, 24 negative controls were incubated: 5 contained pristine GF filters and 19 contained no filter but only medium. After 30 days of incubation, the samples were processed as described above for T-MPN.

RNA extraction and RT-PCR

RNA was extracted using the Power Soil Total RNA Isolation Kit (Mo Bio Laboratories). Yield and purity of extracts were evaluated using a NanoDrop spectrophotometer (Thermo Scientific). RNA extracts were treated with 2 units μl^{-1} Turbo DNAse (Ambion/Applied Biosystems) for 30 min at 37°C, and purified with RNeasy Mini Kit (Qiagen). RT-PCR was performed using the OneStep RT-PCR Kit (Qiagen). 50 µl reactions included 2 µl bovine serum albumin (BSA, 10 µg/µl), 3 µl of each of the primers 8F and 1492R (10 pmol/µl; Loy et al., 2002) and 10 µl of extracted RNA template (between 4 and 14 ng/µl). Reverse transcription at 50°C for 30 min was immediately followed by 20 PCR cycles of 94°C for 40 s, 52°C for 40 s and 72°C for 1.5 min, and final elongation at 72°C for 10 min. As controls for the efficiency of DNase treatment, parallel PCR reactions using non-RT-PCR-treated purified RNA extracts as template were set using the HotStarTaq Master Mix Kit (Qiagen) and did not yield any product. All PCR products were purified using GenElute PCR Clean-Up Kit (Sigma-Aldrich), and used as template for *Desulfotomaculum*-specific 16S rRNA gene PCR with primers DEM116F and DEM1164R as described by Stubner and Meuser (2000). PCR mixtures of 50 µl contained 2 µl BSA (10 µg/µl), 25 µl of 2x Taq Master Mix RED (Ampliqon), 1 µl of each primer (forward and reverse primers at a concentration of 10 µM each) and 2 µl of template cDNA. Thermal cycling was performed as described by Stubner and Meuser (2000), with annealing at 63°C and 30 cycles.

DNA extraction and PCR

DNA was extracted as described previously (Kjeldsen et al., 2007) from ca. 0.3 g sediment samples before incubation (0 h) and after incubation at 50°C (120 h). For DNA extracts obtained after incubation at 50°C, amplification of *Desulfotomaculum* spp. 16S rRNA genes with the DEM116F-DEM1164R primer pair (Stubner and Meuser, 2000) was performed as described above (with 35 cycles). PCR products were purified with GenElute Gel Extraction Kit (Sigma-Aldrich). For the DNA extracted from sediment prior to incubation at 50°C, nested PCR was performed and consisted of 20 cycles with the universal bacterial primers 8F and

1492R, followed by 35 cycles with the DEM primer pair. Products of each step were purified using GenElute PCR Clean-Up Kit (Sigma-Aldrich).

Further PCR analysis of sulfate-reducing *Desulfotomaculum* in sediment samples incubated at 50°C targeted approximately 1.9 kbp-large fragments of *dsrAB*, which encodes the two major subunits of the dissimilatory bisulfite reductase. PCR reactions were performed as described previously (Lund et al., 2010), using primer variant mixtures DSR1Fabc and DSR4Rabcd (Loy et al., 2004) for 40 cycles with an annealing temperature of 52°C. PCR amplicons of the expected size were purified from 1% (w/v) agarose gels (GenElute Gel Extraction Kit, Sigma-Aldrich) prior to cloning.

Cloning and sequence analyses

PCR amplicons were cloned using the pGEM-T Vector System II cloning kit (Promega Biotech), according to the manufacturer's instructions. Plasmids were extracted using GenElute Plasmid Miniprep Kit (Sigma-Aldrich) and inserts were sequenced (Macrogen, Seoul, Korea) using the vector-specific primers M13F and M13R (Messing, 1983). A set of internal sequencing primers (data not shown) was designed to obtain full-length sequences of the retrieved *dsrAB* amplicons. The 16S rRNA sequences were aligned using the SILVA webaligner (Pruesse et al., 2007) and added to the SILVA SSURef version 102 small subunit ribosomal RNA database (Pruesse et al, 2007). The *dsrAB* sequences were added to a *dsrAB* sequence ARB database and aligned according to their inferred amino acid sequences. Phylogenetic sequence analyses used the ARB software package (Ludwig et al., 2004) and bootstrap analysis was done using PHYLIP v3.67 (distributed by J. Felsenstein, Department of Genome Sciences, University of Washington, Seattle, WA, USA). Aligned nucleotide sequence datasets were clustered into operational taxonomic units (OTUs) based on uncorrected distances and the furthest neighbor principle using Mothur (Schloss et al., 2009).

Denaturing gradient gel electrophoresis (DGGE)

For DGGE analyses of *Desulfotomaculum*-derived 16S rRNA gene fragments, RT-PCR (from temperature gradient incubations) or PCR (from tracer MPN) were conducted with primers DEM116F and DEM1164R for 30 cycles. Products were purified and used as template for a second PCR reaction with primers 341F (including a 40 nt 5'-terminal GC-clamp, Muyzer et al., 1993) and 907R (Lane, 1991). Thermocycling consisted of initial denaturation at 94°C for 1 min, 20 cycles of 94°C for 40 sec, 57°C for 40 sec and 72°C for 1 min, and a final elongation step at 72°C for 10 min.

DGGE was performed on 8% acrylamide gels with a 20-80% urea and formamide denaturing gradient (100% denaturant was 7 M urea and 40% (v/v) deionized formamide). Gels were run in 60°C TAE buffer for ca. 15 h at 100 V, then stained with SybrGold (Invitrogen) for visualization under UV light (Gel Doc XR, Bio-Rad).

For the samples derived from T-MPN incubations, visible bands were excised for PCR product re-amplification (without the GC-clamp on primer 341F). Products were purified using GenElute PCR Clean-Up Kit (Sigma-Aldrich) and sequenced using primer 907R. For the samples from the temperature gradient incubations, non-metric multidimensional scaling (NMDS) analysis was performed using metaMDS of the vegan package (Oksanen et al., 2010) on the presence/absence datasets of DGGE bands that occurred in more than one sample. Jaccard distance measures were used. The variables of temperature and incubation time were fitted as z-scores.

Results

Identification of distinct communities of endospore-forming SRB at different temperatures

Germination of thermophilic SRB endospores was induced by incubating pasteurized Aarhus Bay sediment between 28 and 85°C as slurries that were either amended with organic substrates or left unamended (Figure 1A and B, respectively). Under either condition, two temperature optima for sulfate reduction emerged during a five-day incubation period. Broad temperature-activity distributions with optima between 45 and 60°C were apparent in both conditions after 24 and 72 h. After 120 h of incubation, a second optimum at ca. 70°C was observed. 70°C was also the highest temperature at which sulfate reduction could be measured. Sulfate reduction was detected at 28°C, the lowest temperature tested, but only after 72 and 120 hours. The amount of sulfate reduced at temperatures below 40°C was smaller than at higher temperatures. Organic substrate amendment promoted 5 to 10-fold higher sulfate reduction (Figure 1A and B).

The broad temperature distribution of sulfate reduction, from 28 to 70°C, and the occurrence of two optima after 120 h of incubation indicate that distinct populations of endospore-forming SRB were induced at different temperatures. This hypothesis was supported by cDNA-based analysis of the *Desulfotomaculum* community by sequence analysis of 16S rRNA extracted from unamended sediment that was incubated at 32, 46, 57, 61, 63 and 69°C (Figures 1B, C and D). Non-metric multidimensional scaling analysis of shared DGGE bands indicated that temperature was a significant parameter explaining the difference in community ($p=0.001$). Samples obtained after 72 and 120 h of incubation were more similar to each other than to those collected after 24 h (Figure 1D), suggesting a community shift as a function of incubation time. However, the effect of incubation time was not statistically significant ($p = 0.340$).

Based on initial DGGE results (Figure 1C and D), cDNA clone libraries were constructed from samples incubated at 46, 61 and 69°C to identify active *Desulfotomaculum* populations. In order to focus on the effect of temperature, sequences that were retrieved from samples collected after 24 and 120 h of incubation were pooled prior to analysis. The largest diversity, of 11 *Desulfotomaculum* phylotypes, was observed from sediment samples that were incubated at 46°C. At 61 and 69°C, five and one phylotype were

retrieved, respectively (Figure 2 and Table 1). None of the 17 phylotypes occurred in more than one clone library, indicating that different temperatures induced different populations of the overall community of thermophilic SRB endospores.

To assess the effect of organic substrates on the structure of enriched thermophilic *Desulfotomaculum* communities, DNA clone libraries of 16S rRNA genes were constructed from substrate-amended and unamended sediments following 50°C incubation for 120 h. From the unamended incubation, 31 sequences were obtained that clustered into 7 phylotypes, while from the amended incubation 32 sequences were obtained that clustered into 4 phylotypes, of which two (G and R) were also present in the unamended incubation (Figure 2 and Table 1). This suggests that amendment with the chosen substrates promoted higher sulfate reduction rates by stimulating the growth of a limited subpopulation among the thermophilic SRB that are present in Aarhus Bay sediment, rather than by evenly stimulating several SRB populations that would otherwise remain undetected.

To investigate whether the results could be explained by germination of dormant *Desulfotomaculum* endospores, a *Desulfotomaculum*-specific 16S rRNA gene library was constructed from pristine sediment that was not incubated at high temperature. Only one out of 36 cloned sequences from this pre-incubation library clustered with sequences from heated sediment (phylotype D, Figure 2). Detecting this sequence in the pre-incubation library is most likely due to DNA extraction from endospores of this taxon present in pristine sediment and due to the increased sensitivity of the nested PCR approach, as there have been no indications of vegetative cells of thermophiles in pristine Aarhus Bay sediments (Thomsen et al., 2001; Leloup et al., 2009; Webster et al., 2011). The fact that phylotype D increased in relative abundance in clone libraries from 3% to 52% when the sediment was incubated at 50°C (unamended; Figure 2, Table 1) suggests that a massive enrichment of this particular organism occurs at high temperature. The lack of any other putative thermophilic endospore-forming *Desulfotomaculum* spp. in the pre-incubation analysis supports the assumption that these SRB are present as endospores and that incubation at temperatures above *in situ* was necessary to assess the phylogenetic diversity of these thermophiles in cold sediments.

Sequence identity between thermophilic Desulfotomaculum *present in both Aarhus Bay and Svalbard sediments*

Three of the thermophilic *Desulfotomaculum* phylotypes present in Aarhus Bay (D, F and G) were previously discovered in an arctic fjord sediment of northwest Svalbard (Figure 2 and Table 1; Hubert et al, 2010). 16S rRNA sequences of these phylotypes derived from Aarhus Bay and Svalbard sediments share 99 to 100% sequence identity. This relationship is further supported by *dsrAB* sequence comparisons. Our clone libraries based on the general *dsrAB* primer pair DSR1Fabc-DSR4Rabcd indicates that this primer pair fails to target phylotype F members affiliated with the *dsrAB* lineage of *Desulfotomaculum halophilum* and *Desulfotomaculum alkaliphilum* (Figure S1). Therefore a specific *dsrAB* primer pair was designed for targeting members of the latter lineage. For phylotype F, cloned *dsrAB* sequences derived from Aarhus Bay were 99.7% similar to a cloned sequence from Svalbard sediments, with 5 mismatches over 1,654 nucleotides (S4-3-A85; Figure S1; C. Hubert and A. Loy, unpublished). For phylotype G, the *dsrAB* sequence of a thermophilic *Desulfotomaculum* isolated from heated Svalbard sediment (strain Eth-2, Figure S1) and a cloned sequence derived from Aarhus Bay heated sediment (A05, Figure S1) have only one mismatch over 1,937 nucleotides (i.e. 99.9% sequence identity). This single mismatch could be due to random sequencing errors introduced by Taq polymerase. Due to the lack of close cultured relatives, phylotype D members currently remain unidentified at the *dsrAB* level (Figure 2).

Thermophilic SRB endospores in deep sediments of Aarhus Bay

The distribution of thermophilic SRB in deep sediments was quantitatively investigated by T-MPN (Vester and Ingvorsen, 1998). MPN-estimated cell density decreased exponentially from $4\text{-}9\times10^3$ thermophilic SRB endospores per cm^3 of sediment at the surface to 0.9 at 650 cm depth (Figure 3A). The dominant thermophilic SRB in the uppermost and deepest sediments were identified via *Desulfotomaculum*-targeted 16S rRNA gene DGGE using DNA extracted from positive MPN dilutions (Figure 3B). Prominent bands were excised and sequenced, and results are included in Table 1. Only phylotypes G and R were detected in

undiluted surface sediment after 30 days of incubation at 50°C, whereas in the 10^{-3} MPN dilution of surface sediment only phylotype F was detected, indicating its relative abundance *in situ*. Furthermore, only phylotype F was detected following incubation of undiluted sediment (the highest positive MPN dilution) from 650 cm depth (Figure 3B). The 16S rRNA DGGE results were supported by *dsrAB* clone library compositions. Using the group-specific *dsrAB* primer pairs described above, phylotype G was not detected at 650 cm depth, whereas phylotype F was detected in both the deepest and the uppermost sediment (Figure S1). The *dsrAB* cloned sequences from 650 cm sediment depth were 99.7% identical (1,650 out of 1,655 nucleotides) to one of the clusters of phylotype F detected at the surface sediment (Figure S1).

Thermophilic SRB endospores in the water column of Aarhus Bay

Thermophilic SRB were detected at 1 m and 14 m water depth, in both pasteurized and unpasteurized incubations that were inoculated with biomass concentrated by filtration of water from these depths. For 1 m water depth thermophilic sulfate reduction was observed in three out of seven replicates, while at 14 m water depth sulfate reduction was measured for all replicates (Figure 4). The detection of higher sulfate reduction on filters from 14 m may indicate higher abundance of endospores in deeper water; a larger initial community of endospores likely corresponds with a greater diversity of endospores, hence the ability to use a wider variety of substrates and increase the sulfate reduction (Figure 4).

Discussion

Long-distance dispersal of thermophilic endospores

This study shows that the community of dormant thermophilic SRB in Aarhus Bay is much more diverse than previously recognized (Isaksen et al, 1994). In total 23 phylotypes were identified in incubation experiments conducted under different conditions with respect to temperature and organic substrate availability. 19 of these 23 phylotypes have less than 97% 16S rRNA sequence identity to *Desulfotomaculum* type strains and thus probably represent novel species within this genus (Table 1). Given that thermophiles cannot, and presumably could not grow in cold Aarhus Bay sediments, this large diversity must be due to passive influx of SRB endospores from external sources rather than growth and diversification of SRB thermophiles at this location. Of particular interest are phylotypes D, F and G, which were also discovered in the arctic sediment of Smeerenburgfjorden, Svalbard (Table 1; Hubert et al, 2009; 2010). Classification of phylotypes based on a 16S rRNA sequence identity threshold of 99% cannot exclude that sequences from different species are regarded as the same phylotype (Tourova et al., 2001; Stackebrandt and Ebers, 2006). However, the presence of endospores with identical 16S rRNA sequences and, in particular, identical *dsrAB* sequences in Svalbard and Aarhus Bay sediments indicates that the same thermophilic *Desulfotomaculum* species are found in these two geographically distant locations. This supports the hypothesis that these bacteria originate from a common warm, anoxic source environment. Such an environment may be theoretically represented by a single-source habitat with a strong efflux that has resulted in an yearly deposition of ca. 10^8 thermophilic SRB per m^2 in the surface sediments of Svalbard and ca. 10^7 m^{-2} in Aarhus Bay for the last hundred years (Hubert et al, 2009; Figure 3A), despite more than 3,000 km separating these two locations.

While a common source may supply thermophiles to distant locations, the detection of several thermophilic SRB following incubation of Aarhus Bay sediment at different temperatures suggests that multiple source habitats could contribute to the supply of thermophilic endospores to Aarhus Bay. The diversity of thermophilic *Desulfotomaculum* spp. reported here may arise from a combination of different

source environments in distinct geographic locations. Alternatively, different source environments may be at the same geographic location but associated with a habitat or habitats where temperature varies between at least 40 and 70°C. These temperature gradients are common in geothermal aquifers, e.g. the Great Artesian Basin, Australia, from where *Desulfotomaculum australicum* was isolated (Love et al., 1993). *D. australicum* is the closest cultivated relative of phylotype AB, the only phylotype identified in our incubations at 69°C (99% sequence identity, Table 1). Similarly, advection of geofluids (e.g. hydrocarbons, mud volcanoes) from warm deep sediments could provide a transport pathway from warm depths up through the seabed to cold surface environments (Judd and Hovland, 2007; Hubert and Judd, 2010). Alternatively, hydrothermal circulation with diffuse fluid venting, in particular at the Lost City hydrothermal field off the Mid Atlantic Ridge, is also subjected to such temperature gradients; these environments provide conditions to host abundant thermophilic microbial communities (Früh-Green et al., 2003). A large diversity of endospore-forming *Firmicutes*, including *Desulfotomaculum*-related 16S rRNA sequences, have been found in carbonate chimneys at this site (Brazelton et al., 2006) and are closely related to phylotype F (Hubert et al, 2010).

