Diversity of
Mat-forming Sulfide-oxidizing Bacteria
at Continental Margins

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Diese Arbeit ist all denjenigen gewidmet,

die ihre Segel setzen, um neue Welten zu erkunden.

Seien sie sich gewiss, dass auf Sturm immer ruhiges Wasser folgt.

Vertrauen sie auf ihr größtes Gut – ihre Freunde und Familie.

Nutzen sie ihre Schwächen, um neue Stärken zu finden.

Soll Zuversicht ihr Kompass sein!

Summary

In the oceans, microbial mats formed by chemosynthetic sulfide-oxidizing bacteria are mostly found in so-called 'reduced habitats' that are characterized by chemoclines where energy-rich, reduced substances, like hydrogen sulfide, are transported into oxic or suboxic zones. There, these organisms often thrive in narrow zones or gradients of their electron donor (sulfide) and their electron acceptor (mostly oxygen or nitrate). Through the build up of large biomasses, mat-forming sulfide oxidizers may significantly contribute to primary production in their habitats and dense mats represent efficient benthic filters against the toxic gas hydrogen sulfide.

As gradient organisms, these mat-forming sulfide oxidizers seem to be adapted to very defined ecological niches with respect to oxygen (or nitrate) and sulfide gradients. However, many aspects regarding their diversity as well as their geological drivers in marine sulfidic habitats required further investigation. Within this thesis, different thiotrophic mats ranging from shallow coastal waters to the deep sea along the European continental margin were investigated. The focus was on five different sulfide-oxidizing bacteria, including giant filamentous *Beggiatoa* species, giant vacuolated attached filaments (VAF), the giant 'sulfur pearl' *Thiomargarita*, gelatinous-mat-forming *Thiobacterium* species and filamentous-sulfur-excreting *Arcobacter* species. Molecular techniques targeting the diversity of the mat-forming sulfide-oxidizing bacteria were combined with geochemical analyses of prevailing chemical gradients to broaden our knowledge on these conspicuous organisms in marine sulfidic habitats.

In **Chapter 2**, novel observations of the genus *Thiobacterium* are described. *Thiobacterium* species are rod-shaped cells that internally store several granules of elemental sulfur, and that are embedded within a gelatinous matrix. So far, no 16S rRNA gene sequence has been clearly attributed to these organisms. Within this thesis, *Thiobacterium* mats were recovered from a whale bone, deep-sea sediment and a shallow-water cave. Microprofiles of oxygen and sulfide confirmed the dependence of *Thiobacterium* on hydrogen sulfide as energy source. Fluorescence *in situ* hybridization indicated that *Thiobacterium* species belong to the Gammaproteobacteria.

Niche differentiation between mats dominated by either *Beggiatoa* species and VAF, *Thiomargarita* species, or *Arcobacter* species was investigated on the Nile Deep Sea Fan in

the Eastern Mediterranean Sea (**Chapters 3 & 4**). Visual observations from ROV (remotely operated vehicle) dives were combined with *in situ* geochemical measurements and analyses of the morphology as well as phylogeny of the mat-forming sulfide oxidizers. The filamentous sulfide oxidizers were found to thrive at non-overlapping oxygen-sulfide gradients. *Thiomargarita* species populated a highly dynamic and brine-influenced habitat characterized by temporarily changing supplies of oxygen and sulfide. *Arcobacter* species dominated when oxygen-sulfide gradients overlapped in the bottom water.

Investigations on the diversity of mat-forming sulfide oxidizers as well as their geological drivers in different deep-sea thiotrophic mats observed along the Norwegian margin are presented in **Chapter 5**. Small grayish mat patches of 20 cm to 5 m in diameter were compared with extensive white mats covering up to 850 m^2 of seafloor. Whereas between and within gray mats a high diversity of sulfur-storing and non-sulfur-storing thiotrophs was observed, the extensive white mats were composed only of two types of *Beggiatoa* filaments. In combination with obtained geochemical data, it was hypothesized that large continuous mats may develop in stable geochemical gradients selecting for only 1 or 2 types of sulfide-oxidizing bacteria, whereas small patchy mats may be indicators of rather dynamic habitats hosting a variety of competing thiotrophic bacteria.

The occurrence, biomass and size distribution of *Beggiatoa* species in fjord sediments of the arctic archipelago Svalbard are discussed in **Chapter 6**. The obtained results suggest that *Beggiatoa*, although previously unnoticed, occur widespread in these arctic fjord sediments and are well adapted to thriving at seawater temperatures permanently close to the freezing point.

Zusammenfassung

In marinen Ökosystemen findet man von chemosynthetisch lebenden, sulfidoxidierenden Bakterien gebildete Matten vor allem an den Grenzflächen ‚reduzierter Habitate' vor, wo energiereiche, reduzierte Substanzen wie Schwefelwasserstoff auf Sauerstoff oder andere oxidierte Substanzen treffen. Dort leben diese Organismen meist innerhalb eines schmalen Horizontes oder Gradienten, wo ihnen sowohl ihr Elektronendonor (Sulfid) als auch ihr Elektronenakzeptor (meist Sauerstoff oder Nitrat) zur Verfügung stehen. Kommen sie in großer Zahl vor und bilden dabei dichte Matten, können mikrobielle Sulfidoxidierer deutlich zur Primärproduktion in ihren Lebensräumen beitragen und als benthische Filter wirken, indem sie giftiges Schwefelwasserstoffgas aus ihrer Umgebung entfernen.

Als Gradientenorganismen scheint jeder dieser mattenbildenden Sulfidoxidierer an eine spezielle ökologische Nische im Hinblick auf Verfügbarkeit und Konzentration von Sauerstoff (oder Nitrat) und Sulfid angepasst zu sein. Viele Aspekte bezüglich ihrer Diversität und der auf die Mattenbildung einflussnehmenden Umweltfaktoren sind jedoch noch nicht vollständig verstanden und bedürfen weiterer Untersuchungen. Innerhalb der vorliegenden Doktorarbeit wurden verschiedene Sulfidoxidierer-Matten entlang des europäischen Kontinentalrandes untersucht, wobei sowohl Matten in der Tiefsee als auch in flachen Küstengewässern vorkommende Populationen von Sulfidoxidierern analysiert wurden. Im Mittelpunkt standen dabei fünf verschiedene Typen von sulfidoxidierenden Bakterien, und zwar (1) große, filamentöse Bakterien der Gattung *Beggiatoa*, (2) an verschiedene Substrate angeheftete, vakuolierte, große, filamentöse Bakterien (VAF), (3) die großen ‚Schwefelperlen' der Gattung *Thiomargarita*, (4) Bakterien der Gattung *Thiobacterium*, welche gallertartige Matten bilden, und (5) schwefelausscheidende Bakterien der Gattung *Arcobacter*. Um das allgemeine Wissen über die Diversität dieser Organismen und ihre ökologische Anpassung in marinen, sulfidischen Lebensräumen zu erweitern, wurden molekulare und geochemische Analysen miteinander kombiniert.

Neue Erkenntnisse über die Bakterien der Gattung *Thiobacterium* werden in **Kapitel 2** beschrieben. *Thiobacterium*-Zellen sind stäbchenförmig, enthalten mehrere runde Schwefeleinschlüsse und sind von einer gallertartigen Masse umgeben. Bisher wurden

Thiobacterium-Spezies noch nicht eindeutig taxonomisch klassifiziert, da keine 16S rRNA-Gensequenz für sie bekannt ist. In der vorliegenden Doktorarbeit wurden *Thiobacterium*-Matten auf einem Walknochen, auf Tiefseesediment und in einer Unterwasserhöhle gefunden. In einer Matte gemessene Sauerstoff- und Sulfidgradienten bestätigten die Abhängigkeit von *Thiobacterium*-Zellen von Schwefelwasserstoff als Energiequelle, und Fluoreszenz *in situ* Hybridisierung ordnete die untersuchten Zellen den Gammaproteobakterien zu.

Die Besiedlung unterschiedlicher ökologischer Nischen durch verschiedene filamentöse Sulfidoxidierer (*Beggiatoa*, VAF) sowie Bakterien der Gattungen *Thiomargarita* oder *Arcobacter* wurde in der Tiefsee des östlichen Mittelmeeres untersucht (**Kapitel 3 & 4**). Beobachtungen am Meeresgrund mit Hilfe so genannter ROVs (remotely operated vehicles, zu deutsch: ferngesteuerte Fahrzeuge) wurden mit geochemischen *in situ* Messungen und Untersuchungen zur morphologischen und phylogenetischen Diversität der mattenbildenden Sulfidoxidierer in Zusammenhang gesetzt. Dabei zeigte sich, dass die filamentösen Sulfidoxidierer in einem Habitat vorkamen, wo Sauerstoff und Sulfid nicht überlappten. Bakterien der Gattung *Thiomargarita* besiedelten einen hochdynamischen und durch Salzsole beeinflussten Lebensraum mit zeitlich veränderlichem Vorkommen von Sauerstoff und Sulfid. Bakterien der Gattung *Arcobacter* dominierten ein Habitat, wo Sauerstoff und Sulfid dicht über dem Sediment in der Wassersäule aufeinander trafen.