Anthropogenic sources may also be relevant for explaining the occurrence of thermophilic SRB in Aarhus Bay sediment. Local coal-fired power plants and industrial wastewater treatment facilities around the city of Aarhus have established in recent decades, and were previously proposed as the source of thermophiles such as *Desulfotomaculum kuznetsovii* to Aarhus Bay sediment (Isaksen et al, 1994). Another possible source may be North Sea oil production systems that include different high temperature components and harbor diverse thermophilic SRB (e.g. Stetter et al., 1993; Beeder et al., 1994; Nilsen et al., 1996; Dahle et al., 2008). The Dan and Halfdan oil fields located ca. 200 km west of Denmark are characterized by temperatures from ca. 40 to 80°C and host a diverse microbiota (Gittel et al., 2009). *Desulfotomaculum* 16S rRNA sequences originating from these oil fields are the closest known relatives of phylotypes R and S (95%; Figure 2) which became highly enriched in substrate-amended 50°C incubations. Indeed, phylotypes R and S were detected in surface sediment but not in deeper layers (Figure 3B), which is suggestive of a recent, possibly anthropogenic source for these taxa. However, the detection of other thermophilic *Desulfotomaculum* spp. at 650 cm depth, which corresponds to ca. six thousand years of sedimentation

(Jensen and Bennike, 2009), highlights that the presence of thermophiles in this cold sediment is not exclusively due to human industry. Outflow from natural, non-ephemeral environments must contribute to the long-term influx of thermophilic endospores into Aarhus Bay sediment. Whatever the primary origin of these thermophiles is, the presence of thermophilic SRB in the water column of Aarhus Bay (Figure 4) confirms that they are being spread in this region by the water currents, and remain viable despite the cold temperatures and oxic surroundings. The detection of thermophilic SRB in coarse glass fiber filters and not in the subsequent filters of smaller pore size suggests that these thermophiles are transported not as single cells, but attached to larger particles or aggregates.

Long-term deposition and survival of thermophilic SRB endospores in Aarhus Bay sediments

The abundance of thermophilic SRB endospores as detected by our T-MPN method decreases exponentially from ca. 10^4 cells to 1 cell cm^{-3} over 650 cm of sediment. The depth profile shown in Figure 3A may be a result of an increase over time in the influx of endospores to Aarhus Bay sediments during the past 6,000 years. This could be due to an increased flux from a single source or a growing number of sources supplying thermophilic endospores to this location. Nevertheless, it is unlikely that an increased flux of thermophiles is exclusively responsible for the changes of four orders of magnitude in the observed abundance profile in Aarhus Bay. It is much more likely that endospore death on millennial time scales explains the decreasing concentrations.

The depth profile of thermophilic endospore abundance reflects a more rapid decline than is measured for total microbial numbers at this location, which drop by one order of magnitude over the same 650-cm depth interval (4×10^9 to 1×10^8 cells cm^{-3}, Mark Lever, personal communication). Rejuvenation of thermophilic endospore populations through germination, repair and sporulation cycles (Yung et al., 2007) is not expected at the *in situ* temperature of 0 to 15°C (Thamdrup et al., 1994), thus their death and decay-dependent decrease in abundance is most likely not counterbalanced by growth and multiplication. Therefore, unlike vegetative cells, the decrease of thermophilic endospores does not relate to bottom-up controlling factors such as electron donor and electron acceptor availability. Given that the T-MPN method

only counts viable cells that are induced to grow, the observed depth profile may be interpreted as a decrease in the overall viability of endospore populations over time, e.g. as a result of cellular damage (Johnson et al., 2007). Furthermore, populations of endospores may become reduced following stochastic germination events (Paidhungat and Setlow, 2000; Epstein, 2009).

From Figure 3A a population half-life of ca. 400 years can be calculated for thermophilic SRB endospores in Aarhus Bay sediment. Mukamolova and colleagues (2003) speculated that the survival half-life of a microbial cell under optimal conditions, in the absence of nutrient influx, turnover and regeneration, is between 10 and 100 years. The longer half-life calculated in our study may be explained by the higher resistance of endospores as compared to vegetative cells. Rothfuss and colleagues (1997) calculated death rates in a study of deep lake sediments dominated by endospore populations. Their data translates into population half-lives of 525 years for aerobic microorganisms and 575 years for anaerobic microorganisms – even longer than the half-life we have calculated for thermophilic endospores. The difference may be explained by the fact that the endospores studied by Rothfuss et al. (1997) are mesophiles and thus may cycle through reproduction and sporulation *in situ*. Consequently these may not be true half-life estimates. Their MPN method reached its lower detection limit at 400 to 600 cm depth, which corresponded to a sediment age of 8,900 years of age (Rothfuss et al, 1997). The deepest sediment retrieved in our sampling campaign was from 650 cm depth (ca. 6,000 years; Jensen and Bennike, 2009), yet there is no reason to expect that this is the oldest sediment layer in Aarhus Bay where viable thermophilic endospores persist, neither from the perspective of when their deposition started, nor from the perspective of how long these cells might survive. However, the possible disappearance of thermophiles in older sediments could be due to the their relatively low abundance at the time of deposition (Lewis et al., 2008). Alternative methods with improved sensitivity or larger sample volumes should be considered for analyses of deeper sediments.

Conclusion

Thermophilic endospores in cold sediments have been proposed as models for investigating the dispersal of marine microorganisms, as demonstrated for sulfate-reducing *Desulfotomaculum* spp. supplied to Aarhus

Bay. The presence of phylotype F throughout a 650-cm sediment core indicates that this thermophilic endospore population has been deposited in Aarhus Bay sediments for thousands of years. The presence of the same phylotype, along with phylotypes D and G, in surface sediments of both Aarhus Bay and a Svalbard fjord suggests that these results are explained by a passive dispersal over great distances and that these endospores may originate from a common source. The presence of approximately 10^4 thermophilic SRB endospores per cm^3 of surface sediment in both locations indicates a large flux of these cells, and their detection in the water column confirms that this passive dispersal is an ongoing process involving seawater circulation. The molecular and physiological diversity of thermophilic *Desulfotomaculum* spp. in Aarhus Bay points to the likelihood that multiple source environments must be implicated to explain the biogeography of these bacteria.

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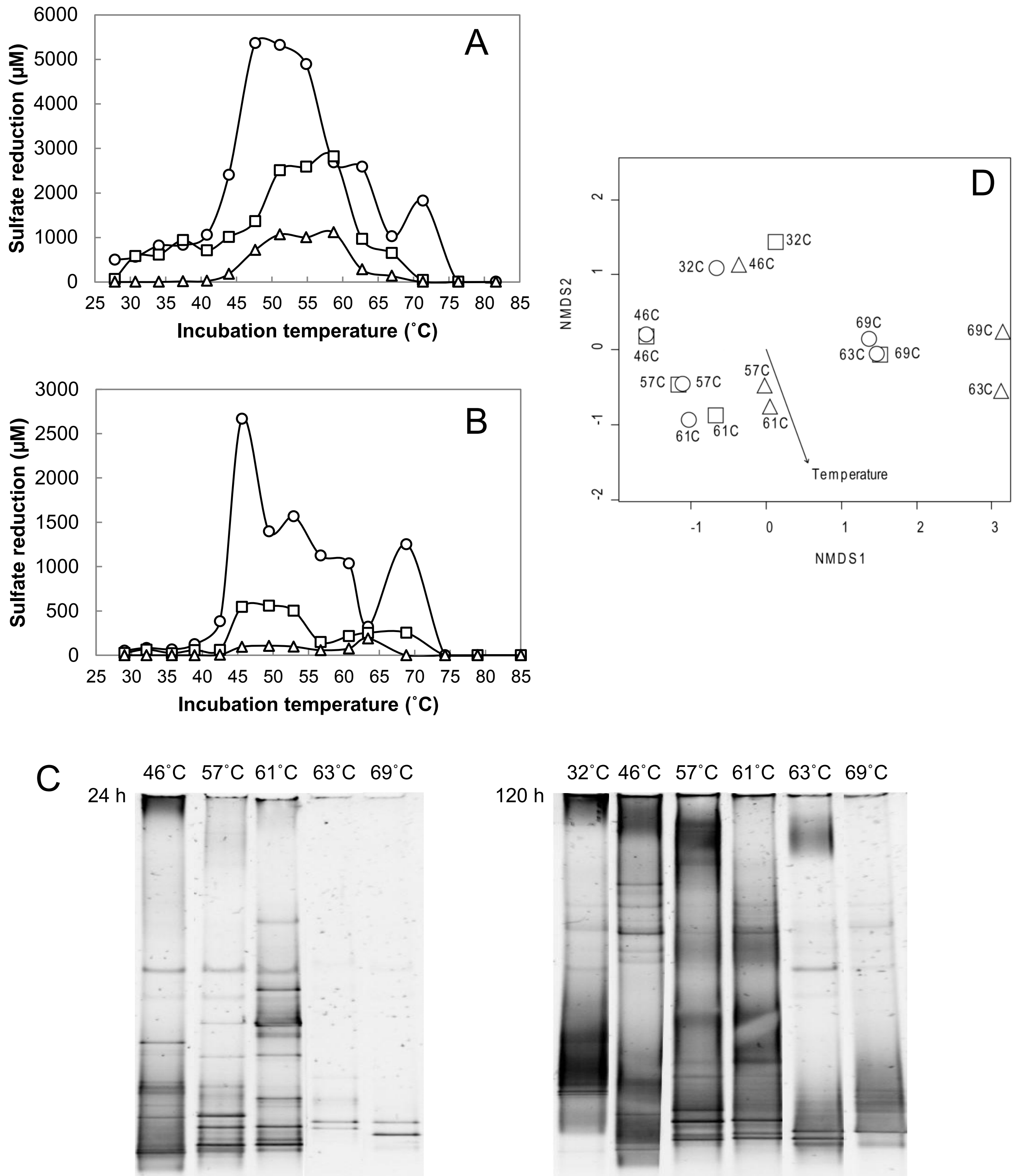
Figure 1. Replicate Aarhus Bay sediment slurries were amended with organic substrates (A) or left unamended (B) and were incubated between 28 and 85°C in a temperature gradient block for up to 120 h. Sulfate reduction (µM sulfate consumed) at each incubation temperature was measured after 24 h (triangles), 72 h (squares) and 120 h of incubation (circles). RNA was extracted from selected unamended samples (B) and DGGE targeting the 16S rRNA of *Desulfotomaculum* spp. was performed. DGGE images for samples from 24 h and 120 h are shown (C); samples from 72 h were similar to 120 h. Non-metric multidimensional scaling analysis (D) was conducted to compare the DGGE patterns at each temperature and incubation time. Symbols in (D) correspond to the ones in (A) and (B).



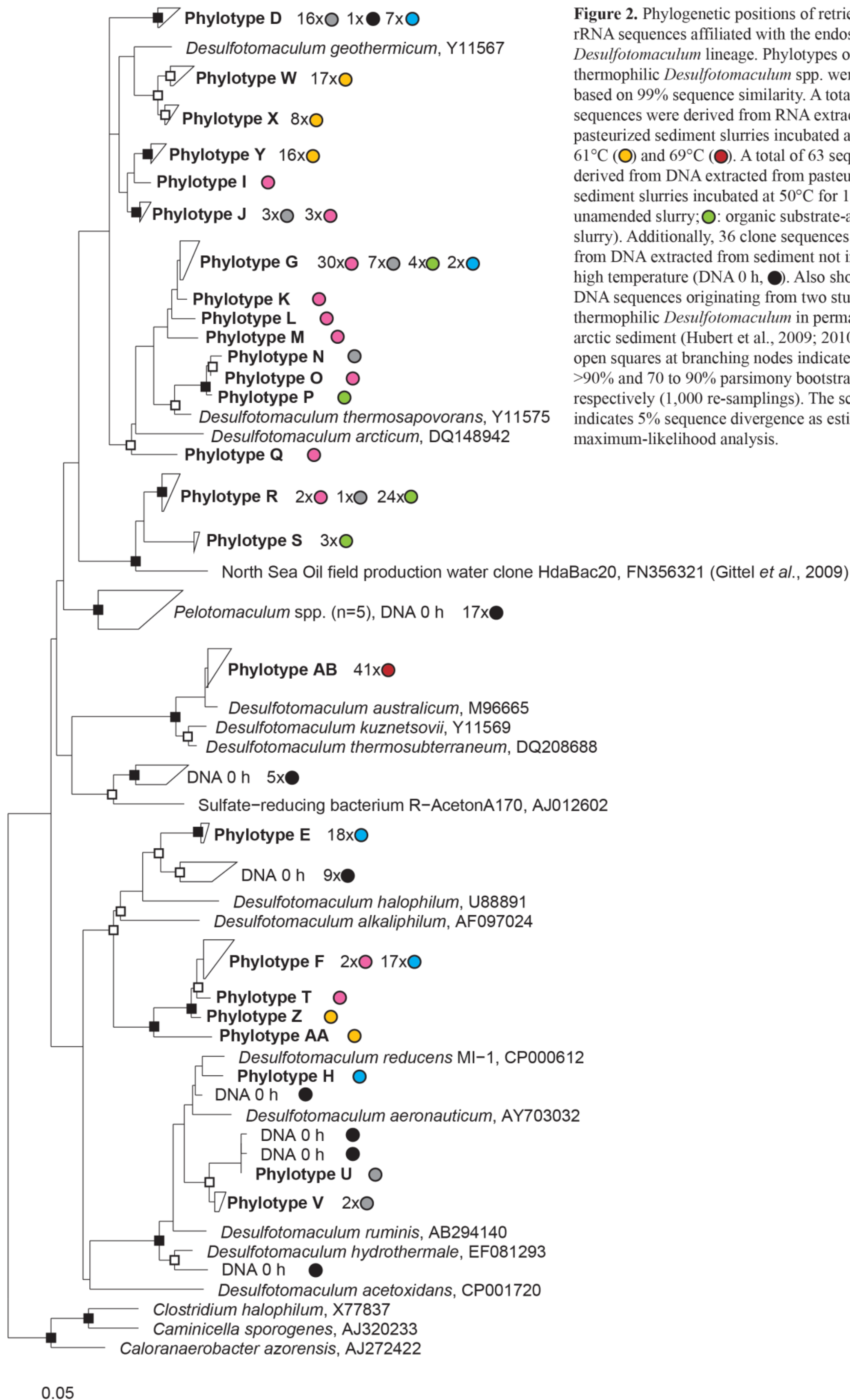


Figure 2. Phylogenetic positions of retrieved 16S rRNA sequences affiliated with the endospore-forming *Desulfotomaculum* lineage. Phylotypes of putative thermophilic *Desulfotomaculum* spp. were defined based on 99% sequence similarity. A total of 128 clone sequences were derived from RNA extracted from pasteurized sediment slurries incubated at 46°C (●), 61°C (●) and 69°C (●). A total of 63 sequences were derived from DNA extracted from pasteurized sediment slurries incubated at 50°C for 120 h (●: unamended slurry; ●: organic substrate-amended slurry). Additionally, 36 clone sequences were derived from DNA extracted from sediment not incubated at high temperature (DNA 0 h, ●). Also shown (●) are DNA sequences originating from two studies of thermophilic *Desulfotomaculum* in permanently cold arctic sediment (Hubert et al., 2009; 2010). Filled and open squares at branching nodes indicate lineages with >90% and 70 to 90% parsimony bootstrap support, respectively (1,000 re-samplings). The scale bar indicates 5% sequence divergence as estimated from maximum-likelihood analysis.

Figure 3. Depth distribution of thermophilic SRB endospores in Aarhus Bay sediment determined by tracer MPN (A). A linear regression of endospore abundance (N) as a function of depth (d in cm) resulted in the following equation: log N(d) = $-61.74\times10^{-4}\times d + 552.38$, with $R^2 = 0.9291$. The sedimentation rate is estimated as 0.108 cm y^{-1} (Jensen and Bennike, 2009). The MPN method of triplicate tenfold dilutions typically gives a 95% confidence limit of 1 order of magnitude (Rothfuss *et al.*, 1997). From the 35 non-inoculated controls, only 3 yielded TRIS% above the background threshold of 0.10 (maximum TRIS% = 0.47, data not shown). DGGE targeting the 16S rRNA of *Desulfotomaculum* spp. was performed (B) with DNA extracted from serial dilutions of sediment from 0-10 and 640-650 cm depth. Bands were excised, sequenced and identified as the phylotypes shown in the figure.