Untersuchungen zur Diversität mattenbildender sulfidoxidierender Bakterien und der die Mattenbildung beeinflussenden Umweltfaktoren in verschiedenen Tiefsee-Lebensräumen vor der Küste Norwegens werden in **Kapitel 5** beschrieben. Kleine, graue Matten von 20 cm bis 5 m Durchmesser wurden dabei mit großflächigen, weißen Matten, die bis zu 850 m^2 des Meeresbodens bedeckten, verglichen. Wohingegen die Diversität an Schwefelbakterien in und zwischen verschiedenen grauen Matten hoch war, zeigten die weißen Matten nur das Vorkommen zweier Arten von *Beggiatoa*. Im Zusammenhang mit geochemischen Untersuchungen wurde die Hypothese aufgestellt, dass große zusammenhängende Matten vor allem in geochemisch stabilen Habitaten entstehen und auf ein oder zwei Arten von Sulfidoxidierern selektieren, während kleine, isolierte Matten ein Anzeichen für eher dynamische Umweltbedingungen sind und eine Vielzahl verschiedener Sulfidoxidiererarten beherbergen.

In **Kapitel 6** werden Untersuchungen zum Vorkommen, der Biomasse und Größenverhältnisse von *Beggiatoa*-Spezies in arktischen Fjordsedimenten (Svalbard) beschrieben. Dabei wurde entdeckt, dass *Beggiatoa*, obwohl bisher unbemerkt, in diesen Sedimenten weit verbreitet sind und gut an Lebensbedingungen nahe dem Gefrierpunkt angepasst scheinen.

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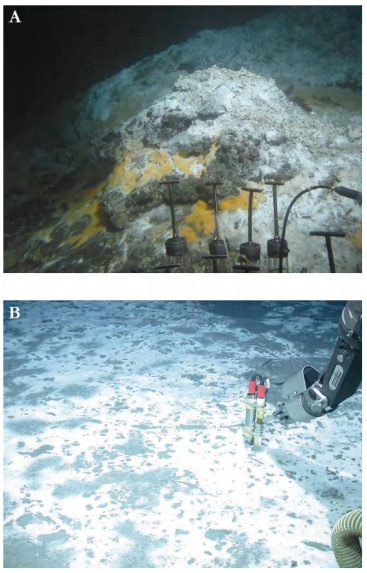
Preface

When I began my research in the field of marine microbiology, I was most fascinated by the fact that there are bacteria that can occur in such high numbers that they form visible microbial mats on the seafloor. Some of these bacteria gain their energy through the oxidation of hydrogen sulfide, a substance known to be toxic for most organisms at higher concentrations. As mat-forming sulfide-oxidizing bacteria usually store or excrete elemental sulfur, an intermediate produced during sulfide oxidation, these microbial mats often appear bright white due to the highly refractive nature of sulfur. In addition, yellow and orange-pigmented mats have also been observed. These sulfide oxidizer mats can be as small as several centimeters to decimeters in diameter, but can also extend hundreds of square meters or even thousands of square kilometers. Such extensive mats have, for example, been found at the deep-sea mud volcano Håkon Mosby off Norway (*Beggiatoa*; Jerosch *et al.*, 2007), in the Namibian shelf region (*Thiomargarita*; Schulz *et al.*, 1999), and at the continental margin off Peru and Chile (mainly *Thioploca*; Jørgensen and Gallardo, 1999).



Microbial mats on the deep-sea floor. (A) White and orange *Beggiatoa* mats at the Guaymas Basin hydrothermal vent field. Source: Woods Hole Oceanographic Institution. (B) White *Beggiatoa* mat at the Håkon Mosby mud volcano. Source: Marum.

Despite many studies on the bacteria forming these conspicuous mats, it seems that they still hide as much of their diversity as they have revealed. Therefore, expanding our knowledge on the diversity of different mat-forming sulfide-oxidizing bacteria as well as on their geological drivers in marine habitats was the focus of this thesis. Within the following chapters I will first give an overview about prominent marine ecosystems where microbial sulfide oxidizer mats have been observed. Then I will focus on their diversity, with an emphasis on the organisms studied within this thesis, i.e. the giant sulfide oxidizers *Beggiatoa*, *Thiomargarita* and VAF as well as the smaller sulfide oxidizers *Thiobacterium* and *Arcobacter*.

Chapter 1

Introduction

1.1 Microbial Mat Ecosystems in the Ocean

In the marine environment, microbial mats formed by sulfide-oxidizing bacteria are mostly found in so-called 'reduced habitats' that are characterized by chemoclines where energy-rich, reduced substances (hydrogen sulfide) meet oxic or suboxic zones. In such zones, sulfide-oxidizing bacteria can derive their energy from chemical redox reactions in which they oxidize one compound (electron donor; reductant) while simultaneously reducing another one (electron acceptor; oxidant). This energy is used to fix CO_2 autotrophically – a process called chemosynthesis. Through the build up of high biomasses, mat-forming sulfide oxidizers may significantly contribute to benthic primary production in their habitats (e.g. Lichtschlag *et al.*, 2010).

Microbial mats formed by sulfide-oxidizing bacteria have been found in areas ranging from shallow coastal waters to the deep-sea floor, but also in continental habitats such as lakes, rivers and hot springs. The most prominent marine mat habitats include hydrothermal vents (Chapter 1.1.1), cold seeps (Chapter 1.1.2), large food falls (Chapter 1.1.3), oxygen minimum zones (Chapter 1.1.4), and eutrophic shallow-water marine sediments (Chapter 1.1.5). There, mat-forming sulfide oxidizers often thrive in narrow zones or gradients where their electron donor (sulfide) and their electron acceptor (mostly oxygen or nitrate) co-exist (Robertson and Kuenen, 2006). Special adaptations allow some species to also thrive in habitats where their reductant and oxidants are spatially or temporarily separated (discussed in Chapter 7).

1.1.1 Hydrothermal Vents

Hydrothermal vents are found from shallow waters to the deep sea, particularly in seafloor spreading zones (Martin *et al.*, 2008) and recently also off-axis (Kelley *et al.*, 2005). Vent fluids carrying methane, hydrogen sulfide, ammonia, hydrogen, CO_2 and dissolved transition metal ions like Fe(II) and Mn(II), originate from subsurface seawater-rock interactions at high temperatures and pressures (Robertson and Kuenen, 2006; Jørgensen and Boetius, 2007; Martin *et al.*, 2008). So-called 'black smokers' that are located directly above deep-subsurface magma chambers emit fluids that can be as hot as 405°C (Martin *et al.*, 2008), whereas diffuse hydrothermal fluids rising up through

Chemosynthesis. In contrast to the process of photosynthesis, where energy for carbon fixation is derived from sun light, chemosynthetic organisms gain their energy through chemical redox reactions. Depending on the nature of the electron donor and the used carbon source, one can distinguish several modes of chemosynthesis.

Obligate chemolithotrophs use inorganic compounds as electron donor and grow autotrophically with CO_2 as only carbon source. *Facultative chemolithotrophs* cover the whole metabolic spectrum and are able to use inorganic and organic substances as both electron donor and carbon source. *Chemolithoheterotrophs* can gain energy from the oxidation of inorganic compounds, but do not use CO_2 as carbon source. *Chemoorganoheterotrophs* use organic compounds as both electron donor and carbon source (Robertson and Kuenen, 2006).

Hot-spot Ecosystems. Hot-spot ecosystems are defined as habitats of high or exceptional diversity within larger areas of low or more common diversity (Jørgensen and Boetius, 2007).

Microbial Mats. Microbial mats are generally referred to as layered, or dense cohesive microbial communities. *Laminated mats* are characterized by a distinct zonation of metabolically different groups of microorganisms, including cyanobacteria, anoxygenic phototrophs (e.g. purple sulfur bacteria), non-photosynthetic sulfide-oxidizing bacteria (e.g. *Beggiatoa* spp. or *Thiovulum* spp.), and sulfate-reducing bacteria (van Gemerden, 1993; Garcia-Pichel *et al.*, 1994; Hinck *et al.*, 2007). Within this thesis, *microbial mats* are referred to as dense communities of sulfide-oxidizing bacteria that are visible on the sediment surface (see also Figure 1.1), and in some cases also occur subsurface in upper sediment layers.

sediments tend to be much cooler (< 25°C) and can be substantially enriched in organic matter (Robertson and Kuenen, 2006). The composition of the vent fluids varies between different vent systems, depending on the geologic and tectonic settings of the vents (Jørgensen and Boetius, 2007). Upon contact of the electron-donor-rich vent fluids with the electron-acceptor-bearing seawater (e.g. oxygen, nitrate or sulfate), a wide range of chemosynthetic reactions becomes possible (Jørgensen and Boetius, 2007). In addition to hydrogen sulfide derived from the vent fluids, sulfide in organic-rich vent sediments may also be produced through microbial sulfate reduction (e.g. in the Guaymas Basin hydrothermal vent field; Elsgaard *et al.*, 1994; Dhillon *et al.*, 2003 and references therein; Kallmeyer and Boetius, 2004).

The discovery of hydrothermal vent ecosystems, approximately 30 years ago, can be considered as a mile-stone in understanding the ecological significance of microbially driven chemosynthesis. Chemolithoautotrophic microorganisms are the primary producers in these habitats, supporting rich (symbiotic) animal communities of

tubeworms, shrimps, clams, snails or crabs (van Dover, 2000; Jørgensen and Boetius, 2007; Dubilier *et al.*, 2008). Dense microbial mats, including such formed by sulfide-oxidizing bacteria, have often been observed at hydrothermal vents (Nelson *et al.*, 1989; Sievert *et al.*, 1999; van Dover, 2000; Kalanetra *et al.*, 2004). At the Guaymas Basin hydrothermal vent field in the Gulf of California, white, yellow and orange *Beggiatoa* mats covering sulfidic sediments and rock chimneys have been found (Figure 1.1A; Jannasch *et al.*, 1989; Nelson *et al.*, 1989). Within bushes of *Riftia* tubeworms, *Beggiatoa* mats being as thick as 60 cm were observed (Nelson *et al.*, 1989), thereby possibly comprising some of the thickest sulfide oxidizer mats described so far. The occurrence of *Arcobacter* mats as well as the existence of *Thiomargarita* cells within white and yellow *Beggiatoa* mats at the Guaymas Basin (S. Grünke, personal observation) further broadens the spectrum of ecological niche adaptations of mat-forming sulfide oxidizers in this habitat.

1.1.2 Cold Seeps

Marine cold seeps are found at both active and passive continental margins, and are related to focused upflow of low-temperature subsurface fluids (e.g. water, gas, oil or brine) and sediments. Similar to hydrothermal vents, cold seep ecosystems are chemosynthetic hot-spots (Jørgensen and Boetius, 2007). A key microbial process at cold seeps is the anaerobic oxidation of methane (AOM) coupled to sulfate reduction (Eq. 1.1) in subsurface sediments producing bicarbonate and energy-rich sulfide (Boetius *et al.*, 2000; Jørgensen and Boetius, 2007):

$$CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O \qquad \text{(Eq. 1.1)}$$

Anaerobic oxidation of methane with sulfate is mediated by microbial consortia of anaerobic methanotrophic archaea (ANME) and sulfate-reducing Deltaproteobacteria of the genera *Desulfosarcina/Desulfococcus* or *Desulfobulbus* (Jørgensen and Boetius, 2007; Knittel and Boetius, 2009). The true nature of the intermediate(s) shuttled from methane oxidation into sulfate reduction, though, is not yet known (Jørgensen and Kasten, 2006; Knittel and Boetius, 2009). AOM-derived bicarbonate can precipitate as calcium carbonate, thereby providing a hard substratum for the development of faunal benthic

communities (Boetius *et al.*, 2000; Foucher *et al.*, 2009). Sulfide serves as electron donor for symbiotic and free-living sulfide oxidizers, the latter including mat-forming sulfide-oxidizing bacteria (Jørgensen and Boetius, 2007; Dubilier *et al.*, 2008). However, studies conducted at hydrocarbon seeps in the Gulf of Mexico have shown that sulfate reduction at cold seeps is not restricted to co-occurrence with AOM, but may potentially also be fueled by the oxidation of higher hydrocarbons or oil (Joye *et al.*, 2004).

The rate with which subsurface fluids rise to the sediment surface seems to be a controlling factor in the absence or presence of certain chemosynthetic communities in cold seep ecosystems. In areas characterized by high fluid-flow microbial conversion of methane may be possible only in the upper few millimeters of the sediment as strong advection counteracts the penetration of electron acceptors like sulfate into deeper layers. Consequently, only low amounts of sulfide, or even no sulfide, is produced. These areas often lack visible fauna. Microbial mats are usually found in habitats characterized by medium fluid-flow. Here, diffusional penetration of sulfate and other electron acceptors deeper into the sediment is possible, allowing for sufficient AOM and sulfate reduction, respective sulfide production. Finally, habitats with low fluid-flow regimes seem to be mostly dominated by specially adapted chemosynthetic seep fauna like bivalves and tubeworms (Torres *et al.*, 2002; Boetius and Suess, 2004; de Beer *et al.*, 2006; Jørgensen and Boetius, 2007).

In the following paragraphs, prominent cold seep systems are shortly described, thereby focusing on cold seeps studied within this thesis, including gas chimneys, pockmarks and mud volcanoes. Common to most are the occurrence of subsurface (methane) gas hydrates, methane seepage, methane-derived carbonates, as well as chemosynthesis-dependent faunal and microbial communities. Occasionally, also the occurrence of brine pools or lakes, or brines flowing along sulfidic mud 'rivers' has been observed. Brines are fluids of high salt content ($\geq$ 5%) that, in the deep sea, are formed when rising fluids interact with subsurface salt deposits (Boetius and Joye, 2009). Brines may carry methane and sulfide, which may create unique ecological niches for typical seep fauna and microbial communities (MacDonald *et al.*, 1990; Charlou *et al.*, 2003; de Lange *et al.*, 2006; Omoregie *et al.*, 2008; Huguen *et al.*, 2009).

Gas chimneys. Gas chimneys are geologically rather inconspicuous on the seafloor and represent subsurface migration pathways for free gas. They can be detected on microbathymetric maps and via acoustic imaging of the seafloor, even when they become dormant (Foucher *et al.*, 2009). Gas chimneys have, for example, been documented on the Nile Deep Sea Fan in the Eastern Mediterranean Sea (Loncke *et al.*, 2004; Dupré *et al.*, 2007) as well as on the Norwegian margin (Foucher *et al.*, 2009).

Within this study, small patches of thiotrophic mats associated with gas chimneys on the Storegga Slide at the Norwegian continental margin were investigated (Chapter 5). These mats varied greatly in appearance and were found to also harbor gelatinous mats of *Thiobacterium* spp. (Chapter 2), a long-known, but so far poorly understood sulfide oxidizer.

Pockmarks. Pockmarks have been found in most oceans and are generally defined as crater-like depressions on the seafloor which can be of various sizes and shapes (Hovland *et al.*, 2002). Pockmarks can be formed by multiple processes. When related to near-surface gas reservoirs (gas chimneys), their formation is thought to be triggered by an initial outburst of escaping gas and fluids. In the following, pockmarks are thought to be characterized by microseepage until gas venting may cease and they eventually become dormant. Pockmarks may be re-activated by a later gas outburst if the shallow gas reservoirs can be recharged (Hovland and Judd, 1988; Hovland *et al.*, 2005; Cathles *et al.*, 2010). Vented gas and fluids usually contain methane, but can also carry higher hydrocarbons.

Within this thesis, a large *Arcobacter* mat associated with sulfidic muds in a pockmark-dominated area of the central province of the Nile Deep Sea Fan off Egypt (Chapter 4) was investigated. Further, small (20 to 50 cm in diameter) thiotrophic mat patches found at complex pockmarks in the Nyegga area off Norway (Figure 1.1B) were studied regarding the mat-associated sulfide oxidizer diversity and prevailing geochemical gradients (Chapter 5).

Mud volcanoes. Submarine mud volcanoes are generally referred to as elevated seafloor structures formed by episodic extrusions of muds and fluids from subsurface reservoirs (Milkov, 2000; Dimitrov, 2002; Judd *et al.*, 2002). They have been found on continental