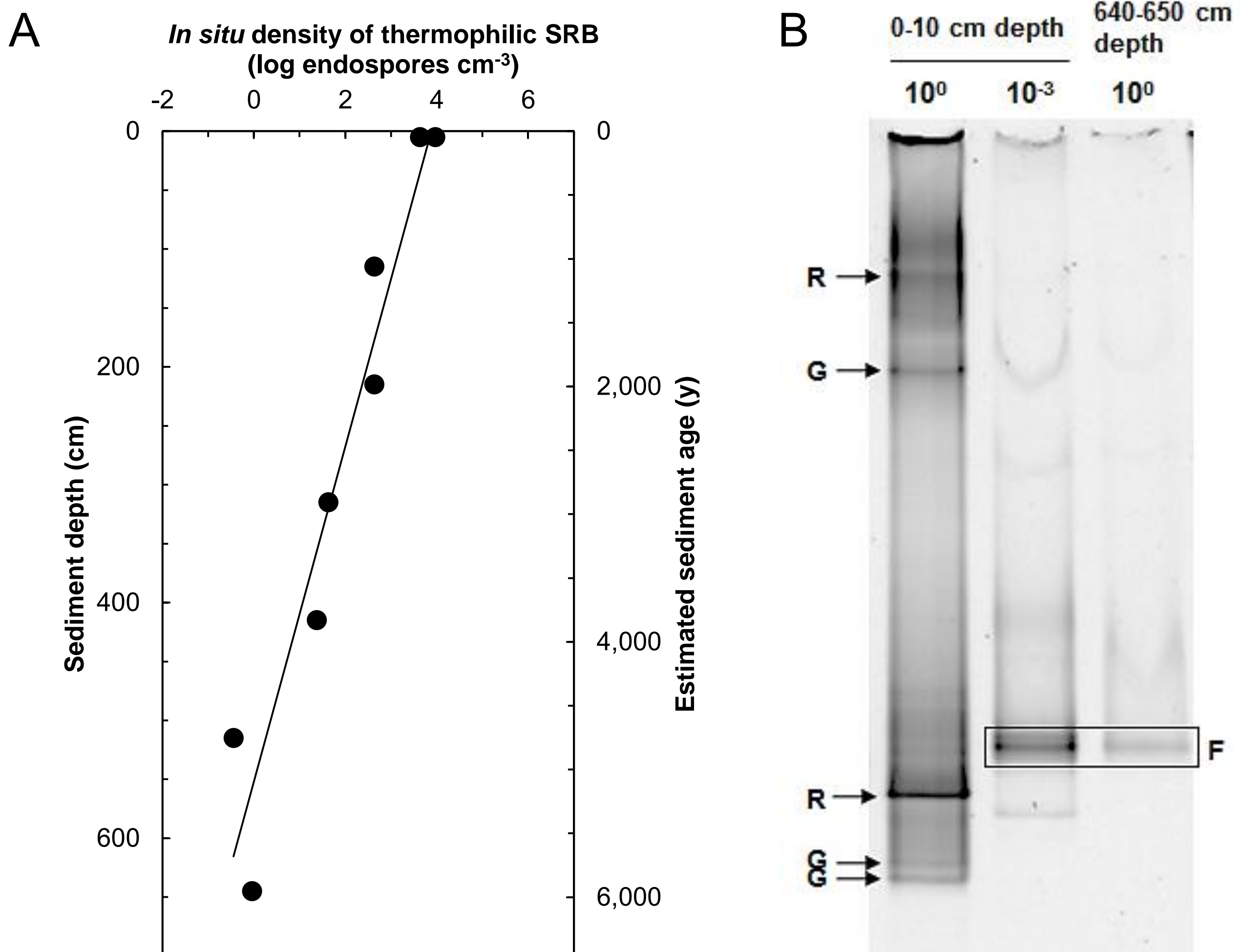


Figure 4. Seawater from 1 m and 14 m water depth in Aarhus Bay was sequentially filtered through glass fiber filters, 0.45 µm and 0.2 µm filters, which were then incubated in sterile sediment medium at 50°C for 30 days. Thermophilic sulfate reduction was detected in incubations of glass fiber filters from both water depths (TRIS% from 2 to 55), as shown in the figure. No sulfate reduction was detected in incubations of 0.45 and 0.2 µm filters (TRIS% = 0.00, data not shown). From the 24 negative control incubations, TRIS% was above background in 1 out of 5 unused glass fiber filters and 3 out of 19 non-inoculated controls (maximum TRIS% = 0.47, data not shown).

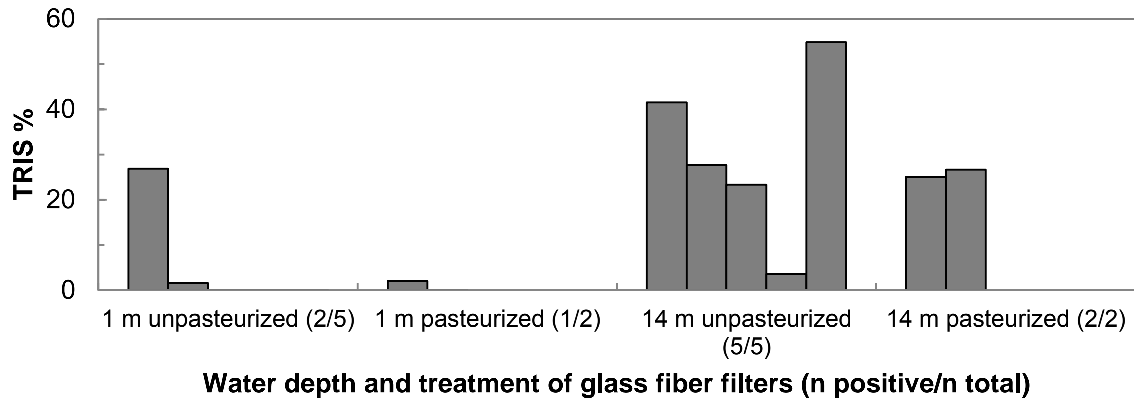


Table 1. *Desulfotomaculum* phylotypes detected in high temperature incubation experiments with sediments from Aarhus Bay and Svalbard. Unless indicated, Aarhus Bay sediment corresponds to 0-10 cm depth and was unamended. Phylogenetic relationships of cloned DNA and cDNA sequences are shown in Figure 2. DGGE bands refer to the analysis shown in Figure 3B.

Phylotype	Closest type strain (accession number), maximum % similarity	Detected in	Incubation temperature (°C)	Detected as	Relative abundance in library (number of clones/total number of clones in library)
D	*D. geothermicum* (Y11567), 96%	Svalbard (Hubert *et al.*, 2009)	50	DNA clone	8% (7/85)*
		Aarhus Bay	50	DNA clone	52% (16/31)
E	*D. halophilum* (U88891), 93%	Svalbard (Hubert *et al.*, 2010)	50	DNA clone	47% (18/38)
F	*D. halophilum* (U88891), 93%	Svalbard (Hubert *et al.*, 2010)	50	DNA clone	45% (17/38)
		Aarhus Bay	46	cDNA clone	5% (2/44)
		Aarhus Bay amended (10^{-3} dilution)	50	DGGE band	-
		Aarhus Bay 650 cm depth amended	50	DGGE band	-
G	*D. thermosapovorans* (Y11575), 97%	Svalbard (Hubert *et al.*, 2010)	50	DNA clone	5% (2/38)
		Aarhus Bay	46	cDNA clone	68% (30/44)
		Aarhus Bay	50	DNA clone	23% (7/31)
		Aarhus Bay amended	50	DNA clone	12% (4/32)
		Aarhus Bay amended	50	DGGE band	-
H	*D. aeronauticum* (AY703032), 96%	Svalbard (Hubert *et al.*, 2010)	50	DNA clone	3% (1/38)
I	*D. geothermicum* (Y11567), 95%	Aarhus Bay	46	cDNA clone	2% (1/44)
J	*D. geothermicum* (Y11567), 94%; *D. thermosapovorans* (Y11575), 94%	Aarhus Bay	46	cDNA clone	7% (3/44)
		Aarhus Bay	50	DNA clone	10% (3/31)
K	*D. thermosapovorans* (Y11575), 96%	Aarhus Bay	46	cDNA clone	2% (1/44)
L	*D. thermosapovorans* (Y11575), 96%	Aarhus Bay	46	cDNA clone	2% (1/44)
M	*D. thermosapovorans* (Y11575), 96%	Aarhus Bay	46	cDNA clone	2% (1/44)
N	*D. thermosapovorans* (Y11575), 95%	Aarhus Bay	50	DNA clone	3% (1/31)
O	*D. thermosapovorans* (Y11575), 97%	Aarhus Bay	46	cDNA clone	2% (1/44)
P	*D. thermosapovorans* (Y11575), 98%	Aarhus Bay amended	50	DNA clone	3% (1/32)
Q	*D. arcticum* (DQ148942), 95%	Aarhus Bay	46	cDNA clone	2% (1/44)
R	*D. geothermicum* (Y11567), 92%; *D. kuznetsovii* (Y11569), 92%	Aarhus Bay	46	cDNA clone	5% (2/44)
		Aarhus Bay	50	DNA clone	3% (1/31)
		Aarhus Bay amended	50	DNA clone	75% (24/32)
		Aarhus Bay amended	50	DGGE band	-
S	*D. kuznetsovii* (Y11569), 91 %	Aarhus Bay amended	50	DNA clone	9% (3/32)
T	*D. alkaliphilum* (AF097024), 93%	Aarhus Bay	46	cDNA clone	2% (1/44)

Table 1. (cont.)

Phylotype	Closest type strain (accession number), maximum % similarity	Detected in	Incubation temperature (°C)	Detected as	Relative abundance in library (number of clones/total number of clones in library)
U	*D. aeronauticum* (AY703032), 96%	Aarhus Bay	50	DNA clone	3% (1/31)
V	*D. reducens* MI-1 (U95951), 96%	Aarhus Bay	50	DNA clone	6% (2/31)
W	*D. geothermicum* (Y11567), 95%	Aarhus Bay	61	cDNA clone	40% (17/43)
X	*D. geothermicum* (Y11567), 95%	Aarhus Bay	61	cDNA clone	19% (8/43)
Y	*D. thermosapovorans* (Y11575), 95%	Aarhus Bay	61	cDNA clone	37% (16/43)
Z	*D. halophilum* (U88891), 90%	Aarhus Bay	61	cDNA clone	2% (1/43)
AA	*D. halophilum* (U88891), 92%	Aarhus Bay	61	cDNA clone	2% (1/43)
AB	*D. australicum* (M96665), 99%	Aarhus Bay	69	cDNA clone	100% (41/41)

* clone library with primers 8F and 1492R. All other libraries were done with *Desulfotomaculum*-targeting primers DEM 116F and 1164R.

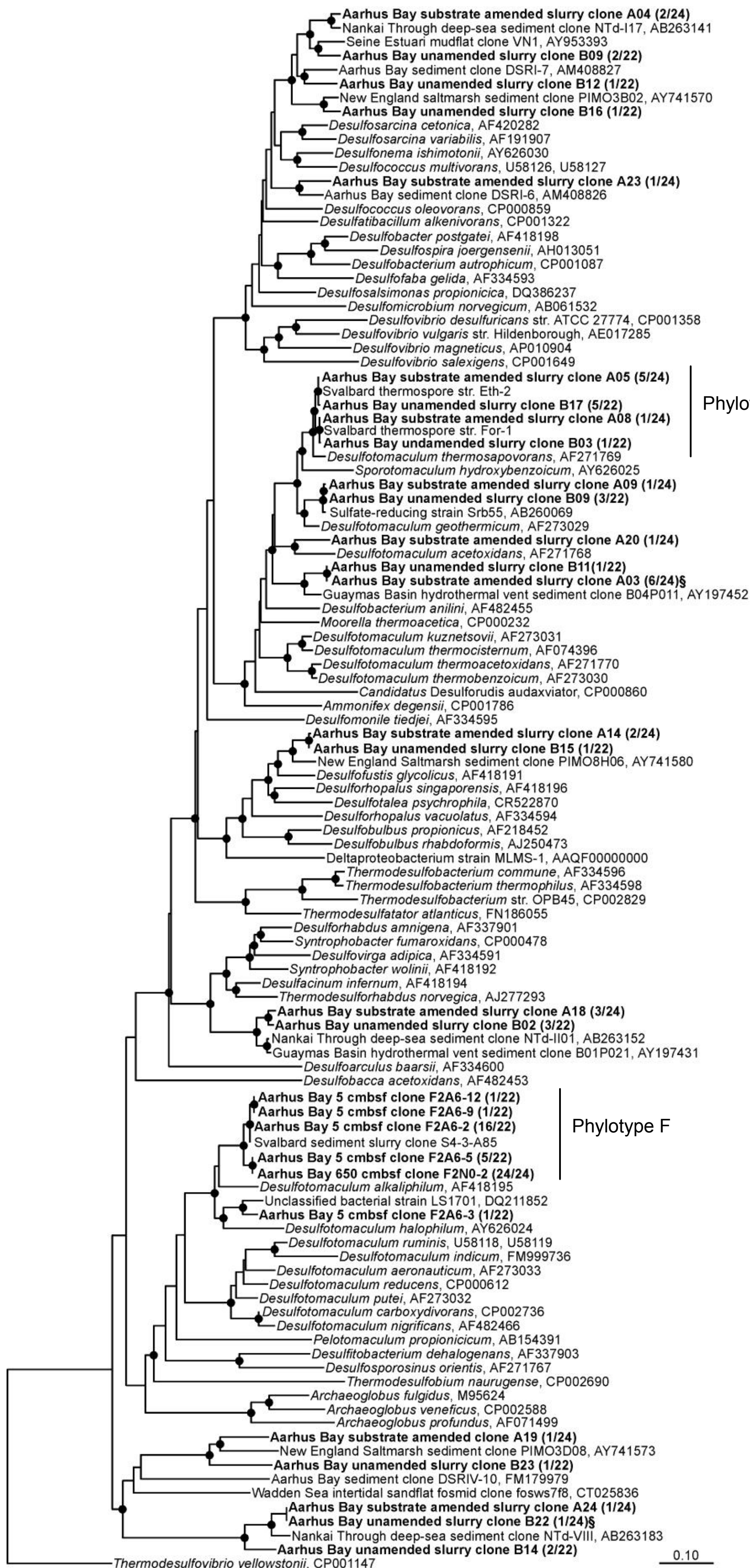


Figure S1. Tree showing the phylogenetic affiliation of representative *dsrAB* clones retrieved from slurries of pasteurized sediment (0-10 cm depth) incubated at 50°C for 120 h with and without organic growth substrate amendment, and *dsrAB* clones retrieved from 5 and 650 cm below the seafloor (cmbsf) with a primer pair designed to target *dsrAB* sequences affiliated with *Desulfotomaculum alkaliphilum* and *D. halophilum*. Numbers in parentheses show the relative abundance of the respective clones within a given clone library. The tree was inferred from FITCH distance matrix analysis of 532 aligned DsrAB amino acid sequence positions with JTT distance correction, global rearrangements, randomized input order of sequences and one category of substitution rates. Closed circles indicate nodes receiving >70% neighbor joining analysis-based bootstrap support (JTT distance correction, 500 re-samplings). The scale bar represents 10% estimated sequence divergence. §Short sequence manually drawn in to the tree (the sequence shares 100% identity with its closest relative(s) in the tree).

Chapter 3

Manuscript II

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A simple model to estimate the abundance of viable endospores of thermophilic sulfate-reducing bacteria

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Abstract

Thermophilic endospore-forming sulfate-reducing bacteria are present in various environments, both as active cells or dormant endospores. Few studies have focused on quantifying their abundance as endospores, and one of the reasons is the lack of adequate methodologies. Here we present a simple model for estimating the abundance of viable endospores of thermophilic sulfate-reducing bacteria (tSRB) in environmental samples based on the measurement of sulfate reduction rates (SRR) and estimation of cell-specific metabolic rates from average growth yield and cell size. We use this model to quantify relatively low abundance populations of tSRB endospores in marine sediment. Resulting numbers are comparable to tracer-enhanced most probable number enumerations, with the advantage that the SRR-based model can specifically quantify multiple communities of tSRB that grow in succession during short (120 h) incubations.

Introduction

Thermophilic endospore-forming sulfate-reducing bacteria have been isolated from several high temperature environments, including hot springs (Liu et al., 1997; Haouari et al., 2008), hot solfataric sediments (Goorissen et al., 2003), deep geothermal groundwater mines (Daumas et al., 1988; Love et al., 1993; Liu et al., 1997; Kaksonen et al., 2006; Chivian et al., 2008), oil and gas fields (Nilsen et al., 1996; Tardy-Jacquenod et al., 1998; Nazina et al., 2005). The ability to form endospores is a particular feature of this sub-group of tSRB and facilitates wide dispersal, leading to the isolation of thermophilic *Desulfotomaculum* strains from low temperature environments such as temperate and arctic marine sediments (Isaksen et al., 1994; Vandieken et al., 2006). Endospores of thermophilic sulfate-reducing bacteria (tSRB) are continually added to marine environments that do not support their germination and growth (Hubert et al., 2009). Quantifying the abundance of tSRB endospores in these environments sheds light into the dispersal rate of microorganisms in the ocean, the potential sources of tSRB endospores to cold marine sediments, and their survival rate during sedimentation (De Rezende et al., in prep.). Furthermore, quantifying endospores of SRB may also be of major importance for industrial systems, in particular oil production facilities, where large

investments are done aiming to eradicate sulfate-reducing populations due to their role in reservoir souring (Voordouw et al., 1996; Gittel et al., 2009; Hubert, 2010).

Few studies have focused on the quantification of tSRB endospores, especially in natural environments, owing in part to the paucity of methods for quantifying metabolically specific populations of endospores in complex matrices such as sediment and soils. The quantification of tSRB endospores has previously been achieved by most-probable-number enumerations (MPN). This method provides reliable minimum estimates of tSRB population sizes but often requires long incubation times of weeks to months (Isaksen et al., 1994; De Rezende et al., in prep.). Quantitative polymerase chain reaction (qPCR) and fluorescence *in situ* hybridization (FISH) are commonly used molecular methods for the quantification of specific groups of SRB, including vegetative cells of sulfate-reducing *Desulfotomaculum* spp. (Hristova et al., 2000; Stubner, 2002; Detmers et al., 2004), which is the largest phylogenetic group of endospore-forming SRB and includes considerable tSRB diversity (e.g. Liu et al., 1997; Stackebrandt et al., 1997; Pikuta et al., 2000; Haouari et al., 2008). However, the thick layers of endospore walls (Setlow, 2007) are resistant barriers to nucleic acid extraction (for qPCR) and prevent cell permeabilization to allow the penetration of oligonucleotide probes (for FISH). Due to these problems, which might result in low detection sensitivity and biases, these methods currently remain inappropriate for the quantification of environmental endospore populations. Recent advances have been made to improve nucleic acid extraction from bacterial endospores (Marentis et al., 2005; Warner et al., 2009; Geissler et al., 2011) and to permeabilize endospore walls for the application of FISH probes (Filion et al., 2009), but these technical improvements have yet to be tested beyond pure cultures of *Bacillus* species.