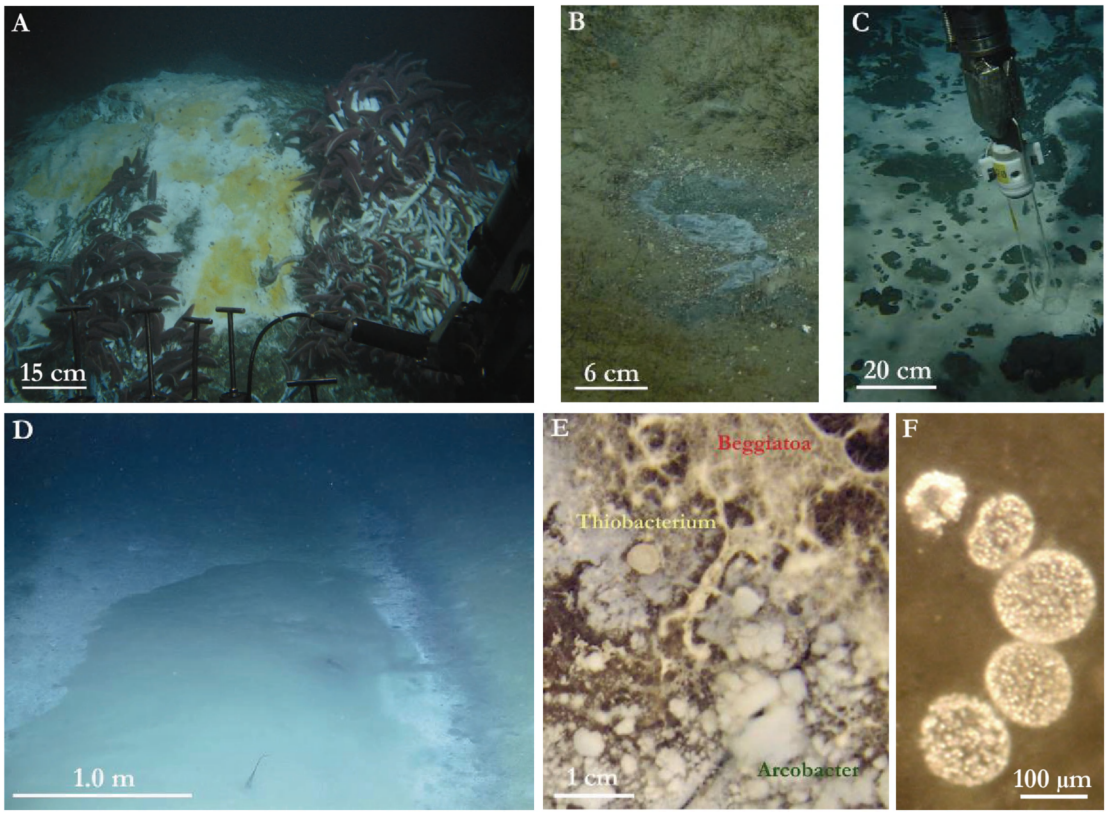


Figure 1.1: Mats formed by sulfide-oxidizing bacteria. (A) White and orange *Beggiatoa* mats at the Guaymas Basin hydrothermal vent field in the Gulf of California. Source: Woods Hole Oceanographic Institution, MA, USA. (B) Small mat patch found at pockmarks in the Nyegga area off Norway. Source: Ifremer, France. (C) White *Beggiatoa* mat at the Håkon Mosby mud volcano off Norway. Source: Ifremer, France. (D) A *Thiomargarita* spp.-dominated white mat was found on top of a sulfidic mud outflow at the flank of the Amon mud volcano off Egypt. Part of the mat is covered with brine. Source: Marum, University Bremen, Germany. (E) Close up on a whale bone that was recovered off Sweden. This bone was only 11 cm in diameter and was densely covered with mats of three different sulfide oxidizers, including *Beggiatoa* spp., *Arcobacter* spp. and *Thiobacterium* spp. (F) *Thiomargarita namibiensis* recovered off Namibia. Picture by Verena Salman, MPI Bremen.

shelves, continental and insular slopes, and in abyssal parts of inland seas. The number of deep-water mud volcanoes was estimated to reach 10^3 to 10^5 (compared to 900 known terrestrial mud volcanoes; Milkov, 2000; Dimitrov, 2002). Gas venting at mud volcanoes is dominated by the emission of methane (Judd *et al.*, 2002). The presence of subsurface (methane) gas hydrates is an important feature of submarine mud volcanoes (Milkov, 2000; Jorgensen and Boetius, 2007).

Free-living sulfide oxidizers forming dense mats of various sizes are often observed at mud volcanoes. A prominent example are the vast white *Beggiatoa* spp. mats present at the Håkon Mosby mud volcano (Figure 1.1C) that had previously been studied regarding chemical gradients and co-occurring meiofauna (de Beer *et al.*, 2006; Niemann *et al.*, 2006; van Gaever *et al.*, 2006; Lichtschlag *et al.*, 2010). Within this thesis, the phylogenetic diversity of the mat-forming species was further investigated (Chapter 5). Furthermore, smaller patches of white mats dominated by *Beggiatoa* spp. and potential vacuolated attached filaments (VAF) occurring near the center of the Amon mud volcano in the Eastern Mediterranean Sea were analyzed (Chapter 4). This study is of special interest, as spatially separated microbial mats formed by either *Thiomargarita* spp. (brine-influenced; Figure 1.1D; Chapter 3) or *Arcobacter* spp. (pockmarks; Chapter 4) could be investigated during the same cruise, expanding our knowledge on occurrence and occupied niches of these very organisms.

1.1.3 Food Falls

Food falls, or large organic falls, such as sunken woods, whale carcasses or kelp create locally restricted, organic-rich oases on the seafloor. In the past, especially whale falls (both in shallow waters and the deep sea) have been investigated. When deposited on the deep-sea floor, whale carcasses go through various successional stages. A sulfophilic stage, fueled by sulfide production in the lipid-rich bones and the organically enriched sediment, may last for years or decades, thereby supporting species-rich and trophically complex communities (Smith and Baco, 2003; Treude *et al.*, 2009). Microbial sulfate reduction was postulated to be the key process in hydrogen sulfide production (Treude *et al.*, 2009). As whale falls may occur anywhere on the seafloor and are not restricted to mid-ocean ridges or ocean margins such as vents and seeps, sunken whale remains are thought to provide dispersal 'stepping stones' for sulfide-dependent chemosynthetic communities over large areas of the deep-sea floor (Smith *et al.*, 1989; Bennett *et al.*, 1994; Smith and Baco, 2003).

Microbial mats formed by sulfide-oxidizing bacteria have often been observed on whale bones and the surrounding sediment. For example, thick yellow mats formed by filamentous sulfide oxidizers have been observed at a deep-sea whale fall in the Santa

Cruz Basin (Treude *et al.*, 2009). White and yellow mats, also dominated by filamentous bacteria, were found to colonize whale bones investigated in the Santa Catalina Basin (Bennett *et al.*, 1994; Deming *et al.*, 1997). In Monterey Canyon, white filaments expanded within 8 months on the whale skeleton and the adjacent sediment up to 15 m^2 (Goffredi *et al.*, 2004). On recovered whale bones off Sweden that were kept in aquaria flushed with filtered seawater (Glover *et al.*, 2005), mats of *Beggiatoa* spp., *Arcobacter* spp. and *Thiobacterium* spp. could be observed in close association on the very same, only 11 cm in diameter, whale bone (Figure 1.1E; partly included in Chapter 2).

1.1.4 Oxygen Minimum Zones

Oxygen minimum zones (OMZs) are characterized by persistent low oxygen concentrations (< 0.5 ml L^{-1}, < 22 μM). They generally form in areas where high surface productivity, as e.g. caused by strong upwelling, is coupled to high remineralization rates of sinking organic matter, the latter involving depletion of oxygen within the water column and the production of reduced compounds. Hydrogen sulfide is mainly derived from microbial sulfate reduction. The largest OMZs have been found in the Eastern Pacific Ocean (e.g. coastal upwelling areas off Peru and Chile), off southwest Africa (e.g. the Namibian shelf and slope), in the Arabian Sea and in the Bay of Bengal (Levin, 2003).

Extensive populations of sulfide-oxidizing bacteria have been observed on the Chilean and Peruvian shelf (Gallardo, 1977; Maier *et al.*, 1990) and in the Namibian shelf (Schulz *et al.*, 1999), harboring giant sulfur bacteria of the genera *Beggiatoa*, *Thioploca* and *Thiomargarita* (Figure 1.1F). Owing to the oxygen depletion, these organisms needed to develop special strategies for thriving in these areas (discussed in Chapter 7.1).

1.1.5 Shallow-water Marine Sediments

The top 200 m of the ocean are referred to as marine shallow waters. Shallow-water marine sediments are usually rich in organic matter. Microbial sulfate reduction in deeper sediment layers produces sulfide that fuels both symbiotic (Dubilier *et al.*, 2008) and free-living sulfide oxidizers. Thereby, sulfide is mainly produced through dissimilatory sulfate reduction, in which sulfate-reducing bacteria use sulfate as their terminal electron

acceptor while respiring short-chain fatty acids or other small organic compounds derived from organic matter remineralization (Eq. 1.2):

$$2[CH_2O] + SO_4^{2-} \rightarrow 2HCO_3^- + H_2S \qquad \text{(Eq. 1.2)}$$

Due to the availability of high amounts of organic matter, oxygen may become depleted rapidly in the top few millimeters, whereas sulfide is produced in deeper, anoxic sediment layers. Hence, the electron donor (sulfide) and main electron acceptor (oxygen) are spatially separated, and sulfide-oxidizing bacteria need to develop special strategies to survive under such conditions. Vacuolated species of the known mat-forming sulfide oxidizers *Beggiatoa* may shuttle between oxic and anoxic sediment layers by their gliding motility and use internally stored nitrate as alternative electron acceptor (Sayama, 2001; Mußmann *et al.*, 2003; Teske and Nelson, 2006). As such, they might play an important role in the cycling of nitrogen, either through the production of dinitrogen or ammonia, the latter potentially contributing to coastal eutrophication (Sayama, 2001). Within this thesis, coastal sediments off Svalbard (Arctic) were investigated regarding the occurrence, biomass and size distribution of the giant sulfur bacterium *Beggiatoa* (Chapter 6).

1.2 Sulfur-Oxidizing Bacteria

Biological oxidation of sulfide or other reduced sulfur compounds (also referred to as 'sulfur oxidation') is known to be performed by microorganisms of the domains Archaea and Bacteria. Eukaryotic organisms, including typical seep and vent fauna like tubeworms, mussels, clams and shrimps, benefit only indirectly from sulfur oxidation through their symbiotic association with sulfur-oxidizing bacteria. Symbiotic sulfur oxidizers can either occur as epibionts (living on the surface of their host) or endobionts (living inside their host), and are supplied by various behavioral and physiological strategies of their host species (Dubilier *et al.*, 2008).

Among the Archaea, aerobic sulfur oxidation seems to be restricted to members of the order Sulfolobales (Friedrich *et al.*, 2001; Friedrich *et al.*, 2005; and references therein). Prominent examples include organisms of the genera *Acidianus* and *Sulfolobus*.

Within the Bacteria, sulfur oxidation is widely distributed among taxonomically different groups (Figure 1.2). Generally, one can distinguish between anaerobic phototrophic and aerobic chemosynthetic sulfur oxidizers.

Phototrophic sulfur bacteria use inorganic sulfur compounds as electron donors while fixing CO_2 in anoxygenic photosynthesis. They are generally divided into the green sulfur bacteria (family *Chlorobiaceae* within the phylum Chlorobi) and the purple sulfur bacteria (families *Chromatiaceae* and *Ectothiorhodospiraceae* within the Gammaproteobacteria), but a number of other bacteria including filamentous anoxygenic phototrophs (family *Chloroflexaceae* within the phylum Chloroflexi), purple non-sulfur bacteria (Alpha- and Betaproteobacteria) or heliobacteria (family *Heliobacteraceae* within the phylum Firmicutes) and phototrophic acidobacteria are also assigned to this group (Frigaard and Dahl, 2008).

The chemosynthetic sulfur oxidizers gain their energy through chemical redox reactions by using sulfide or other reduced sulfur compounds as electron donors. They encompass diverse phylogenetic lineages and include known sulfur-oxidizing animal symbionts as well as several free-living bacteria. Prominent among the free-living sulfur oxidizers are the so-called 'colorless sulfur bacteria'. The term 'colorless' refers to the absence of photopigments in these organisms, even though high cytochrome content may cause colored appearance of colonies or dense cultures (Robertson and Kuenen, 2006). Traditionally assigned to the group of colorless sulfur bacteria were, for example, bacteria of the genera *Thiobacillus*, *Thiomicrospira*, *Thiovulum*, *Thiothrix*, *Thiobacterium*, *Beggiatoa*, *Thioploca*, *Macromonas, Thiospira* or *Achromatium*. However, some of these genera are included in the colorless sulfur bacteria mainly based on their repeated occurrence in sulfidic habitats or the appearance of internal sulfur inclusions, but their nutritional status is still uncertain and may range from chemolithoautotrophy to merely detoxifying sulfide oxidation (La Rivière and Schmidt, 2006; Robertson and Kuenen, 2006). In addition, other bacteria also capable of growth on reduced sulfur compounds are known, including members of the genera *Arcobacter*, *Sulfurimonas*, *Sulfurovum*, *Halothiobacillus*,

Paracoccus, *Alcaligenes* or *Pseudomonas* (Robertson and Kuenen, 2006; Sievert *et al.*, 2008; and references therein).

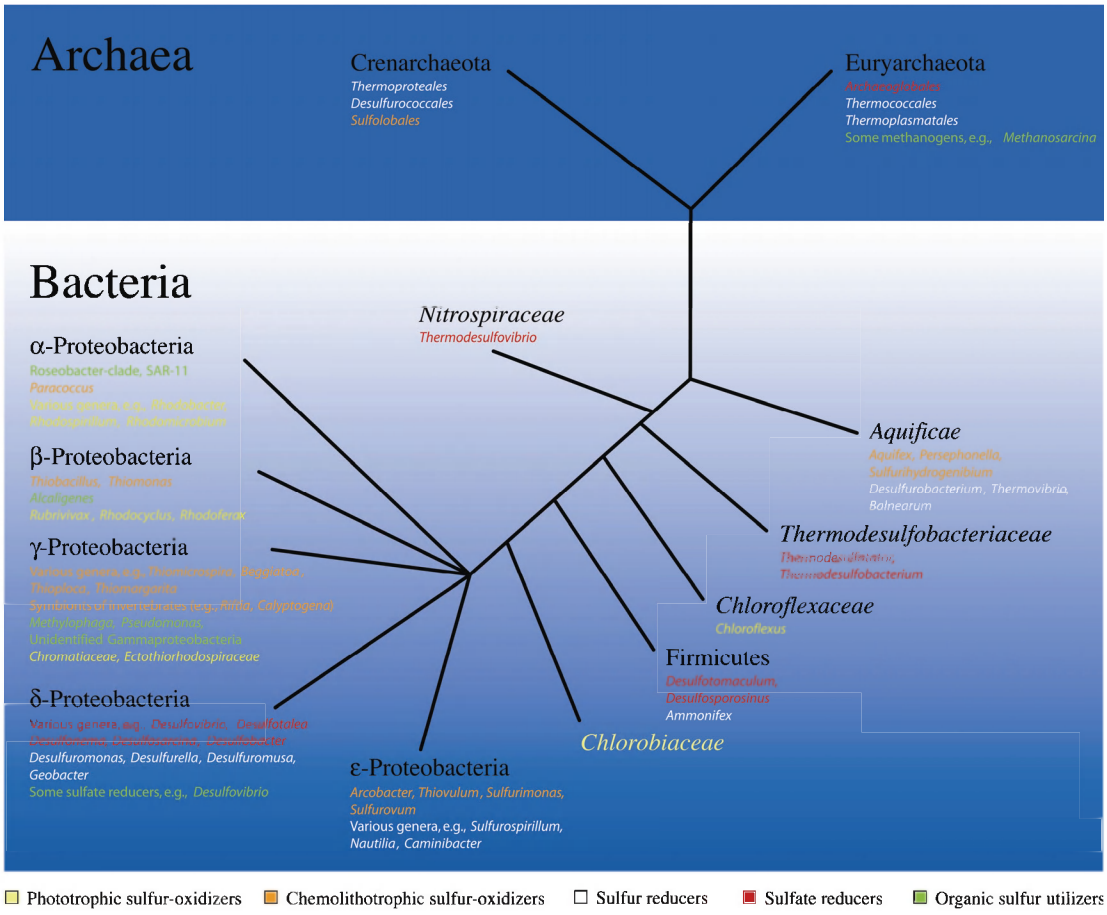


Figure 1.2: Schematic phylogenetic tree giving an overview of different organisms involved in microbial sulfur oxidation (and sulfate reduction). Picture taken from Sievert *et al.* (2007a).

The capability to occur in dense populations and to form microbial mats in marine reduced habitats has been observed for various sulfide-oxidizing bacteria. Prominent examples are members of the genera *Beggiatoa* (Teske and Nelson, 2006), *Thiomargarita* (Schulz, 2006), *Thioploca* (Gallardo, 1977), *Thiobacterium* (La Rivière and Schmidt, 2006), *Thiovulum* (Jørgensen and Revsbech, 1983; Bernard and Fenchel, 1995), *Achromatium* (Dando *et al.*, 1995) and *Arcobacter* (Sievert *et al.*, 1999; Sievert *et al.*, 2007b), as well as vacuolated attached filaments (Kalanetra *et al.*, 2004). Very often, one type (genus) of

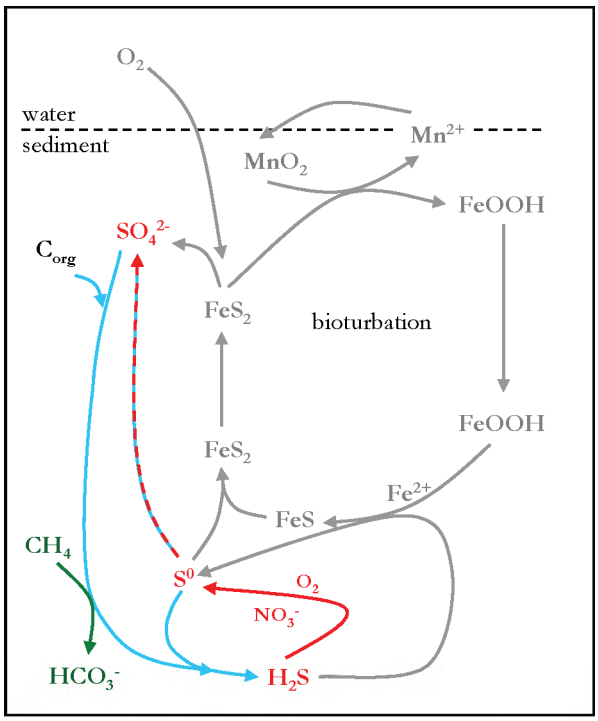
sulfide oxidizer dominates a certain mat, but also mixed mats comprising several of these conspicuous bacteria exist (Nelson *et al.*, 1989; Glud *et al.*, 2004; de Beer *et al.*, 2006). In this context, mat succession (or more precisely, species succession) within a microbial mat seems an important issue when analyzing niche differentiation between different mat-forming sulfide oxidizers (Bernard and Fenchel, 1995).