Other methods have been developed for enumerating endospores in environmental samples, based on dipicolinic acid (DPA) measurement. DPA is a specific component of bacterial endospores and appears to be involved in the maintenance of dormancy (Powell, 1953). In the presence of terbium chloride and UV excitation, terbium-DPA complexes can be detected by fluorescence (Hindle and Hall, 1999). Indirect fluorescence detection of DPA has been combined with high-performance liquid chromatography to quantify endospores in deep sediment cores of tidal flats in the North Sea, revealing an abundance of 10^5 to 10^7 endospores per gram of dry sediment. This represented up to 10% of total cell counts in deeper sediment

layers (Fichtel et al., 2008). However, since DPA seems to be universally present in bacterial endospores, the method cannot be directly applied for specific quantification of tSRB endospores. Another method based on the indirect fluorescence detection of DPA has been developed for genus-specific single-cell quantification of viable endospores. Micro-EVA (microscopy-based endospore viability assay) revealed 1 to 2 germinable *Clostridium* endospores per ml of Greenland ice cores and 66 to 157 per g of Atacama Desert soil. The method is based on the release of DPA from endospores stimulated to germinate by the addition of D-alanine, which triggers the germination of *Clostridium* endospores while inhibiting *Bacillus* endospore germination (Yang and Ponce, 2011). The knowledge of specific germinants is therefore essential to the use of micro-EVA. Little is known about the factors that induce the germination of tSRB endospores, and there may be no single chemical factor that stimulates the germination of only and all tSRB endospores.

In the present work, we demonstrate a simple method to estimate the abundance of tSRB endospores in a temperate marine sediment. We incubated surface sediment from Aarhus Bay, Denmark, at 50°C and monitored the exponential increases of sulfate reduction for five days. An average cell-specific sulfate reduction rate (csSRR) was calculated for different tSRB communities that developed during the incubation, allowing the initial abundance of each group of tSRB endospores to be estimated.

Materials and Methods

Sediment samples

Marine sediment was sampled in January 2009 from station M5 (56°06’20”N, 10°27’48”E, 28 m water depth) in Aarhus Bay, located on the transition between the Baltic Sea and the North Sea. Surface sediment (0-10 cm) was collected with a box corer (Hessler and Jumars, 1974) and stored at 4°C in gas-tight plastic bags (Hansen et al., 2000) for three months before treatment. The sediment temperature at this site regularly varies between 0 and 15°C throughout the year (Thamdrup et al., 1994).

Sediment slurry incubation with radiotracer at 50°C

Surface sediment slurries were prepared by homogenizing sediment with sterile anoxic synthetic seawater medium (pH 7.0) at a 1:2 (w/w) ratio under a constant flow of N_2 gas. The medium was prepared according to Widdel and Bak (1992) with the following changes in salt concentration (per liter): 15 g NaCl, 2.0 g $MgCl_2 \cdot 6H_2O$, 0.2 g $CaCl_2 \cdot 2H_2O$, 2.8 g Na_2SO_4 (i.e. 20 mM sulfate). The anoxic sediment slurry was divided into two separate master slurries of ca. 400 ml in 500 ml butyl rubber-stoppered serum bottles. One slurry was amended with formate, lactate, acetate, succinate, propionate, butyrate and ethanol (each to a final concentration of 1 mM), and the other slurry was left unamended.

Substrate-amended and unamended master slurries were pasteurized at 85°C for 30 min and immediately incubated at 50°C. During this incubation, slurries were sub-sampled regularly between 0 and 120 h of incubation. At each sub-sampling event, duplicate aliquots (3 ml) were transferred under N_2 into Hungate tubes (16×125 mm) containing 150 kBq carrier-free ^{35}S-sulfate, and incubated in parallel with the master slurry at 50°C for a further 1 to 1.5 h. Microbial activity was subsequently terminated and sulfide was preserved by injecting ca. 6 ml 20% (w/v) zinc acetate solution into the Hungate tubes and transferring to 50 ml centrifuge tubes which were immediately stored at -20°C. Sulfate reduction rates for each Hungate tube incubation were determined via single-step cold chromium distillation and liquid scintillation counting of the reduced ^{35}S-sulfur compounds and unreacted ^{35}S-sulfate tracer (Kallmeyer et al., 2004).

Results and Discussion

Estimation of initial sulfate reduction rates by fitting to an exponential function

Sulfate reduction in both organic substrate-amended and unamended sediment slurries was detected after 15 h of incubation at 50°C (Figures 1A and 1C, respectively). The initial lag phase represents the time necessary for germination of endospores and the time needed for the population to grow to a sufficiently high cell density that produces measurable amounts of reduced ^{35}S-sulfur compounds. The occurrence of two exponential phases of increasing SRR within the 120 h of incubation is due to the sequential exponential growth phases of distinct populations of thermophilic SRB associated with the consumption of different substrates, which we have observed previously (Hubert et al., 2010). The sulfate reduction rates (SRR) are proportional to the increasing population during the exponential growth phase. By fitting the measured SRR to an exponential function (Equation 1) the sulfate reduction rate at any time point in the experiment can be extrapolated, including the initial phase (before 15 h of incubation) when sulfate reduction was below detection limit:

$$SRR(t) = SRR_0 \times e^{kt} \qquad \text{Eq. 1}$$

SRR is the sulfate reduction rate (in nmol ml^{-1} hour^{-1}), t is time (hours), SRR_0 is the sulfate reduction rate at time zero and k is the growth constant. Different exponential phases were treated separately in both the amended and unamended incubation (see Figure 1). The doubling time (T_2) for SRR during an exponential phase also represents the doubling time of the responsible tSRB population, and can be calculated according to $T_2=\ln(2)\times k^{-1}$ (Table 1). For the initial analysis we assumed that sulfate reduction started at time zero, which implies that we assume that all cells germinated instantly at time zero. We will later consider the implications of this assumption.

Estimation of cell-specific sulfate reduction rates

The number of new SRB cells generated during an exponential phase can be calculated based on the amount of sulfate reduced during exponential growth, the growth yield ($Y_{sulfate}$, pg biomass generated per nmol reduced sulfate) and the amount of biomass per cell (bm_{cell}, pg cell^{-1}). The accumulated amount of sulfate reduced for a given time interval can be found by integrating Equation 1. Multiplying the total amount of sulfate reduced within a time interval by the growth yield gives the biomass production (pg ml^{-1}). Dividing the amount of biomass produced by the amount of biomass contained in a single tSRB cell (bm_{cell}) results in the number of cells produced per ml slurry during the time period in question (Equation 2):

$$amount\ of\ sulfate\ reduced = \int_0^t SRR_0 \times e^{kt} = SRR_0 \times \tfrac{1}{k} e^{kt}$$

$$biomass\ produced = SRR_0 \times \tfrac{1}{k} e^{kt} \times Y_{sulfate}$$

$$number\ of\ cells\ produced = SRR_0 \times \tfrac{1}{k} e^{kt} \times Y_{sulfate} \times {bm_{cell}}^{-1} \qquad \text{Eq. 2}$$

For our experiment, we assumed an average growth yield of 8230 pg biomass per nmol sulfate based on values reported for pure cultures of SRB (Table S1). The biomass per cell (bm_{cell}) is estimated by multiplying cell volume, wet density and dry weight. A cell volume of 1.0 µm^3 can be assumed as this is the median reported cell size among 60 SRB pure cultures, including all described endospore-forming species (Table S1). Assuming a cellular wet density of 1.0 pg µm^{-3} and that cellular dry weight is 30% of cell weight (Habicht et al., 2005) results in a bm_{cell} of 0.3 pg cell^{-1}. The assumptions of growth yield and biomass per cell are uncritical within the range of Table S1, as the variation of these parameters is smaller than the uncertainty derived from the extrapolation of SRR.

The cell-specific sulfate reduction rate (csSRR) of different tSRB populations can be calculated by dividing SRR (Equation 1) by the total number of cells at a given time point. The total number of vegetative cells present during the incubation corresponds to the number of cells produced by cell division (Equation 2)

plus the number of cells present originally. Considering a time point well into the exponential phase, the contribution of the original number of cells to the total becomes negligible. We can therefore calculate csSRR by dividing the sulfate reduction rate (Equation 1) by the number of cells generated during the experiment according to Equation 2. The results are presented in Table 1.

To quantify the confidence in the estimated parameters from each population we generated 10,000 pseudo datasets by drawing randomly with replacement from the original data (bootstrapping). We performed the entire procedure of fitting an exponential function, calculating cell numbers, csSRR and doubling times for each bootstrap sample. We then calculated 90% confidence limits of sulfate reduction rates, cell numbers, doubling times and csSRR as the 5% and 95% percentiles of the 10,000 estimates.

Estimation of the initial abundance of tSRB

Once csSRR is established, the total number of vegetative tSRB cells at any time point can be estimated by diving Equation 1 by csSRR. Results are shown in Figures 1B and 1D. At the beginning of the experiment, the total number of vegetative cells is approximately equal to the number of germinated tSRB endospores. It may be unlikely that all tSRB endospores germinate instantaneously. The duration of the germination process has been studied for mesophilic *Bacillus* and *Clostridium* species, and it varies among individual endospores within a population; however, most endospores stimulated to germinate in an experiment typically conclude the process in less than two hours (e.g. Zhang et al., 2010; Kong et al., 2011; Yang and Ponce, 2011). In the heated sediment experiments presented here, the endospores that germinated into the faster- and the slower-growing populations of tSRB that produce the two exponential phases in SRR must have germinated between the start of the incubation ($t_0 = 0$ h) and the earliest detection of SRR. Sulfate reduction was first measured at 15 h for the faster-growing populations (P1) and at 40 h for the slower-growing populations (P2), therefore these times were taken as maximum limits to provide a conservative estimate of the initial number of tSRB endospores (shaded areas in Figures 1B and 1D; Table 1). Uncertainty surrounding the precise germination time and the regression and extrapolation lead to the initial abundance of tSRB endospores being estimated within a range of two orders of magnitude for the more abundant,

slower-growing populations (P2) and three orders of magnitude for the less abundant, faster-growing populations (P1; Table 1). Improved constraints on the germination time for different populations may narrow these uncertainties to one order of magnitude.

Validation of the model

Estimating the initial number of tSRB by calculating csSRR provides the opportunity to compare the obtained values of csSRR against measured values from pure cultures of SRB. For all four populations considered here, the calculated csSRR (Table 1) are within the range reported for pure cultures of SRB, from 0.04 to 18.8 fmol cell^{-1} h^{-1}, with an average of 2.0 fmol cell^{-1} h^{-1} (Detmers et al., 2001). It has been suggested that csSRR of active SRB populations in sediments are likely 10 to 100-fold lower than for exponentially growing pure cultures (Sahm et al., 1999); however our sediment slurry experiments do not reflect conditions *in situ*, since the thermophilic populations develop from germination of endospores and grow exponentially upon incubation. Therefore they resemble more closely the conditions of experiments with pure cultures. This is also reflected by the calculated doubling times, which are similar to pure cultures of SRB and to other incubation experiments that stimulate the germination and growth of tSRB endospores (1.5 to 6.9 h; Hubert et al., 2010), further supporting the model.

Another validation of the estimated number of tSRB endospores is obtained by comparing the present results with the abundance estimated for the same Aarhus Bay sediment by tracer-enhanced most probable number enumerations (T-MPN). This was performed in similar incubations at 50°C, with the same type of medium and amendment of organic substrates (Figures 1A and 1B). T-MPN indicated between 4.3×10^3 and 9.3×10^3 tSRB endospores cm^{-3} sediment (De Rezende et al., in prep.), thus within the interval estimated for $P2_A$, the dominating population in these conditions.

Furthermore, we have previously estimated the abundance of tSRB endospores in arctic sediments of Svalbard by performing experiments similar to the ones described here and using the average literature value of csSRR of 2.0 fmol cell^{-1} h^{-1} (Hubert et al., 2009). By applying the present model on the data of the

previous report, a csSRR value of 2.3 fmol cell^{-1} h^{-1} is obtained, thus supporting the estimate of at least 10^5 tSRB endospores per cm^3 of sediment in Smeerenburgfjorden, Svalbard.

Comparison of physiological parameters of the different tSRB populations and the effect of organic substrate amendment

The model presented also allows an interpretation of the relative abundance of the different tSRB populations present in the same sediment sample. The initial abundance of the different populations ranged from 2×10^{-1} ($P1_U$) to 7×10^5 tSRB endospores per cm^3 of sediment ($P2_A$; Table 1). The distinct exponential phases and the difference in abundance and doubling time between the first (P1) and second (P2) tSRB populations suggest that they are distinct species with different physiological characteristics. The slower-growing populations in the amended and unamended incubations ($P2_A$ and $P2_U$) have nearly identical abundance estimates and doubling times (Table 1), even though SRR are on average 3 times higher in the amended incubation (Figure 1). This suggests that these populations are largely similar, and that substrate amendment promoted higher rates by stimulating faster growth of germinated tSRB rather than stimulating distinct tSRB, which is consonant to diversity studies of the same sediment in similar incubations (De Rezende et al., in prep.). The faster-growing tSRB population in the unamended incubation ($P1_U$) was the least abundant, on average two orders of magnitude lower than the faster-growing tSRB population in the amended incubation ($P1_A$; Table 1). This difference suggests that substrate amendment stimulated the germination and growth of a larger diversity of faster-growing tSRB endospores that grew to a larger final abundance (ca. 100-fold higher) during an exponential phase that ended within 30 h of incubation (Figure 1).

Advantages of the model

Our SRR-based model only detects the tSRB endospore populations that germinate and grow under the incubation conditions, similarly to MPN and other cultivation-dependent methods. However, the SRR-based model provides several advantages in relation to the MPN method. First, it allows the estimation of the

abundance, csSRR and doubling times for distinct populations that may develop in succession during the incubation, hence information about the composition and physiology of the tSRB community may be inferred. Second, the calculation of csSRR indicates that the estimated abundance of tSRB endospores by the SRR-based model is a more realistic minimum estimate than MPN enumeration, since the T-MPN estimates for this sediment are in the lower end of the estimated abundance by the SRR-based model. Third, estimates can be obtained in a much shorter time frame of only a few days, while the MPN procedure usually requires incubation times from weeks to months.

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Figure 1. Sediment slurries were amended with organic substrates or left unamended and incubated at 50°C for up to 120 h. Sulfate reduction rates (SRR; triangles and circles) are presented in panels A and C. Triangles indicate SRR data used in the model proposed. The lines represent 90% confidence intervals. Average doubling times (T_2) of each exponential phases are presented in A and C. Panels B and D show the estimated number of thermophilic SRB cells (left y-axis) during each of the exponential phases of the amended and unamended incubations, respectively. Shaded areas correspond to the period in which tSRB endospores must have germinated. The corresponding estimated numbers of tSRB endospores in the original sediment are plotted on the right y-axis.

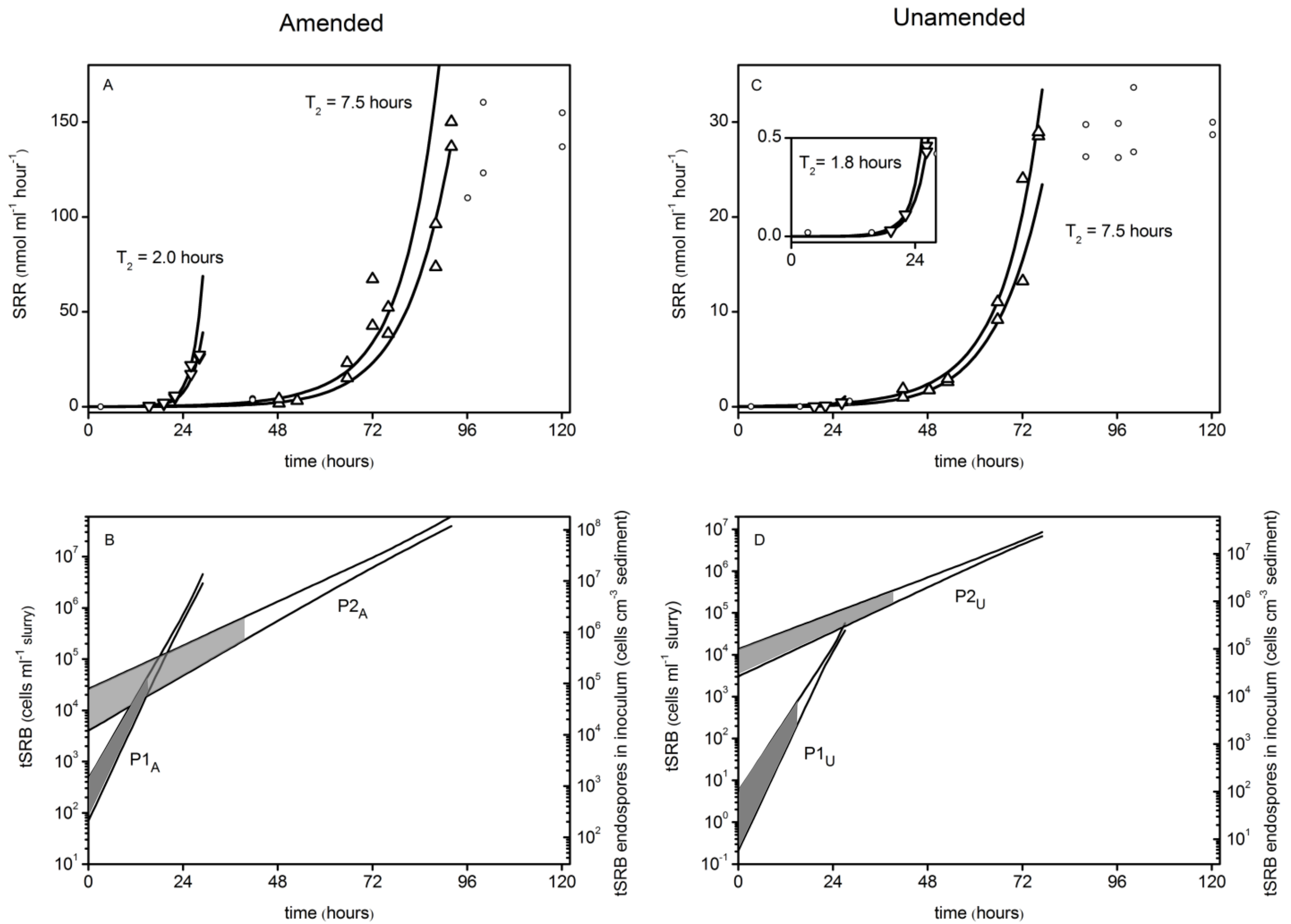

Table 1. Calculated parameters for the first and second populations (P1 and P2, respectively) in the amended and unamended incubations (index A and U, respectively). Values are presented as average and 90% confidence interval. The estimated number of tSRB endospores in the sediment is presented as minimum and maximum considering germination at 0 and 15 h, respectively.

Thermophilic SRB population	**SRR_0 (10^{-3} nmol ml^{-1} h^{-1})**	**k**	**T_2 (h)**	**csSRR (fmol $cell^{-1}$ h^{-1})**	**Estimated number of tSRB endospores (cm^{-3} sediment)**
$P1_A$	2.2 (1.1 - 6.3)	0.34 (0.30 - 0.38)	2.0 (1.8 - 2.3)	13.8 (12.1 - 15.2)	7×10^1 - 5×10^4
$P2_A$	34.6 (15.9 - 92.5)	0.09 (0.08 - 0.10)	7.5 (6.6 - 8.6)	3.7 (3.2 - 4.2)	4×10^3 - 7×10^5
$P1_U$	0.018 (0.004 - 0.071)	0.39 (0.33 - 0.46)	1.8 (1.5 - 2.1)	15.4 (13.3 - 18.6)	2×10^{-1} - 8×10^2
$P2_U$	23.0 (12.4 - 46.6)	0.09 (0.08 - 0.10)	7.5 (6.8 - 8.5)	3.7 (3.3 - 4.1)	3×10^3 - 4×10^5

Supplemental material

Table S1. Growth properties reported for endospore-forming SRB and other sulfate-reducing microorganisms. Minimum and maximum biovolumes are calculated as ellipsoidal volumes: $\pi\times(\text{width}/2)^2\times(\text{length})$. §This species has been suggested to be reclassified to *Clostridium* spp. (Stackebrandt et al. 1997). *Temperature range. Optimum temperature was not reported.

Organism name	Temperature optimum (°C)	Min width (µm)	Min length (µm)	Max width (µm)	Max length (µm)	Min biovolume (µm^3)	Max biovolume (µm^3)	$Y_{sulfate}$ (pg nmol^{-1}), electron donor	Reference
Archaeoglobus fulgidus	83	0.4	0.4	1.2	1.2	0.03	0.9	800-4000, lactate	König and Stetter (1989); Habicht et al. (2005)
Desulfacinum hydrothermale	60	0.8	1.5	1.0	2.5	0.5	1.3		Sievert and Kuever (2000)
Desulfacinum infernum	60	1.0	2.5	1.0	3.0	1.3	1.6		Rees et al. (1995)
Desulfobacter postgatei	37	1.0	1.7	2.0	3.5	0.9	7.3	4800, acetate	Widdel and Pfennig (1984); Rabus et al. (2006)
Desulfococcus multivorans	28-35	1.4	1.4	2.3	2.3	1.4	6.4	6200, benzoate	Rabus et al. (2006)
Desulfocurvus senezii	37	0.3	1.0	0.3	1.3	0.05	0.1		Klouche et al. (2009)
Desulfonauticus autotrophicus	58	0.6	4.0	1.2	4.0	0.8	3.0		Mayilraj et al. (2009)
Desulfonauticus submarinus	45	0.4	5.0	0.5	6.0	0.3	0.8		Audiffrin et al. (2003)
Desulfosoma caldarium	57	1.0	2.0	1.5	2.0	1.0	2.4		Baena et al. (2011)
Desulfosporosinus acidiphilus	30	0.8	4.0	1.0	7.0	1.3	3.7		Alazard et al. (2010)
Desulfosporosinus auripigmenti	25-30	0.4	0.4	0.4	0.4	0.03	0.03		Stackebrandt et al. (2003)
Desulfosporosinus hippei	28	1.0	1.2	1.0	1.2	0.6	0.6		Vatsurina et al. (2008)
Desulfosporosinus lacus	30	0.6	2.3	0.7	3.8	0.4	1.0		Ramamoorthy et al. (2006)
Desulfosporosinus meridiei	37	0.7	4.0	1.1	13.0	1.0	8.2		Robertson et al. (2001)
Desulfosporosinus orientis	37	0.7	3.0	1.0	5.0	0.8	2.6	8500-12400, H_2	Widdel (2006)
Desulfosporosinus youngiae	32-35	0.7	3.0	1.2	7.0	0.8	5.3		Lee et al. (2009)

Table S1. (cont.)

Organism name	Temperature optimum (°C)	Min width (µm)	Min length (µm)	Max width (µm)	Max length (µm)	Min biovolume (µm^3)	Max biovolume (µm^3)	$Y_{sulfate}$ (pg nmol^{-1}), electron donor	Reference
Desulfothermus naphthae	60-65	0.8	2.0	1.0	3.5	0.7	1.8		Kuever et al. (2005)
Desulfothermus okinawensis	50	0.6	2.5	0.9	5.0	0.5	2.1		Nunoura et al. (2007)
Desulfotomaculum acetoxidans	34-36	1.0	3.5	1.5	9.0	1.8	10.6		Sneath (1986)
Desulfotomaculum aeronauticum	37	0.5	2.2	0.8	5.5	0.3	1.8		Hagenauer et al. (1997)
Desulfotomaculum alcoholivorax	44-46	0.8	2.0	1.0	8.0	0.7	4.2		Kaksonen et al. (2008)
Desulfotomaculum alkaliphilum	53	0.6	3.0	0.7	3.5	0.6	0.9		Pikuta et al. (2000)
Desulfotomaculum antarcticum	20-30	1.0	4.0	1.2	6.0	2.1	4.5		Sneath (1986)
Desulfotomaculum arcticum	44	1.0	3.0	1.0	3.0	1.6	1.6		Vandieken et al. (2006)
Desulfotomaculum australicum	68	0.8	3.0	1.0	6.0	1.0	3.1		Love et al. (1993)
Desulfotomaculum carboxydivorans	55	0.5	5.0	1.5	15.0	0.7	17.7		Parshina et al. (2005)
Desulfotomaculum geothermicum	54	0.5	2.0	0.8	3.0	0.3	1.0		Daumas et al. (1988)
Desulfotomaculum gibsoniae	35-37	1.5	4.0	2.5	7.0	4.7	22.9	6600-12600, butanol	Kuever et al. (1999)
Desulfotomaculum guttoideum§	31	1.0	2.3	1.0	2.3	1.2	1.2		Widdel (2006)
Desulfotomaculum halophilum	35	0.5	3.0	0.5	6.0	0.4	0.8		Tardy-Jacquenod et al. (1998)
Desulfotomaculum hydrothermale	55	1.0	3.0	1.0	6.0	1.6	3.1		Haouari et al. (2008)
Desulfotomaculum kuznetsovii strain P60	62-63	1.0	2.8	1.1	4.2	1.5	2.7		Isaksen et al. (1994)
Desulfotomaculum kuznetsovii type strain	60-65	1.0	3.5	1.4	5.0	1.8	5.1		Widdel (2006)
Desulfotomaculum luciae	50-70*	1.0	3.0	1.0	3.0	1.6	1.6		Liu et al. (1997)
Desulfotomaculum nigrificans	55	0.5	2.0	0.7	4.0	0.3	1.0		Widdel (2006)
Desulfotomaculum putei	64	1.0	2.0	1.1	5.0	1.0	3.2	10360, lactate	Liu et al. (1997); Davidson et al. (2009)
Desulfotomaculum ruminis	37	0.5	2.0	0.7	4.0	0.3	1.0		Widdel (2006)
Desulfotomaculum salinum	60-65	0.9	2.0	1.3	5.0	0.8	4.4		Nazina et al. (2005)
Desulfotomaculum sapomandens	38	1.2	5.0	2.0	7.0	3.8	14.7		Cord-Ruwisch & Garcia (1985)
Desulfotomaculum solfataricum	60	1.5	3.5	1.5	5.0	4.1	5.9		Goorissen et al. (2003)

Table S1. (cont.)

Organism name	Temperature optimum (°C)	Min width (µm)	Min length (µm)	Max width (µm)	Max length (µm)	Min biovolume (µm^3)	Max biovolume (µm^3)	$Y_{sulfate}$ (pg nmol^{-1}), electron donor	Reference
Desulfotomaculum sp. strain WW1	65	0.8	2.0	0.8	5.0	0.7	1.7		Balk et al. (2007)
Desulfotomaculum thermoacetoxidans	55-60	0.7	2.0	0.7	5.0	0.5	1.3		Min and Zinder (1990)
Desulfotomaculum thermobenzoicum	62	1.5	5.0	2.0	8.0	5.9	16.8		Tasaki et al. (1991)
Desulfotomaculum thermocisternum	62	0.7	2.0	1.0	5.2	0.5	2.7		Nilsen et al. (1996)
Desulfotomaculum thermosapovorans	50	1.5	5.0	2.0	8.0	5.9	16.8		Fardeau et al. (1995)
Desulfotomaculum thermosubterraneum	61-66	0.8	3.0	1.0	10.0	1.0	5.2		Kaksonen et al. (2006)
Desulfovibrio halophilus	35	0.6	2.5	0.6	5.0	0.5	0.9		Caumette et al. (1991)
Desulfovibrio hydrothermalis	35	0.5	1.0	1.0	2.0	0.1	1.0		Alazard et al. (2003)
Desulfovibrio inopinatus	30	1.0	4.0	1.5	12.0	2.1	14.1	17800, lactate	Reichenbecher and Schink (1997); Rabus et al. (2006)
Desulfovibrio thermophilus	65	0.5	2.0	0.5	2.0	0.3	0.3		Widdel and Pfennig (1984)
Desulfovibrio vexinensis	37	0.5	3.0	0.5	5.0	0.4	0.7		Klouche et al. (2009)
Desulfovibrio vulgaris	34-37	0.5	3.0	1.0	5.0	0.4	2.6	8300, H_2	Widdel and Pfennig (1984); Rabus et al. (2006)
Desulfovirgula thermocuniculi	69-72	0.8	2.0	1.0	6.0	0.7	3.1		Kaksonen et al. (2007)
Desulfurispora thermophila	59-61	0.6	2.0	1.0	9.0	0.4	4.7		Kaksonen et al. (2007)
Strain NaphS2		1.3	1.3	1.3	1.9	1.2	1.7	6400, naphtalene	Galushko et al. (1999); Rabus et al. (2006)
Thermodesulfatator atlanticus	65-70	0.3	1.0	0.8	6.1	0.05	1.8		Alain et al. (2010)
Thermodesulfatator indicus	70	0.4	0.8	0.5	1.0	0.1	0.1		Moussard et al. (2004)
Thermodesulfobacterium hveragerdense	55-74	0.5	2.8	0.5	2.8	0.4	0.4		Sonne-Hansen & Ahring (1999)
Thermodesulfobacterium hydrogeniphilum	75	0.4	0.5	0.5	0.8	0.0	0.1		Jeanthon et al. (2002)
Thermodesulfovibrio islandicus	45-70	0.4	1.7	0.4	1.7	0.1	0.1		Sonne-Hansen & Ahring (1999)

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CHAPTER 4

MANUSCRIPT III

Hubert, C., A. Loy, M. Nickel, C. Arnosti, C. Baranyi, V. Brüchert, T. Ferdelman, K. Finster, F. M. Christensen, J. R. de Rezende, V. Vandieken, B. B. Jørgensen. A Constant Flux of Diverse Thermophilic Bacteria into the Cold Arctic Seabed. *Science.* **325:**1541-1544.

less of the level of taxonomic resolution. In this regard, genetic analyses have shown the existence of widespread, although disjunct, distribution patterns for several cryptic species of the marine diatom *Skeletonema* (*23*). Similar results have been reported for other microbial plankton groups, such as picoeukaryote algae and foraminifera (*17*, *24*). Alternatively, our results could reflect the convergence or parallelism of morphological traits among genetically unrelated taxa in disjunct oceanic regions.

Despite enormous environmental variability linked to glacial-interglacial climates of the Pleistocene, our analysis reveals that marine diatom communities have evolved slowly through gradual changes over the past 1.5 My of Earth's history (Fig. 2 and table S2). These patterns of community stability for extensive periods of geological time are probably associated with the great dispersal ability of marine diatoms (*25*, *26*) and highlight the potential of microbial plankton communities for recovering from past and future climatic variations. This conclusion implies that there are few or no biogeographical traces of historical climate change in contemporary communities of marine diatoms.

Models of evolution of species commonly assume that tectonic barriers and water mass fronts act as effective isolating mechanisms (*9*). This is a necessary condition that precedes the delineation of biogeographic provinces sensu stricto (*6*, *9*) and controls the development of global species richness. Our analysis, however, indicates that, even at the largest spatial scale, the geographic distribution of marine planktonic diatoms does not seem to be limited by dispersal. These results, together with recent genetic evidence for high rates of inter- and intra-oceanic gene flow in planktonic protists and widespread oceanic distributions of cryptic "sibling" species (*17*, *23*, *27*), suggest that the geographic isolation of marine diatoms cannot be maintained for long periods. Our results strongly support the hypothesis that environmental selection rather than dispersal dominates diatom community structure. To the extent that marine diatoms are a model microbial taxonomic group, our results imply that the biodiversity and macroevolutionary patterns at the microbial level fundamentally differ from those of macroscopic animals and plants, negating the idea that all living things follow similar ecological and evolutionary rules (*6*).

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A Constant Flux of Diverse Thermophilic Bacteria into the Cold Arctic Seabed

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Microorganisms have been repeatedly discovered in environments that do not support their metabolic activity. Identifying and quantifying these misplaced organisms can reveal dispersal mechanisms that shape natural microbial diversity. Using endospore germination experiments, we estimated a stable supply of thermophilic bacteria into permanently cold Arctic marine sediment at a rate exceeding 10^8 spores per square meter per year. These metabolically and phylogenetically diverse *Firmicutes* show no detectable activity at cold in situ temperatures but rapidly mineralize organic matter by hydrolysis, fermentation, and sulfate reduction upon induction at 50°C. The closest relatives to these bacteria come from warm subsurface petroleum reservoir and ocean crust ecosystems, suggesting that seabed fluid flow from these environments is delivering thermophiles to the cold ocean. These transport pathways may broadly influence microbial community composition in the marine environment.

Microbial diversity surveys have revealed that species richness is determined by many low-abundance taxa—the so-called rare biosphere (*1*–*3*). In the ocean, certain members of this relatively unexplored biosphere comprise a dormant microbial "seed bank" that can be transported passively over great distances (*1*). Quantitatively tracking the migration of indicator taxa can highlight key factors that influence patterns of biogeography and may help evaluate the extent to which microorganisms exhibit a cosmopolitan distribution (*4*). Endosporulation allows certain bacteria to persist as dormant cells in hostile environments, explaining discoveries of viable thermophilic *Firmicutes* in inhospitably cold habitats (*5*–*10*). Quantitative studies of this phenomenon are scarce, and the origin and distribution of thermophiles in cold environments remain enigmatic (*6*–*11*). Thermophilic sporulating taxa such as certain *Desulfotomaculum* spp. may constitute only 0.001% of marine microbial populations (*8*, *12*). Like the rare taxa, spores are less prone to viral lysis or predation, and are not detected by traditional diversity surveys (*1*, *2*). A spore-forming *Desulfotomaculum* strain that can only grow between 26° and 47°C was recently isolated from permanently cold Svalbard fjord sediment in the Arctic (*10*). The present study assessed thermophile diversity, abundance, and distribution in Svalbard sediments to reveal insights into mechanisms governing biogeography in the marine environment.

Pristine sediment was sampled from Smeerenburgfjorden (80°N; fig. S1) and incubated over an experimental temperature range (*13*), which revealed two distinct sulfate-reduction regimes

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(Fig. 1A). The first has a temperature optimum (T_{opt}) of 22°C and a maximum (T_{max}) of 32°C, consistent with earlier studies in Svalbard sediments (*14*, *15*) where the in situ temperature is –2° to +4°C year-round. This temperature-activity profile is characteristic of psychrophilic sulfate-reducing bacteria (SRB) that are well adapted to cold conditions (*14*, *16*), which explains their activity and abundance (up to 16%) in this environment (*17*). A second T_{opt} of 56°C (Fig. 1) indicates a previously unrecognized thermophilic community with a T_{max} above 60°C. Pasteurization at 80°C killed the psychrophiles but did not adversely affect the thermophiles (Fig. 1B), indicating that the latter exist as endospores in situ and only germinate after heating. This was supported by successful polymerase chain reaction targeting the spore-forming SRB genus *Desulfotomaculum* only if the sediment was incubated at 50°C before DNA extraction.