All mat-forming bacteria discussed in this thesis are free-living, chemosynthetic sulfide/sulfur oxidizers, including gammaproteobacterial species of the genera *Beggiatoa* and *Thiomargarita* as well as a novel group of vacuolated attached filaments (order Thiotrichales), epsilonproteobacterial species of the genus *Arcobacter,* and *Thiobacterium* (no 16S rRNA gene sequence available).

1.3 Microbial Sulfur Oxidation

Generally, marine sulfide-oxidizing bacteria are not only competing amongst each other for sulfide, but to some extent also against chemical sulfide oxidation (La Rivière and Schmidt, 2006). Part of the sulfide is chemically oxidized or precipitated in the form of iron sulfide (FeS) or pyrite (FeS_2). These sulfide minerals cause a blackish appearance of the sediment that has often been observed beneath microbial sulfide oxidizer mats, e.g. at cold seeps, or contribute to the so-called 'black smoke' at hydrothermal vents. However, analyses conducted with the mat-forming sulfur bacterium *Beggiatoa* have shown that biological sulfide oxidation may be up to 3 orders of magnitude faster than chemical conversion of sulfide, thus allowing for efficient competition with chemical sulfide oxidation (Jørgensen and Revsbech, 1983; Nelson *et al.*, 1986). Nevertheless, sulfide removal from sediments is not necessarily dominated by mat-forming bacteria, as shown for *Beggiatoa* spp.-containing coastal sediments (Preisler *et al.*, 2007). In Figure 1.3 the principle cycle of sulfur within marine sediments is illustrated as well as important reactions of chemosynthetic (biological) sulfide/sulfur oxidation that mat-forming bacteria may use to gain energy for growth in their natural environments (Eq. 1.3-1.10).

The Fate of Sulfide in Marine Sediments



sulfate reduction/disproportionation
anaerobic oxidation of methane (AOM)
microbial sulfide oxidation
chemical sulfide oxidation/precipitation

Figure 1.3: The principle sulfur cycle within marine sediments (modified after Jørgensen and Kasten, 2006).

Hydrogen sulfide is produced through microbial reduction of sulfate coupled to the remineralization of organic matter. Part of the sulfide is chemically oxidized or precipitated in the form of iron sulfides, the latter causing a gray-black appearance of the sediment. Sulfur intermediates, such as elemental sulfur or thiosulfate, may be disproportionated by certain bacteria, thereby providing a shunt in the sulfur cycle (Jørgensen and Kasten, 2006). In cold seep ecosystems, sulfate reduction is tightly linked to the anaerobic oxidation of methane (AOM).

Mat-forming sulfide-oxidizing bacteria oxidize sulfide to elemental sulfur and mostly further to sulfate by using oxygen (at the sediment surface). Vacuolated, gliding species of the giant sulfur bacteria *Beggiatoa* and *Thioploca* may also use internally stored nitrate for sulfide oxidation in deeper sediment layers (Teske and Nelson, 2006).

Important Reactions in Chemosynthetic Microbial Sulfide/Sulfur Oxidation

with oxygen

$$2HS^- + O_2 \rightarrow 2S^0 + 2OH^- \quad \text{(Eq. 1.3)}$$

$$2S^0 + 3O_2 + 2H_2O \rightarrow 2SO_4^{2-} + 4H^+ \quad \text{(Eq. 1.4)}$$

$$HS^- + 2O_2 \rightarrow SO_4^{2-} + H^+ \quad \text{(Eq. 1.5)}$$

with nitrate

$$4HS^- + NO_3^- + 6H^+ \rightarrow 4S^0 + NH_4^+ + 3H_2O \quad \text{(Eq. 1.6)}$$

$$4S^0 + 3NO_3^- + 7H_2O \rightarrow 4SO_4^{2-} + 3NH_4^+ + 2H^+ \quad \text{(Eq. 1.7)}$$

$$HS^- + NO_3^- + H_2O + H^+ \rightarrow SO_4^{2-} + NH_4^+ \quad \text{(Eq. 1.8)}$$

$$5HS^- + 8NO_3^- \rightarrow 5SO_4^{2-} + 4N_2 + H_2O + 3OH^- \quad \text{(Eq. 1.9)}$$

$$5HS^- + 2NO_3^- + 7H^+ \rightarrow 5S^0 + N_2 + 6H_2O \quad \text{(Eq. 1.10)}$$

Microbial (dissimilatory) oxidation of reduced sulfur compounds follows several different enzymatic pathways (Friedrich *et al.*, 2005; Meyer and Kuever, 2007; Frigaard and Dahl, 2008; and references therein). Based on pioneering genetic studies with the phototrophic sulfur bacterium *Allochromatium vinosum* and the autotrophic alphaproteobacterium *Paracoccus pantotrophus*, generally two pathways of sulfur oxidation are distinguished among neutrophilic sulfur bacteria (Friedrich *et al.*, 2005; Frigaard and Dahl, 2008; Sievert *et al.*, 2008). While the Sox pathway is driven by a multienzyme complex catalyzing the complete oxidation of reduced sulfur compounds to sulfate, the other pathway has elemental sulfur and sulfite as intermediates (Sievert *et al.*, 2008 and references therein). Formation of sulfur granules is generally mediated by a sulfide:quinone reductase (SQR). Oxidation of sulfur (or sulfide) to sulfite can be catalyzed by reverse-acting dissimilatory sulfite reductase (DsrAB). Sulfite oxidation to sulfate is likely catalyzed by reverse-operating enzymes of the sulfate-reduction pathway and proceeds either directly involving a sulfite:acceptor oxidoreductase, or via the intermediate adenosine-5'-phosphosulfate (APS) using APS reductase (Apr) and ATP sulfurylase (Meyer and Kuever, 2007; Sievert *et al.*, 2008; and references therein). Functional gene assays used for identifying sulfur bacteria from environmental samples mainly focused on *aprBA* (Meyer and Kuever, 2007) and *soxB* genes (Meyer *et al.*, 2007). However, these genes were found to be influenced by lateral gene transfer, why currently the potential of *dsrAB* as phylogenetic marker for sulfur-oxidizing bacteria is under investigation (Loy *et al.*, 2009).

Autotrophic CO_2 fixation in sulfur bacteria mainly proceeds via the reductive pentose phosphate cycle (Calvin-Benson-Bassham cycle, CBB) or the reductive tricarboxylic acid cycle (rTCA; Friedrich *et al.*, 2001; Sievert *et al.*, 2008). Each process is catalyzed by characteristic enzymes, and genes encoding those are often used as diagnostic markers for characterizing the respective CO_2-fixation process in bacteria. RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase, is the key enzyme for the CBB cycle and has been found in a variety of symbiotic and free-living sulfur-oxidizing bacteria, including known mat-forming organisms of the genera *Beggiatoa* and *Thioploca* (McHatton *et al.*, 1996; Otte *et al.*, 1999; Teske and Nelson, 2006; Sievert *et al.*, 2008). Other mat-forming sulfide oxidizers like marine *Arcobacter* spp. showed no RuBisCO

activity (based on studies with the coastal strain '*Candidatus* Arcobacter sulfidicus') and are thought to fix CO_2 mainly via the rTCA cycle (Wirsen *et al.*, 2002; Sievert *et al.*, 2008). Besides functional gene analyses, ^{13}C-based isotopic analyses have been shown to be a valuable tool when investigating carbon fixation in uncultured, environmental organisms. Within microbial sulfide oxidizer mats, depleted carbon isotopic signatures can e.g. reach values of -43‰ (van Gaever *et al.*, 2006 and references therein).

Through the build up of huge biomasses, marine mat-forming sulfide-oxidizing bacteria can significantly contribute to primary production in their habitats (Boetius and Suess, 2004; Lichtschlag *et al.*, 2010). Marine chemolithotrophic *Beggiatoa* can gain a biomass of 8.4 to 15.9 g dry weight per mol sulfide oxidized, corresponding to 0.35 to 0.66 mol C per mol sulfide oxidized, assuming that 50% of the dry weight is carbon (Nelson *et al.*, 1986; Hagen and Nelson, 1997). Lichtschlag and colleagues estimated for *Beggiatoa*-dominated mats at the Håkon Mosby mud volcano (4.2×10^7 *Beggiatoa* filaments per m^2 with a wet biomass of 3.8 g m^{-2}) an annual sulfide-based chemosynthetic biomass production of 1.5 to 2.8 mol C m^{-2}, equaling up to 8.4×10^4 mol C per year for the whole mat-covered habitat and reflecting a complete turnover of thiotrophic biomass within four days (Lichtschlag *et al.*, 2010 and references therein). As such, these organisms can support a heterotrophic food web (van Gaever *et al.*, 2006) by acting as important primary producers and at the same time providing efficient benthic filters against the toxic gas hydrogen sulfide.