Psychrophilic and thermophilic communities were investigated further by incubating homogenized surface sediment at 50°C (*13*). Sulfate reduction rates (SRR) quickly dropped to below the detection limit (Fig. 2A) due to stimulation and subsequent death of vegetative psychrophiles as the sediment warmed up (compare to Fig. 1A). Thermophilic SRR increased exponentially between 20 and 96 hours. The transition from SRR below detection (up to 16 hours) to the onset of the exponential phase (at 20 hours) suggests a lag phase during which conditions became favorable for germination of thermophilic SRB spores. Endospore germination requires appropriate nutrients and substrates (*18*) such as volatile fatty acids (VFA) that are produced by microbial fermentation. VFA are typical electron donors for SRB and were generated rapidly during incubation at 50°C (Fig. 2B), creating conditions suitable for thermophilic sulfate reduction.

Several lines of evidence demonstrate that the VFA production was biologically mediated and not due to thermal alteration of the sediment organic matter. VFA did not accumulate in autoclaved sediment incubated in parallel at 50°C (concentrations never exceeded 0.3 mM). Amendment with the fluorescently labeled polysaccharides pullulan or arabinogalactan revealed extracellular enzymatic hydrolysis in sediment incubated at 50°C, whereas no hydrolysis was detected in sediment-free controls. Clone library analyses of 16*S* ribosomal RNA (rRNA) genes revealed enrichment at 50°C of different bacterial groups related to *Desulfotomaculum*, *Caminicella*, and *Caloranaerobactor-Clostridiisalibacter-Thermohalobacter* lineages within the *Firmicutes* phylum (Fig. 3) [see supporting online material (SOM) and table S3]. The latter lineages are represented by thermophilic anaerobes that convert carbohydrates and proteins to VFA, such as *Caminicella sporogenes* (*19*). Hydrolysis and fermentation at 50°C were therefore likely due to thermophilic spores that germinated at high temperature in the presence of complex natural organic substrates in the sediment.

VFA production at 50°C stimulated growth of a thermophilic *Desulfotomaculum* population

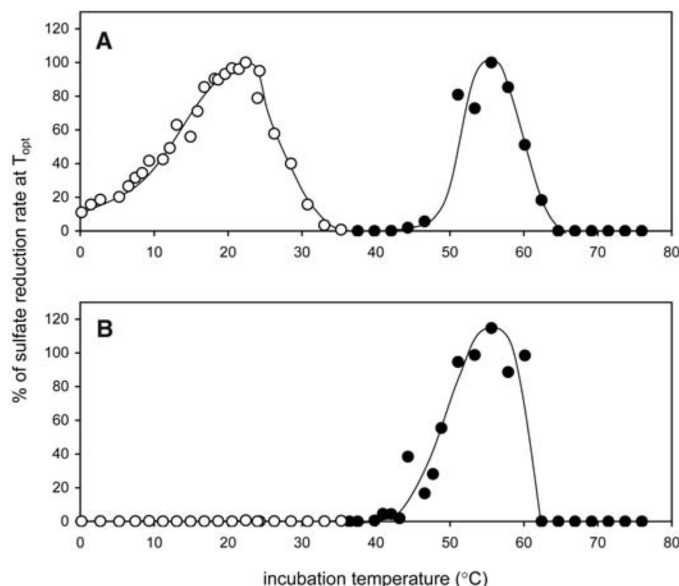

Fig. 1. Temperature-gradient incubations. SRR were measured in Smeerenburgfjorden sediment samples incubated between 0° and 76°C. Distinct SRB populations were active between 0° and 32°C, and between 41° and 62°C. Replicates were either untreated (**A**) or pasteurized for 1 hour at 80°C (**B**) before incubation. Open and closed symbols correspond to experiments below and above 35°C, respectively. Incubations in the thermophilic range resulted in much higher SRR (fig. S3); therefore, data are plotted as the percentage of the maximum SRR in (A) at either T_{opt}. Pasteurization killed the psychrophilic community and resulted in slightly higher activity by the thermophilic community [115% at the T_{opt} (B)].

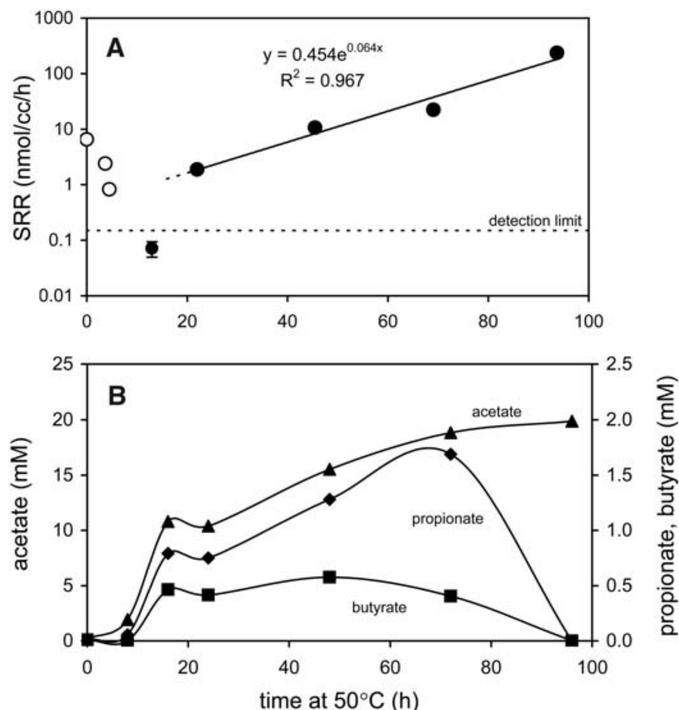

Fig. 2. Sediment incubation at 50°C. SRR (**A**) and concentrations of VFA (**B**) in Smeerenburgfjorden sediment (0- to 3-cm depth) incubated at 50°C. SRR represent 4- to 8-hour incubations with $^{35}SO_4^{2-}$ radiotracer. Open symbols correspond to the psychrophilic SRB community that was killed as the temperature increased to beyond their T_{max} (compare to Fig. 1A). SRR were below detection during the 10 to 16 hours time interval ($n = 2$; vertical bar: standard error). Sulfate reduction by 20 to 24 hours indicates germination of *Desulfotomaculum* spores, which subsequently catalyzed an exponential increase in SRR. These data constrain the earliest onset of the exponential phase to 16 hours, as indicated by the dashed extension of the exponential trendline. The exponential phase corresponded to the consumption of butyrate and propionate, which increased steeply, together with acetate (B), before the onset of thermophilic sulfate reduction.

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(Fig. 3), resulting in an exponential increase in SRR (Fig. 2A). The *Desulfotomaculum* cell density at the onset of sulfate reduction can be estimated by dividing the bulk rate (1.3 nmol cm^{-3} $hour^{-1}$ at 16 hours based on the exponential function in Fig. 2A) by a mean cell-specific sulfate reduction rate of 2.0 fmol $cell^{-1}$ $hour^{-1}$ [based on 33 pure cultures during exponential growth (*20*), and representative of thermophilic *Desulfotomaculum* spp.; see SOM]. This calculation estimates a population of 6.3×10^5 SRB cm^{-3} at 16 hours, which corresponds to the in situ spore density given that SRB growth did not occur before 16 hours (Fig. 2A).

Exponential increases in SRR were also measured across a series of intact sediment cores (0- to 23-cm depth) incubated at 50°C (*13*). These data indicate similar numbers of thermophilic *Desulfotomaculum* spores, on the order of 10^5 cm^{-3}, at all depths (fig. S2). Thermophiles thus constitute a stable component of the rare biosphere (~0.01%) in this Arctic marine habitat (*17*). Abundant taxa in Smeerenburgfjorden sediment are psychrophilic *Cytophaga*, *Flavobacteria*, *Gammaproteobacteria*, and *Deltaproteobacteria* (*17*) that catalyze hydrolysis, fermentation, and sulfate reduction during degradation of organic matter (*15*, *21*). We induced the same processes at 50°C, which stimulated SRR about 30-fold higher than in sediment incubated at 4°C (fig. S3). It is interesting that the thermophilic bacterial community has metabolic potential that mirrors the dominant metabolic processes occurring in situ. However, these thermophilic spores make no contribution to local biogeochemical cycling (Fig. 1). Our experience with thermophiles cultivated from Smeerenburgfjorden that have no detectable metabolic activity below 25°C supports this. Thermophilic spores must therefore be supplied externally and can be considered akin to particles accumulating in the sediment.

To estimate the particle flux of thermophilic spores into the sediment, we determined an age model by measuring the ^{210}Pb activity as a function of depth (see SOM). This revealed a sediment accumulation rate of 0.19 cm $year^{-1}$ in Smeerenburgfjorden (figs. S2 and S4). Given a stable abundance of about 10^5 spores cm^{-3} with depth (fig. S2), the constant supply of thermophiles to this site is 2×10^8 m^{-2} $year^{-1}$. Other fjords along the west coast of Svalbard have comparable thermophilic SRB densities in surface sediments (see SOM; fig. S1). The fjords and the adjacent coastal shelf in this area cover about 1000 km^2 (fig. S1), hence an extrapolation of our estimate corresponds to an annual supply of $\sim 10^{17}$ thermophiles in this Arctic region. These large numbers highlight not merely the occurrence of thermophiles in the Arctic but rather their unexpected quantity and the consistency of their flux. The warm, anaerobic environment where these bacteria originate must have sufficient geographic distribution, magnitude, and source strength to support the population sizes indicated by our data.

A limited number of marine habitats meet these criteria. Deeply buried sediments hundreds of meters below the sea floor represent warm habitats for anaerobic microbes (*22*). Advective flow of hydrocarbons or other fluids from these sediments can penetrate the sea floor (e.g., at mud volcanoes) and transport microscopic particles or cells up into the cold ocean (*23*). Seafloor pockmarks and active cold seeps are known around west Svalbard (*24*) (fig. S1) and are often

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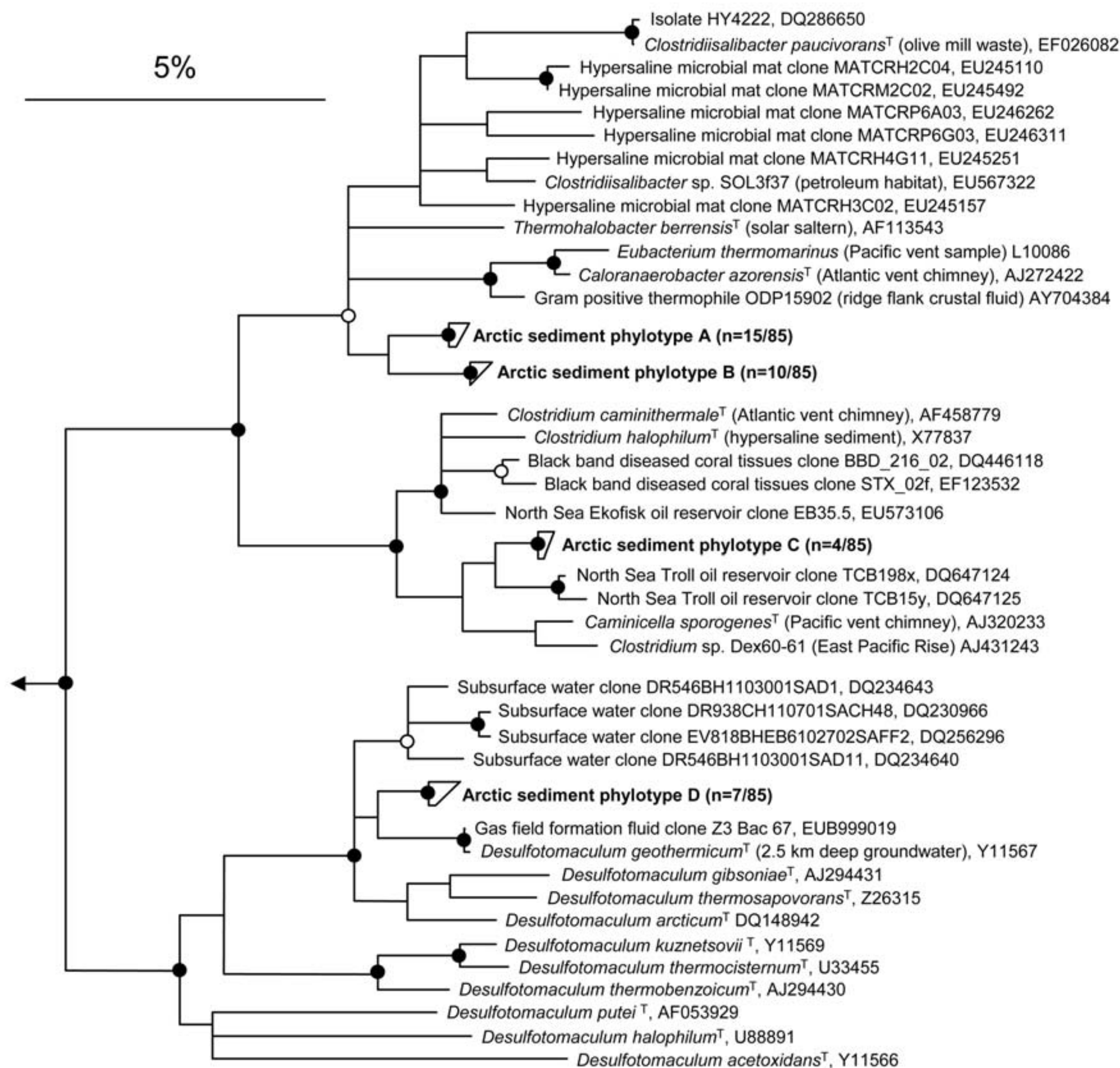

Fig. 3. Phylogenetic analysis of putative spore-forming thermophilic bacteria in Svalbard sediment. Clone libraries constructed before and after incubation at 50°C differed significantly due to the enrichment of thermophilic *Firmicutes* (see SOM, tables S2 and S3). Dominant phylotypes (i.e., >5% of clones) from the 50°C sediment library are indicated in boldface with relative abundances shown in parentheses. These phylotypes were not detected before 50°C incubation. The consensus tree of 16*S* rRNA gene sequences of selected *Firmicutes* includes next relatives for each phylotype (top 10) plus additional type strains (for *Desulfotomaculum*). Habitats of origin for closest relatives are indicated. Filled and open circles indicate lineages with >90% and 80 to 90% parsimony bootstrap support (100 resamplings), respectively. The scale bar indicates 5% sequence divergence as estimated from maximum-likelihood analysis.

associated with deep gas or oil-bearing deposits (*23*). 16*S* rRNA gene sequence comparisons revealed that taxa enriched in our 50°C experiments (Fig. 3) are most closely related to bacteria from subsurface petroleum reservoirs or oil production facilities (94 to 96% similarity; table S3). Another source of thermophiles could be nearby mid-ocean ridge spreading centers (fig. S1). Large volumes of fluids circulating through ocean crust (*25*) could transport cells away from warm anoxic niches in this seafloor habitat (*26*) and suspend them in abyssal currents. The closest relatives to the Arctic thermophiles also include an anaerobic thermophile isolated from deep, hot crustal fluid (94% similarity; table S3) (*27*).

Petroleum-bearing sediments and fractured ocean crust both host anaerobic heterotrophic microbial communities (*26*, *28*). Areas of discharge connecting these habitats to the water column are widespread, and both processes expel large volumes of fluid into the oceans (*23*, *25*). A combination of different point sources could explain the diversity and distribution of thermophilic taxa in Arctic sediments (Fig. 3 and fig. S1). Although our observations suggest that seabed fluid flow governs the biogeography of thermophilic spore formers, these passive dispersal mechanisms are unlikely to act only on these particular bacteria. Permeable conduits through sediments and ocean crust pass through several microbial niches with changing local temperature and geochemistry (*22*, *25*, *26*). Widespread seeding of the oceans by geofluids from deep biosphere habitats may therefore contribute broadly to the high microbial diversity observed in the marine environment.

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Three-Dimensional Structural View of the Central Metabolic Network of *Thermotoga maritima*

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Metabolic pathways have traditionally been described in terms of biochemical reactions and metabolites. With the use of structural genomics and systems biology, we generated a three-dimensional reconstruction of the central metabolic network of the bacterium *Thermotoga maritima*. The network encompassed 478 proteins, of which 120 were determined by experiment and 358 were modeled. Structural analysis revealed that proteins forming the network are dominated by a small number (only 182) of basic shapes (folds) performing diverse but mostly related functions. Most of these folds are already present in the essential core (~30%) of the network, and its expansion by nonessential proteins is achieved with relatively few additional folds. Thus, integration of structural data with networks analysis generates insight into the function, mechanism, and evolution of biological networks.