1.4 Objectives

As outlined in the previous sections, mat-forming sulfide-oxidizing bacteria are intriguing, diverse, highly adapted microorganisms with important biogeochemical functions. As 'gradient organisms' they seem to occupy very defined and specialized ecological niches on the seafloor, regarding the availability and concentration of their electron donor sulfide and their electron acceptor oxygen (or nitrate). As such, it seems intriguing how these bacteria manage to occur in such high numbers on the seafloor, forming visible, dense microbial mats.

Many aspects regarding the diversity of mat-forming sulfide oxidizers, as well as the geological drivers favoring massive populations of these organisms require further investigation. The main objectives of this thesis therefore were to expand our knowledge on these conspicuous organisms by the application of molecular techniques targeting their diversity, and the *in situ* and *ex situ* investigation of geochemical gradients and parameters that potentially might help to explain the dominance of each sulfide oxidizer in the respective mat habitat. In particular, this thesis aimed to answer the following questions:

I) How diverse are mat-forming sulfide-oxidizing bacteria within one habitat?
- e.g. within a small mat patch as compared to an extensive mat?
- e.g. within a seep system?

II) What are the prevailing gradients of oxygen and sulfide in these habitats?

III) Is there a correlation between the observed diversity and these gradients as well as other sample site specific properties (niche differentiation)?

The molecular techniques I used mainly included the construction of 16S rRNA gene based clone libraries to expand the existing 'database' of known mat-forming sulfide-oxidizing species, as well as fluorescence *in situ* hybridization (Amann and Fuchs, 2008) for further exploring phylogenetic relatedness of the bacteria under investigation. Further, I collaborated with scientists from other research disciplines to obtain

geochemical data characterizing the investigated mat habitats, allowing me to infer on the ecological niches occupied by the different sulfide oxidizers. The main study sites within this thesis were located along the European continental margin and included shallow-water marine sediments off the arctic archipelago Svalbard, the Håkon Mosby mud volcano, seep-related structures in the Storegga and Nyegga area, a shallow-water cave off Greece, and cold seeps on the Nile Deep Sea Fan (Figure 1.4).

Figure 1.4: Main study sites in this thesis.

1.5 Publication Outline

Novel observations of *Thiobacterium*, a Gammaproteobacterium producing gelatinous mats

Stefanie Grünke, Anna Lichtschlag, Dirk de Beer, Marcel Kuypers, Tina Lösekann-Behrens, Alban Ramette, Antje Boetius

(The ISME Journal advance online publication 11.03.2010, doi10.1038/ismej.2010.23)

This study of marine sulfidic habitats provides three novel observations of conspicuous gelatinous mats formed by *Thiobacterium* spp. on a whale bone, in deep-sea sediment and in a shallow-water cave. Sampling, macroscopic and microscopic analyses of the *Thiobacterium* mats were done by S. Grünke, H. Røy, A. Boetius and D. de Beer. Staining and phylogenetic analyses of all mats, including the construction of clone libraries, sequence analysis and fluorescence *in situ* hybridization, were done by S. Grünke. Statistical analyses were done by S. Grünke with the help of A. Ramette. An additional single cell sorting approach was carried out by T. Lösekann-Behrens. *Ex situ* microprofiles were recorded and analyzed by A. Lichtschlag and D. de Beer. Experiments regarding the composition of the cave gelatinous mats were done with the help of M. Kuypers. The manuscript was written by S. Grünke with support and input by all co-authors.

A novel, mat-forming *Thiomargarita* population associated with a sulfidic fluid flow from a deep-sea mud volcano

Anne-Christin Girnth, Stefanie Grünke, Anna Lichtschlag, Janine Felden, Katrin Knittel, Frank Wenzhöfer, Dirk de Beer, Antje Boetius

(18.04.2010 – In review with Environmental Microbiology)

This study reports on a novel *Thiomargarita* population discovered on a sulfidic mud outflow at the flank of the Amon mud volcano (Nile Deep Sea Fan, Eastern Mediterranean Sea). Sampling and onboard microscopic analyses of the *Thiomargarita* mat

were done by S. Grünke and A. Boetius. Morphological examination of the *Thiomargarita* cells, clone library construction, sequence analysis, tree reconstruction and fluorescence *in situ* hybridization experiments were carried out by A.-C. Girnth as part of a master thesis at the Leibniz University Hannover (Germany) and the MPI Bremen (Germany). Technical support was given by S. Grünke and K. Knittel. *In situ* microsensor measurements were performed by A. Lichtschlag and D. de Beer. Measurements with a recording current meter (RCM) were conducted by F. Wenzhöfer. Rates of sulfate reduction and anaerobic oxidation of methane as well as total cell counts were determined by J. Felden and A. Boetius. The manuscript was written by A.-C. Girnth and S. Grünke with support by all co-authors.

Niche differentiation among mat-forming, sulfide-oxidizing bacteria at cold seeps of the Nile Deep Sea Fan (Eastern Mediterranean Sea)

Stefanie Grünke, Janine Felden, Anna Lichtschlag, Anne-Christin Girnth, Dirk de Beer, Frank Wenzhöfer, Antje Boetius

(18.04.2010 – In preparation for Geobiology)

This study discusses the niche differentiation between *Beggiatoa* spp. and vacuolated attached filaments, *Thiomargarita* spp. and *Arcobacter* spp. forming mats in cold seep habitats on the Nile Deep Sea Fan. Sampling and microscopic analyses onboard were conducted by S. Grünke, D. de Beer and A. Boetius. Phylogenetic analyses, including the construction of clone libraries and sequence analysis were done by S. Grünke and A.-C. Girnth. *In situ* microsensor measurements were performed and analyzed by A. Lichtschlag and D. de Beer. Benthic chamber measurements were conducted by J. Felden and F. Wenzhöfer. Rates of sulfate reduction were determined by J. Felden and A. Boetius. Pore-water sampling and measurements as well as measuring elemental sulfur concentrations were done by A. Lichtschlag. Microbial abundances were determined by A. Boetius and S. Grünke. The manuscript was written by S. Grünke with support and input by all co-authors.

Diversity of mat-forming sulfide oxidizers in deep-sea thiotrophic bacterial mats associated with cold seeps of the Norwegian continental margin

Stefanie Grünke, Anna Lichtschlag, Dirk de Beer, Janine Felden, Antje Boetius

(18.04.2010 – In preparation for Applied and Environmental Microbiology)

This study compares different thiotrophic bacterial mats, including small isolated mat patches and extensive microbial mats at different cold seep systems on the Norwegian continental margin. Phylogenetic analyses, including the construction of clone libraries, sequence analysis and tree reconstruction, were done by S. Grünke. Sampling and microscopic analyses onboard were conducted by A. Boetius, D. de Beer and S. Grünke. *In situ* and *ex situ* microsensor measurements were performed and analyzed by A. Lichtschlag and D. de Beer. Rates of sulfate reduction and total cell counts were determined by J. Felden and A. Boetius. All other geochemical analyses were carried out by A. Lichtschlag. Microbial abundances were determined by A. Boetius. The manuscript was written by S. Grünke with support and input by all co-authors.

Filamentous sulfur bacteria, *Beggiatoa* spp., in arctic marine sediments (Svalbard, 79°N)

Bo Barker Jørgensen, Rita Dunker, Stefanie Grünke, Hans Røy

(Submitted to FEMS Microbiology Ecology 07.12.2009)

This study investigated the occurrence of *Beggiatoa* spp. in shallow coastal sediments of the arctic archipelago Svalbard. Sampling, biomass determination and geochemical measurements were performed by B.B. Jørgensen, R. Dunker and H. Røy. Phylogenetic analyses were performed by S. Grünke. The manuscript was written by B.B. Jørgensen with support and input by all co-authors.

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Chapter 2

Novel Observations of *Thiobacterium*, a Gammaproteobacterium producing Gelatinous Mats

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Abstract

The genus *Thiobacterium* includes uncultivated rod-shaped microbes containing several spherical grains of elemental sulfur and forming conspicuous gelatinous mats. Due to the fragility of mats and cells, their 16S rRNA genes have not been phylogenetically classified. This study examined the occurrence of *Thiobacterium* mats in three different sulfidic marine habitats: a submerged whale bone, deep-water seafloor and a submarine cave. All three mats contained massive amounts of *Thiobacterium* cells and were highly enriched in sulfur. Microsensor measurements and other biogeochemistry data suggest chemoautotrophic growth of *Thiobacterium*. Sulfide and oxygen microprofiles confirmed the dependence of *Thiobacterium* on hydrogen sulfide as energy source. Fluorescence *in situ* hybridization indicated that *Thiobacterium* spp. belong to the Gammaproteobacteria, a class that harbors many mat-forming sulfide-oxidizing bacteria. Further phylogenetic characterization of the mats led to the discovery of an unexpected microbial diversity associated with *Thiobacterium*.