The advent of genome sequencing has enabled development of computational and experimental tools to investigate complete biological systems, but it has also highlighted the difficulty in integrating complex information for the hundreds to thousands of different molecules that compose even the smallest biological networks. Such integration presents many challenges, especially when assembling data from diverse fields, such as biochemistry and structural biology, that use different operational languages and conceptual frameworks. Biochemistry has traditionally focused on individual reactions and pathways, but recent advances in genomics have led to more rapid growth in the reconstruction and modeling of metabolic networks on a genome-wide scale (*1*–*3*). Thus, biochemical reactions, pathways, and networks can now be described in the context of entire cells, thereby enabling more realistic simulations of the behavior of metabolic networks in a growing number of organisms (*4*–*7*). Nevertheless, metabolism is still generally defined in terms of the chemical names and identity of substrates, products, and reactions. It does not explicitly consider the three-dimensional structures of its components, although such knowledge is required for a comprehensive understanding, not only of the individual reactions, but more importantly, of metabolic networks as a whole. Without such knowledge, we cannot rigorously define enzyme mechanisms or

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Supporting Online Material for

A Constant Flux of Diverse Thermophilic Bacteria into the Cold Arctic Seabed

Casey Hubert,* Alexander Loy, Maren Nickel, Carol Arnosti, Christian Baranyi, Volker Brüchert, Timothy Ferdelman, Kai Finster, Flemming Mønsted Christensen, Júlia Rosa de Rezende, Verona Vandieken, Bo Barker Jørgensen

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A Constant Flux of Diverse Thermophilic Bacteria into the Cold Arctic Seabed

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Materials and Methods

Location and sampling. Marine sediment was sampled during research cruises to Svalbard fjords aboard R/V *Farm* during summer months of 2003 to 2006. Smeerenburgfjorden is situated on the northwest coast of Svalbard (79°43'N, 11°05'E; see fig. S1) and the water depth at our sampling station is 210 m. Surface sediment (0-10 cm) was also sampled from Magdalenefjorden (79°34'N, 11°04'E), Kongsfjorden (78°55'N, 12°16'E), Nordfjorden (78°31'N, 15°04'E), Isfjorden (78°11'N, 14°34'E) and van Mijenfjorden (77°46'N, 15°04'E), as indicated in fig. S1. Sediments were obtained using a Haps corer (Kanneworf and Nicolaisen, 1983) with a 120 mm diameter core liner, allowing ship board sub-sampling into cores or gas-tight plastic bags (Hansen *et al*., 2000) for incubation experiments. A longer Rumohr gravity corer (Meischner and Rumohr, 1974) was used to obtain a 36 cm deep core from Smeerenburgfjorden that was cut into 2 cm sections to determine the sediment accumulation rate at this site.

Temperature gradient incubations. A sediment slurry (1:2 w/w) was prepared by mixing surface sediment with anoxic synthetic seawater (Widdel and Bak, 1991) and amended with volatile fatty acids (VFA). After overnight stirring at 4°C under N_2, aliquots (10 ml) were transferred to Hungate tubes and sealed with rubber stoppers. Tubes were incubated in temperature gradient blocks (TGB), which were 200×15×15 cm insulated aluminum blocks cooled at one end and

heated at the other to create a gradient from 0° to 76°C that increased incrementally between incubation slots. Half of the tubes were pasteurized in an 80°C water bath for one hour then immediately transferred to the TGB. Following pre-incubation in the TGB, 100 to 500 kBq of anoxic ^{35}S-labeled sulfate radiotracer solution was added to each tube. Sulfate reduction was terminated 4 to 8 hours later by transferring slurries into 20 ml zinc acetate solution (20% w/v) and freezing. Sulfate reduction rates (SRR) were determined by cold chromium distillation according to Kallmeyer *et al*. (2004). Triplicate slurries were incubated at several experimental temperatures resulting in standard deviations that were on average 12.7% of mean SRR.

Homogenized sediment incubations at 4° and 50°C. Bags of anoxic Smeerenburgfjorden sediment collected from 0 to 3 cm depth were incubated at 4° and 50°C with sub-samples removed for pore water analyses and determining SRR after 0, 8, 16, 24, 48, 72 and 96 h. Pore water was obtained from homogenized sediment sub-samples with a pore water press under N_2 pressure through GF/F filters. For SRR, sediment was dispensed into 10×1 cm cylindrical glass tubes that were sealed with a rubber stopper at one end and with a plastic syringe piston at the other end. Approximately 100 kBq of anoxic radiotracer solution was injected into the glass tubes, which then incubated at 4° or 50°C in parallel with the ongoing sediment bag incubations. Sulfate reduction was terminated 4 to 8 hours later (end-point times as listed above). Rates and concentrations determined at time zero for the bag incubated near in situ temperature (4°C) were inferred as initial data (time zero) for the 50°C incubation. For incubations at 50°C involving amendments with organic substrates, sediment slurries (1:2 w/w) were used. Thermophilic strains were isolated at 50°C by dilution to extinction in media inoculated with Smeerenburgfjorden sediment, followed by successive transfers in agar shake tubes.

Intact sediment core incubations. Sediment sub-cores (26 mm diameter) were taken from intact Haps cores immediately after retrieval. Replicate cores were incubated at 50°C and duplicate cores were removed on a daily basis (at similar

times as for the bag incubations). Radiotracer was injected for the final 4 to 8 hours of incubation, after which sulfate reduction was stopped and SRR were determined.

Analytical methods. Pore water was extracted regularly from sediment incubations for various analyses. Concentrations of VFA were measured by UV absorbance at 400 nm after derivitization with p-nitrophenyl hydrazine (Albert and Martens, 1987; Finke and Jørgensen, 2008). Hydrolytic potential was determined by amending sediment slurries with fluorescently labelled polysaccharides and analysing hydrolysis rates in 50°C incubations using gel permeation chromatography (Arnosti, 2003) relative to thermal controls (the same polysaccharides incubated at 50°C without sediment). Dissolved inorganic carbon (DIC) was determined by fixing 1.8 ml porewater aliquots with 20 µl of saturated $HgCl_2$ solution in glass vials capped with Viton septa without headspace. These were stored at 4°C until analysis by flow injection with conductivity detection (Hall and Aller, 1992). For ammonium analyses 1.5 ml aliquots were stored frozen prior to spectrophotometric determination with indophenol at 630 nm (Grasshoff *et al*., 1999). Porewater for sulfate analyses was preserved with zinc acetate (2% w/v) and measured by non-suppressed ion chromatography (Waters, column IC-PakTM, 50 x 4.6 mm) and conductivity detection using sodium phthalate eluent, pH 4.7, buffered with sodium tetraborate (Ferdelman *et al*., 1997).

16S rRNA gene surveys and phylogenetic analysis. DNA was extracted from sediment before and after incubation at 50°C using a previously described protocol (Zhou *et al*., 1996) or the Power Soil kit (MoBio). The presence of *Desulfotomaculum* was tested for using genus-specific 16S rRNA gene-targeted primers (116F and 1164R) (Stubner and Meuser, 2000). For clone libraries, bacterial 16S rRNA gene products obtained with PCR primers 8F and 1492R were cloned using the Topo TA cloning kit (Invitrogen). Taq cycle sequencing of clones was performed with an ABI3130xl sequencer (Applied Biosystems). After removing chimeras, 16S rRNA gene sequences (*E. coli* positions 100-1380) with identities ≥99% (approximately defining species level phylotypes; Stackebrandt

and Ebers, 2006) were grouped in operational taxonomic units (OTUs/phylotypes) using the furthest neighbour algorithm of DOTUR (Schloss and Handelsman, 2005). Treeing was performed using a 50% conservation filter for *Bacteria*, and the neighbour-joining, maximum-likelihood and maximum-parsimony methods in ARB (Ludwig *et al*., 2004). Polytomies were introduced in the consensus tree when branching patterns were inconsistent for the different trees. Two maximum likelihood trees (AxML, PHYML) of all clone sequences (n=181) were used for unweighted UniFrac analysis (Lozupone *et al*., 2006) to determine differences in the composition of bacterial communities before and after incubation at 50°C.

Determination of excess ^{210}Pb activity. The distribution of excess ^{210}Pb activity in a Rumohr core obtained from Smeerenburgfjorden was used to constrain sediment burial rates. Unsupported (excess) ^{210}Pb activity was calculated as the difference between total ^{210}Pb activity and ^{210}Pb supported by in situ ^{226}Ra decay. ^{226}Ra was determined as the activity of its short-lived daughter products ^{214}Pb and ^{214}Bi. Freeze-dried sediment samples (4 to 19 grams) obtained at 2 cm sediment depth intervals were stored in polysulfone screw-top jars (diameter 45 mm) and kept for at least 20 days in order that secular equilibrium between the parent isotope ^{226}Ra and short-lived daughter products ^{222}Rn, ^{214}Pb, and ^{214}Bi was reached. Activities were determined by non-destructive gamma spectroscopy using an ultra-low-level germanium gamma detector (Canberra, EURISYS coaxial type N). Depending on the expected activity, individual samples were counted for 1 to 4 days. Activities of the isotopes ^{210}Pb (46.4 keV), ^{214}Pb (295.2 and 352 keV) and ^{214}Bi (609.3 keV) were corrected for detector efficiency and intensity obtained from calibration with a uranium-thorium ore reference standard (DL-1a, Canadian Certified Reference Materials Project). ^{210}Pb self-absorption was checked individually for every sample (Cutshall *et al*., 1983) using a 10 kBq ^{210}Pb source (AEA Technology). ^{137}Cs and ^{40}K were additionally quantified at the 667 keV and 1460 keV lines respectively.

Supporting Online Text (Results and Discussion)

Svalbard. A map indicating our main sampling location in Smeerenburgfjorden is shown in fig. S1. Incubations at 50°C were also performed with sediment from other locations along the west coast of Svalbard (described below and shown in fig. S1).

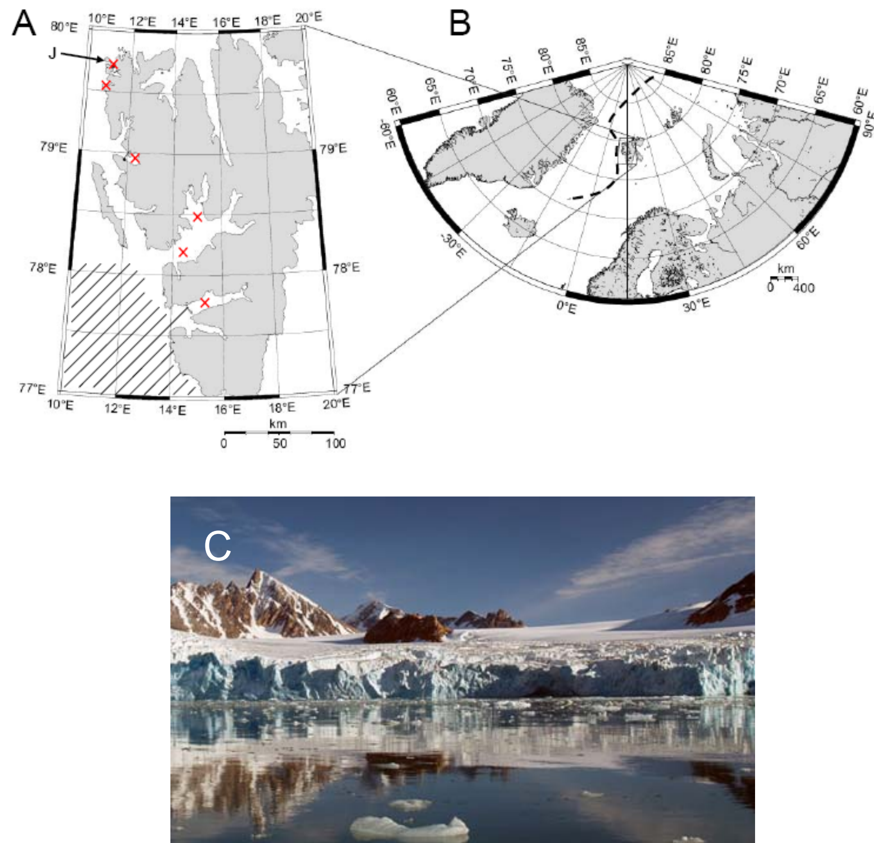

Figure S1. Svalbard fjords. The main sediment sampling location was station J in Smeerenburgfjorden (C) located at 79°43'N, 11°05'E ("J" indicated by the arrow in A). Sediment in Smeerenburgfjorden and other Svalbard fjords is permanently cold with year round in situ temperatures between –2° and +4°C. Exponential increases in SRR were observed in 50°C incubations with sediment from several locations, which are indicated (×) in (A) and correspond to estimated in situ densities of thermophilic SRB >10^4 cm^{-3} (see SOM Text below). The northern extent of a recent study of submarine gas seepage (Damm *et al.*, 2005) is indicated by the diagonal lines in (A). The approximate location of the mid-ocean spreading ridge is indicated by the dashed line (B).

Estimating thermophilic *Desulfotomaculum* abundance in Svalbard sediments. Estimating thermophilic SRB abundance in heated sediments depends on an exponential increase in SRR. This can be described by a function that allows the rate at the beginning of this increase to be extrapolated. In our main 50°C incubation experiment (Fig. 2A) this was 1.3 nmol cm^{-3} $hour^{-1}$ after 16 hours. The exponential response is presumably catalyzed by a growing SRB population that reduces sulfate at a constant cell-specific sulfate reduction rate (cs-SRR). Detmers *et al*. (2001) reported cs-SRR values for 33 pure cultures during exponential growth, and we used their mean value (2.0 fmol $cell^{-1}$ $hour^{-1}$) to estimate 6.3×10^5 SRB cm^{-3} present at 16 hours. Among the 33 pure cultures analyzed, thermophilic *Desulfotomaculum* spp. had cs-SRR values near the maximum and minimum reported (Detmers *et al*., 2001). *Desulfotomaculum geothermicum* – isolated from geothermal groundwater (Daumas *et al*., 1988) and the closest described relative to the thermophilic *Desulfotomaculum* lineage we detected in Smeerenburgfjorden – has a cs-SRR of 0.3 fmol $cell^{-1}$ h^{-1} (Detmers *et al*., 2001); for the data in Fig. 2A this results in a higher in situ SRB density estimate of 4.3×10^6 spores cm^{-3} (fig. S2). On the other hand, Detmers *et al*. (2001) report a high cs-SRR of 12.9 fmol $cell^{-1}$ $hour^{-1}$ for *Desulfotomaculum thermocisternum* (from a hot North Sea oil reservoir; Nilsen *et al*., 1996), which when applied here gives a lower estimate of 1.0×10^5 spores cm^{-3} in situ (fig. S2). The mean cs-SRR value therefore represents the range for thermophilic *Desulfotomaculum*. This range is indicated in the depth profile in fig. S2. Dilution to extinction experiments were performed by inoculating pasteurized sediment into growth medium amended with VFA. These provided minimum estimates of the most probable number (MPN) of thermophilic SRB in the range of 10^1 to 10^2 cm^{-3} in the Arctic and 10^1 to 10^4 cm^{-3} in temperate sediments (Isaksen *et al*., 1994). This confirmed the presence of thermophilic spores, yet for all sediments tested MPN counts were lower than the minimum estimate using cs-SRR (i.e., applying 12.9 fmol $cell^{-1}$ $hour^{-1}$). The MPN approach is known to underestimate actual numbers by several orders of magnitude (Sahm *et al*., 1999), as is evident here given the extremely high cs-SRR required for an in situ SRB density much below 10^5 cm^{-3} (Fig. 2A; fig. S2).

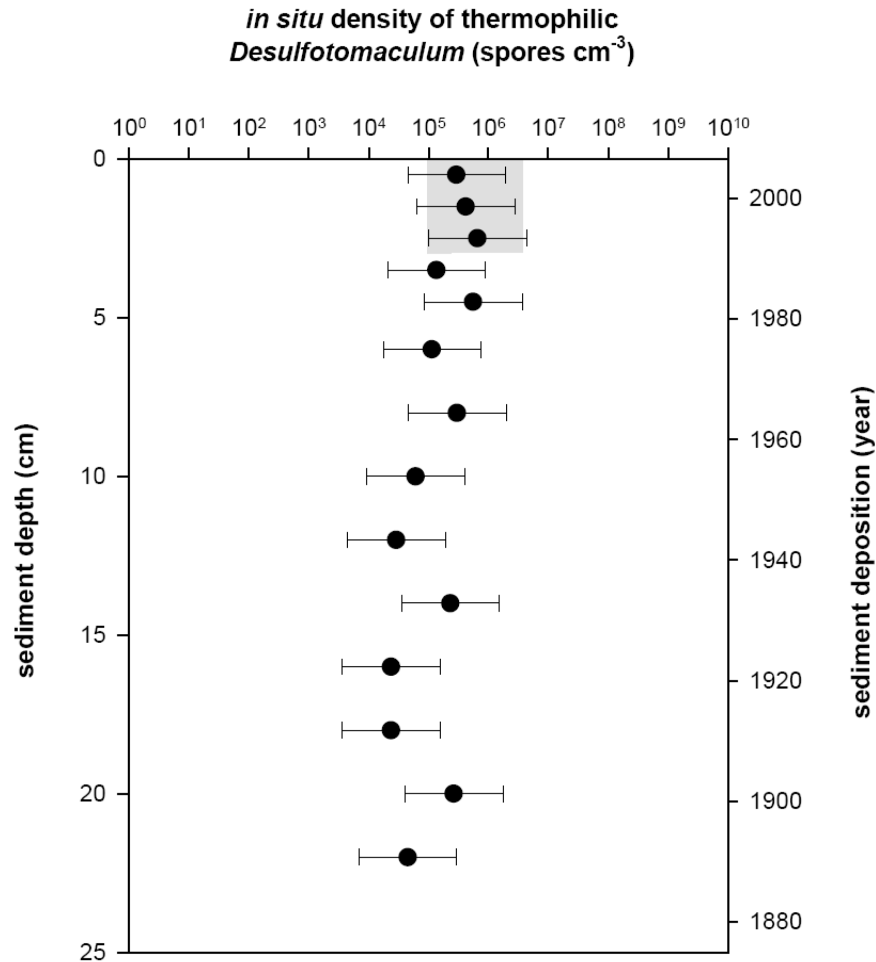

Figure S2. Depth distribution of *Desulfotomaculum* spores. The in situ abundance of thermophilic SRB was estimated from exponential increases in sulfate reduction rates (SRR) measured in intact sediment cores incubated at 50°C (table S1). Plotted values were estimated using a mean cs-SRR as described in the SOM Text. Horizontal bars indicate minimum and maximum cell density estimates based on cs-SRR values reported for thermophilic *Desulfotomaculum* spp. (Detmers *et al.*, 2001) as described in the SOM Text. The same estimate for the 0-3 cm sediment incubation (Fig. 2A) is indicated by the shaded area and agrees with estimates from the sediment cores. A sediment age model for Smeerenburgfjorden determined from excess ^{210}Pb activity as a function of depth (see SOM Text and fig. S4) is shown on the right y-axis and corresponds to a sediment accumulation rate of 0.19 cm year^{-1}.

Estimating thermophilic SRB abundance in other Svalbard sediments. Slurries prepared with surface sediment from Magdalenefjorden, Kongsfjorden, Isfjorden, Nordfjorden and van Mijenfjorden (fig. S1) were amended with VFA and sulfate radiotracer. Incubation at 50°C resulted in exponential increases in sulfate reduction, allowing estimates of thermophilic SRB abundance in situ, which were $>10^4$ cm^{-3} in each sediment (based on the approach outlined above). This confirms that our observations are not unique to Smeerenburgfjorden.

Microbial activity at 4° and 50°C in sediment incubations. Bags of homogenized sediment (ca. 1 kg) were incubated at 4° and 50°C. Sulfate reduction rates (SRR) at 4°C were always 5-9 nmol cm^{-3} $hour^{-1}$ (fig. S3A). This is slightly higher than in situ SRR (table S1), which is attributed to enhanced organic substrate availability for microbial metabolism in homogenized sediment (Hansen *et al*., 2000). At 50°C SRR increased exponentially to levels much higher than those measured at 4°C (fig. S3A, B). As described in the Main Text, hydrolysis and fermentation in the early hours of 50°C incubations preceded the sulfate reduction phase (Fig. 2). This is evident from comparing DIC and ammonium plots with the plot of SRR for the 50°C incubation (fig. S3B, D, F). Production of 5 mM DIC in the first 24 hours did not correspond to any sulfate reduction; however subsequent production of 15 mM DIC (24-96 hours) coincided with thermophilic SRB activity. Ammonium concentrations increased most rapidly during the first 24 hours of the 50°C incubation (fig. S3F), coinciding with the period of rapid VFA production (Fig. 2B). Small changes in DIC and ammonium were observed in sediments incubated at 4°C (fig. S3C, E).

Sulfate reduction in sediment core incubations. Replicate sub-cores from Smeerenburgfjorden were incubated at 4° and 50°C. In situ SRR were determined at 4°C and are shown in table S1. In the 50°C experiment SRR were determined at 1-2 cm intervals during a time-course similar to the bag incubation with homogenized sediment (Fig. 2A; fig. S3B). Functions describing exponential increases in sulfate reduction at each depth are shown in table S1 and were used to estimate initial spore numbers (as described in the Main Text and SOM Text and plotted in fig. S2).

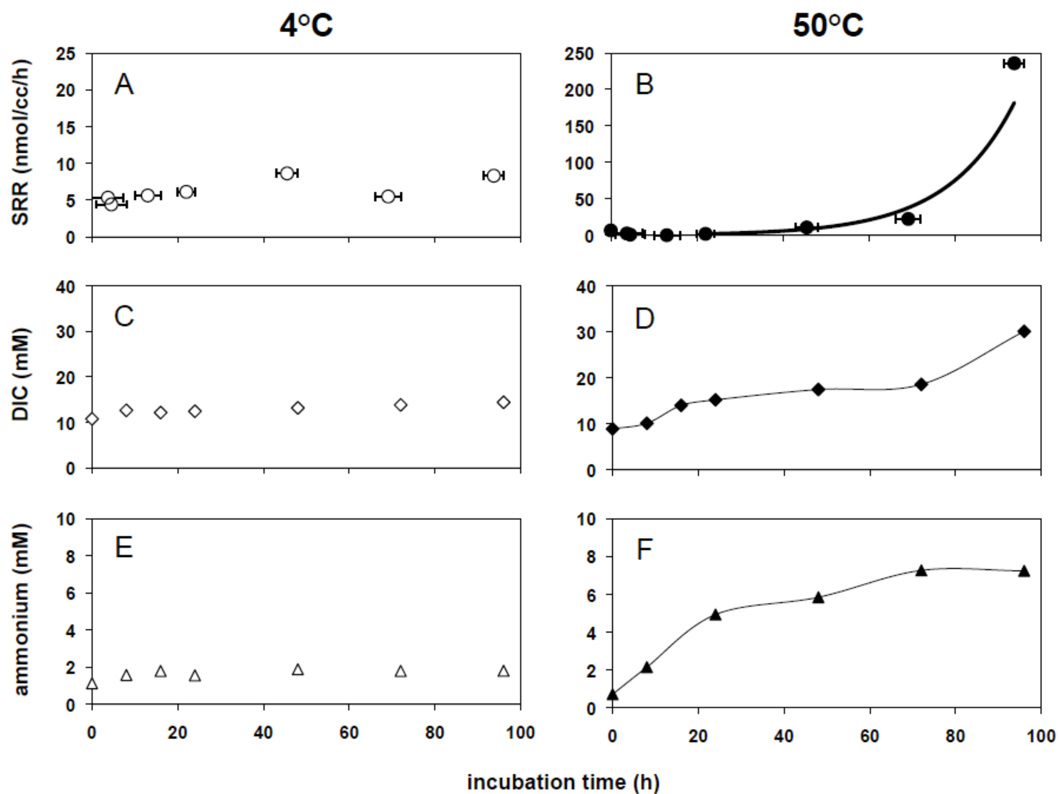

Figure S3. Time course bag incubations. Smeerenburgfjorden sediment (from 0 to 3 cm depth) was incubated in anoxic bags at 4°C (A, C, E) and 50°C (B, D, F). SRR (A, B) and concentrations of DIC (C, D) and ammonium (E, F) were monitored as a function of incubation time. The data and trendline for SRR at 50°C (B) are re-plotted from Fig. 2A for comparison purposes.

Table S1. Sulfate reduction at 50° and 4°C in different sediment depths.

Depth (cm)	SRR at 50°C ($ae^{b(t)}$)	R^2	interval[a]	$SRR_{onset.}$[b]	in situ SRR[c]
0-1	$0.207\ e^{0.064(t)}$	0.98	24-86 h	0.58	0.53
1-2	$0.251\ e^{0.075(t)}$	0.91	48-86 h	0.83	1.12
2-3	$0.416\ e^{0.071(t)}$	0.99	48-86 h	1.30	2.51
3-4	$0.063\ e^{0.090(t)}$	0.90	48-86 h	0.27	1.70
4-5	$0.459\ e^{0.055(t)}$	0.92	48-104 h	1.11	1.91
5-7	$0.068\ e^{0.075(t)}$	0.94	48-104 h	0.23	1.98
7-9	$0.240\ e^{0.057(t)}$	0.94	48-104 h	0.60	1.03
9-11	$0.045\ e^{0.062(t)}$	0.94	68-124 h	0.12	0.26
11-13	$0.019\ e^{0.068(t)}$	0.97	68-124 h	0.06	0.30
13-15	$0.245\ e^{0.039(t)}$	0.95	68-124 h	0.46	0.99
15-17	$0.020\ e^{0.054(t)}$	0.99	86-124 h	0.05	0.58
17-19	$0.019\ e^{0.057(t)}$	0.96	86-124 h	0.05	0.69
19-21	$0.259\ e^{0.044(t)}$	0.98	48-86 h	0.52	0.76
21-23	$0.039\ e^{0.052(t)}$	0.82	68-124 h	0.09	0.66
0-3 (bag)	$0.454\ e^{0.064(t)}$	0.97	20-96 h	1.26	6.82[d]

[a] Exponentially increasing SRR were measured during these intervals in 50°C incubations.

[b] The sulfate reduction rate (nmol cm^{-3} $hour^{-1}$) extrapolated to the onset of the exponential phase (i.e., after 16 hours, as described in the SOM Text).

[c] Mean sulfate reduction rate (nmol cm^{-3} $hour^{-1}$) from duplicate cores incubated at 4°C.

[d] Mean sulfate reduction rate during the 4°C bag incubation (fig. S3A).

16S rRNA gene surveys and phylogenetic analysis. Clone libraries were constructed before (time zero) and after (56 hours) incubation at 50°C. Good's coverage for the two libraries was 46% and 65%, respectively (table S2). Both p-test and UniFrac significance testing showed that the two bacterial communities differed significantly (p-value <0.001). Lineage-specific UniFrac analysis (using approximately phylum level distance; Lozupone *et al*., 2006) indicated that only members of the phylum *Firmicutes* contributed significantly to this difference (p-value < 0.001). Out of 85 clone sequences in the 50°C sediment library, 40 grouped within the *Firmicutes* phylum (Fig. 3), as opposed to only one out of 96 clone sequences in the library constructed prior to incubation at 50°C. The low proportion of *Firmicutes* in the initial clone library agrees with previous in situ molecular community analyses of Svalbard fjord sediments (Ravenschlag et al., 1999; 2000; 2001). Phylotypes represented by 0 clones before, and >5% of clones following incubation at 50°C, were considered to represent Arctic thermophiles enriched at high temperature (indicated in Fig. 3 and table S3). To evaluate putative physiologies and possible sources of Arctic thermophiles, next relatives to these dominant phylotypes were determined using the SINA aligner of SILVA (Pruesse *et al*., 2007) and the sequence match option in RDP II (Cole *et al*., 2007) (Fig. 3 and table S3).

Table S2. Clone libraries before and after sediment incubation at 50°C.

Library designation	Before 50°C (0 h)	After 56 h at 50°C	Both
Number of clones	96	85	181
Number of OTUs	68	41	99
Number of singletons[a]	52	30	68
Good's Coverage	46%	65%	62%

[a] OTUs represented by only one sequence in the clone library

Table S3. *Firmicutes* enriched at 50°C and their closest relatives.

OTU[a]	Number of clones	Next relative (accession number), max. 16S rRNA identity
A	15 (18%)	Hypersaline microbial mat clone (EU245157), 94.5% [b]*Clostridiisalibacter* sp. SOL3f37 (EU567322), 93.6%
B	10 (12%)	[c]Gram positive thermophile ODP15902 (AY704384), 94.1%
C	4 (5%)	[d]North Sea Troll oil reservoir clone (DQ647124), 96% [e]*Caminicella sporogenes*^{T} (AJ320233), 94%
D	7 (8%)	Gas field formation fluid clone (EU999019), 95.7% [f]*Desulfotomaculum geothermicum*^{T} (Y11567), 95.6%

[a] OTU designations as indicated in Fig. 3.

[b] Isolated from petroleum contaminated soil at an oil production facility.

[c] Isolated from Juan de Fuca ridge flank crustal fluid (Huber *et al*., 2006); this strain was more closely related than any environmental clones in the databases (Fig. 3).

[d] Formation water from a high temperature oil reservoir (Dahle et al., 2008).

[e] Isolated from a hydrothermal vent site (Alain *et al*., 2002).

[f] Isolated from 2.5 km deep geothermal groundwater (Daumas *et al*., 1988).

Smeerenburgfjorden ^{210}Pb and estimation of sediment burial rate. Distributions of total ^{210}Pb and ^{226}Ra versus depth are shown in fig. S4A. Background activities of ^{226}Ra, and therefore of ^{226}Ra-supported ^{210}Pb, are constant at 2.2 ± 0.2 dpm cm^{-3}. ^{210}Pb activities decrease exponentially from a surface peak activity of 20.9 dpm cm^{-3} with increasing depth and approach the values of secular equilibrium with respect to ^{226}Ra of 2.2 at depths below 30 cm. The ^{210}Pb profile exhibits distinct deviations from a smooth exponential decrease. These deviations correlated with distinct shifts in the ^{40}K, porosity, and bulk density distributions (data not shown).

Reaction-transport models using unsupported (excess) ^{210}Pb ($^{210}Pb_{xs}$) to estimate sediment burial rates assume that sediment depth and age are equivalent, and that the flux of ^{210}Pb is known or at least constant over time (Robbins, 1978). Superficially, the Smeerenburgfjorden sediment does not appear to meet these criteria, given the variable sedimentation and sediment mixing caused by burrowing infauna. Changes in porosity, bulk sediment density and ^{40}K, along with the deviations in the $^{210}Pb_{xs}$ profile suggest that the rate of sedimentation has varied over time. That is, variable and lateral inputs of glacier derived "^{210}Pb-old" sediments dilute the flux of $^{210}Pb_{xs}$-bearing particles to the sediment-water interface. We assume that the flux of $^{210}Pb_{xs}$ has remained constant over the past 100 years, but the rate of overall sediment input may have varied. The so-called constant flux model (Robbins, 1978) can be used to establish a sediment age model whereby the age for any given layer can be estimated from the following relationship:

$$t = \frac{1}{\lambda} \ln \left[1 - \frac{\Gamma_z}{\Gamma_\infty} \right]$$

where: t = age of bottom of layer z in years

λ = decay constant for ^{210}Pb = 0.0311 y^{-1}

Γ_z = inventory of $^{210}Pb_{xs}$ below depth z

Γ_∞ = total inventory of $^{210}Pb_{xs}$ in sediment

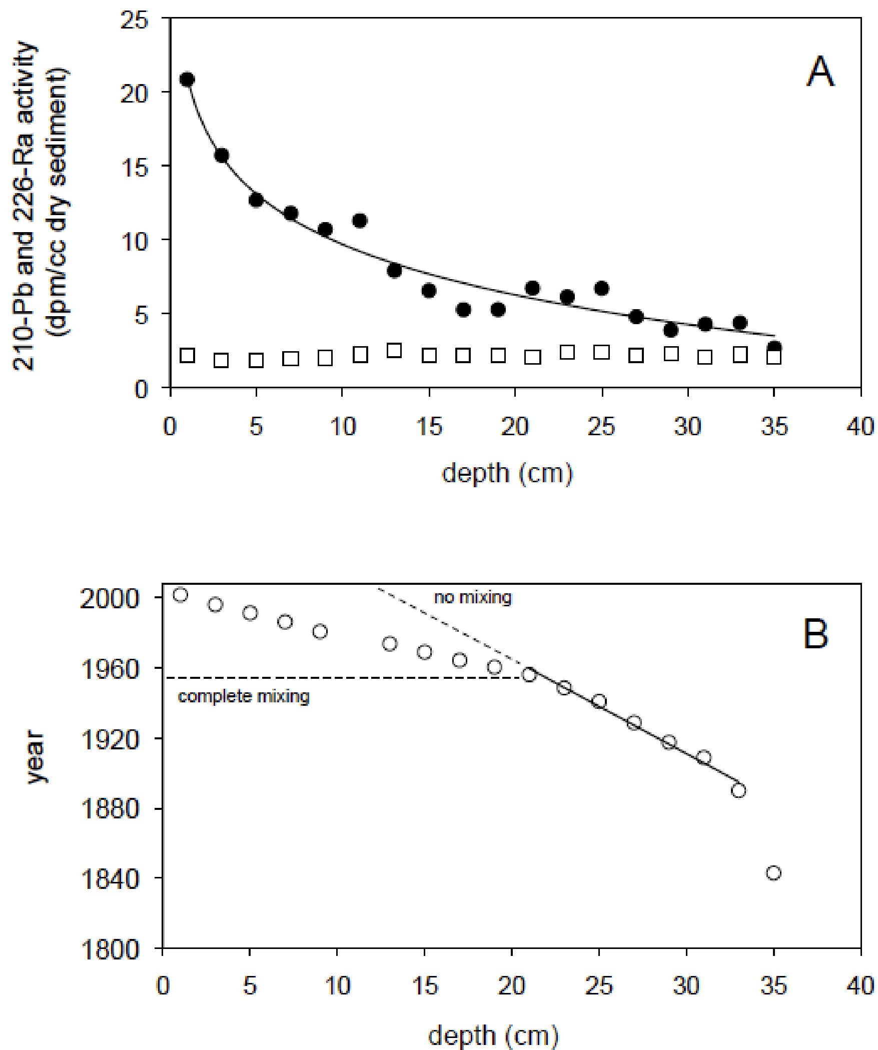

Figure S4. Depth profiles of ^{210}Pb and ^{226}Ra and sediment age model. Activities of total ^{210}Pb (●) and ^{226}Ra (□) measured in freeze-dried sediment (2 cm depth intervals) from Smeerenburgfjorden (A). The exponential decrease in ^{210}Pb activity displays some unevenness, indicating that the surface sediment (down to 20 cm) was partially, but not completely mixed by burrowing infauna (Jørgensen *et al*., 2005). From these data, unsupported (excess) ^{210}Pb activity was calculated and used in a constant flux model (Robbins, 1978) to construct a sediment age profile (B). The steeper slope below 20 cm depth (where there is no influence of bioturbation) allows a burial rate of 0.19 cm year^{-1} to be calculated for Smeerenburgfjorden.

Bioturbation in Smeerenburgfjorden sediments is assumed to be small enough not to violate entirely the assumption of depth-age equivalency. Qualitative observations (during core sectioning) of tube-dwelling polychaetes and other fauna are similar to those observed in surface sediments of other Svalbard fjords (Jørgensen *et al*., 2005). The $^{210}Pb_{xs}$ data for the top 20 cm indicate that bioturbation results only in partial mixing of this sediment layer, as decay still proceeds with depth (fig. S4A). The stable profile of *Desulfotomaculum* spore abundance down to 23 cm (fig. S2) is thus not a function of mixing (the sediment is not completely mixed; fig. S4B) and corresponds to a stable input of spores to the sediment. Mixing is assumed to approach zero below 20 cm, and data from this zone can be used to estimate a sediment burial rate of 0.19 cm $year^{-1}$ (fig. S4B).

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