1. Introduction

The genus *Thiobacterium* was first described by Molisch in 1912 (Molisch, 1912). In the years following this discovery, the conspicuous sulfur-storing, non-motile rods embedded in a gelatinous matrix were found in other marine and continental locations world-wide. They occur in thermal and sulfur springs (Devidé, 1952; Lackey and Lackey, 1961; Lackey *et al.*, 1965; Vouk *et al.*, 1967; Anagnostidis, 1968; Scheminzky *et al.*, 1972; Fjerdingstad, 1979), but also in sulfidic marine and brackish waters (Molisch, 1912; Lackey *et al.*, 1965; Seki and Naganuma, 1989). So far two different morphologies of the gelatinous mats have been described: a spherical or bladder-like shape (Molisch, 1912; Devidé, 1952; Vouk *et al.*, 1967; Anagnostidis, 1968; Scheminzky *et al.*, 1972; Seki and Naganuma, 1989) and a dendroid shape (Lackey and Lackey, 1961; Lackey *et al.*, 1965). First experiments on the chemical and elemental composition of the gelatinous material led to the assumption that it consists of an allophane-sulfur-hydrogel (Vouk *et al.*, 1967; allophane is an amorphous hydrous aluminum silicate). In contrast to the varying shapes and sizes of the gelatinous material, the morphology of the rod-shaped microbes was very consistent in most studies. An experiment targeting the physiology of *Thiobacterium* suggested that under aerobic conditions these organisms may express a eurythermally mesophilic and slightly halophilic behavior (Seki and Naganuma, 1989). Most importantly, the enrichment study indicated that *Thiobacterium* cells are themselves forming the gelatinous masses, most likely to retain a favorable spatial position in their habitat with access to the sulfide and oxygen sources.

Despite all past observations, knowledge on the genus *Thiobacterium* is still rather poor, because none of its members have been cultivated. By morphological analogy and ecological context, it was associated with the family Thiotrichaceae of the Gammaproteobacteria in Bergey's manual of systematic bacteriology (Kuenen, 2005) and the Encyclopedia of Life (http://www.eol.org/pages/97513). Its classification as one genus *Thiobacterium* is based on consistent morphological observations of rod-shaped cells with chain-like inclusions of up to 20 spherical sulfur granules (Lackey and Lackey, 1961), embedded in gelatinous matter (Kuenen, 2005). However, no 16S rRNA gene sequence has yet been attributed to a *Thiobacterium* sp., and the taxonomic positioning of this genus within the group of sulfur bacteria is uncertain. Furthermore, the energy

sources, niche-selection and ecological role of *Thiobacterium* remain unknown. The main problems in the investigation of these mat-forming bacteria are their rarity and the fragility of mats, colonies and cells (Molisch, 1912; Lackey and Lackey, 1961; Vouk *et al.*, 1967; Seki and Naganuma, 1989).

This study investigated *Thiobacterium* mats from three sulfidic marine habitats, including a minke whale bone collected offshore Sweden, deep-sea sediments from the Storegga Seeps off Norway, and a shallow-water cave in Greece. To broaden our knowledge on the genus *Thiobacterium* and its ecological role, (i) the geochemical gradients within the gelatinous masses were analyzed, (ii) the cells were microscopically and phylogenetically characterized, and (iii) the overall microbial community composition associated with the gelatinous mats was examined.

2. Materials and Methods

2.1 Site description and sample collection

All sampling locations and prevailing environmental conditions are described in Table 1. Whale bones were recovered from the carcass of a female minke whale that was previously implanted at 125 m depth in Kosterfjord, Sweden (58°53.1'N, 11°6.4'E; Glover *et al.*, 2005; Dahlgren *et al.*, 2006). Since their recovery, the bones had been maintained at 7-8°C in aquaria flushed with filtered seawater (Glover *et al.*, 2005). Sampling of a small spherical *Thiobacterium* mat was achieved by pipetting.

Deep-sea *Thiobacterium* mats were obtained from the Storegga area off Norway during the "VICKING" expedition with the RV *Pourquoi Pas?* and the ROV *Victor 6000* (IFREMER) in June/July 2006 from a small seep structure covered with a whitish mat (Dive 275-05; core CT-02; 64°45.27'N, 4°58.87'E). Aboard the ship, the *Thiobacterium*-containing core was immediately transferred to a cold room. Sampling occurred directly after the dive through mechanical disruption with a pipette.

The gelatinous mats of the shallow-water cave ('Blue Pot Cave'; 39°10.66'N, 20°12.54'E) off Paxos (Greece) were first discovered by Paul Bowbeer (Oasi-Sub-Diving) and Dr. Thomas Beer. Initial samples of the spheres, native and fixed in 4% formaldehyde, reached the MPI Bremen in September 2006. A second sampling in

Paxos took place in August 2007. The partially sun-lit cave is located at approx. 23 m depth. Whole gelatinous spheres and cave water were sampled into sterile tubes and transported in a cool chamber to the on-site laboratory. Subsampling of the gelatinous mats was done either by preserving whole spheres for biogeochemical analyses, or by dissecting single spheres with a sterile scalpel for microscopic, phylogenetic and FISH analyses.

Table 1: Physicochemical characteristics of habitats in which *Thiobacterium* spp.-resembling microbes and gelatinous mats occurred.

Location[a]	Habitat	H_2S[b]	pH	Temp.	Salinity	Depth[c]	Reference
Trieste (Italy)	Marine water	Assumed	-	-	-	1 m	Molisch (1912)
Dubrovnik (Croatia)	Sulfur spring[d]	Detected	-	-	-	1 m	Devidé (1952)
Venice (Florida)	Sulfur spring[d]	~5 µM	7.2	29°C	17‰	1 m	Lackey and Lackey (1961) Lackey *et al.* (1965)
Titusville (Florida)	Polluted water[d]	-	-	12.5°C	17‰	Shallow	Lackey and Lackey (1961) Lackey *et al.* (1965)
Cedar Keys (Florida)	Tide pools[d]	-	-	-	17‰	1 m	Lackey *et al.* (1965)
Bad Gastein (Austria)	Thermal spring	~15-23 µM	8.7-9.3	43.5-45.5°C	-	0 m	Vouk *et al.* (1967)
Amélie-Les-Bains (France)	Thermal spring	~103 µM[e]	9.1[e]	22°C; 45°C	-	0 m	Scheminzky *et al.* (1972)
Greece	Hot springs	Detected	-	-	-	0 m	Anagnostidis (1968)
North Fiji Basin[f]	Hydrothermal vent	-	7.5	-	27.8‰	2671 m	Seki and Naganuma (1989)
Tjärnö (Sweden)	Whale bone	Detected	-	7-8°C[g]	34.3-34.7‰[g]	aq	this study
Storegga Seeps (Norway)	Marine sediment	Detected	7.8-7.9	-1°C	34.1-35.5‰	745 m	this study
Paxos (Greece)	Marine cave	b.d.[h]	8.0-8.2	15-20°C	39‰	23 m	this study

[a]Includes observations on *Thiobacterium* that could clearly be attributed to the genus under investigation. Publications providing only scarce morphological information (Miyoshi, 1897; Caldwell and Caldwell, 1974; Gugliandolo and Maugeri, 1993; Yang *et al.*, 2008), or describing microbes that appear morphologically deviant from *Thiobacterium* (Miyoshi, 1897; Naganuma *et al.*, 1990; Hedoin *et al.*, 1996; Mosso *et al.*, 2002) are not listed.
[b]Hydrogen sulfide was occasionally detected only qualitatively, or its presence was mentioned without presenting data on measured concentrations. Hydrogen sulfide concentrations were estimated for Venice (Florida) from 0.162 ppm H_2S (Lackey and Lackey, 1961), for Bad Gastein (Austria) from 0.5-0.8 mg kg^{-1} H_2S (Vouk *et al.*, 1967) and for Amélie-Les-Bains (France) from 3.5 mg l^{-1} H_2S (Scheminzky *et al.*, 1972).
[c]If no explicit value for depth was given, this is now indicated by a substitute value of 1 m. A value of 0 m resulted from the observation of gelatinous structures directly on rock surface overflown by water from thermal springs (Vouk *et al.*, 1967; Scheminzky *et al.*, 1972), or where structures were found on the water surface (Anagnostidis, 1968). The minke whale bones from Tjärnö were kept in an aquarium, which is indicated by aq.
[d]Mixture of seawater/freshwater.
[e]Values determined for a nearby source.
[f]One *Thiobacterium* sp. was isolated from water as single rods, and formation of the gelatinous matrix was observed during a laboratory life cycle experiment. Salinity and pH were determined by Seki and Naganuma (1989) for the seawater used in enrichment cultures (collected from the Strait of Georgia, Canada).
[g]Values obtained from Glover *et al.* (2005).
[h]Hydrogen sulfide was detected qualitatively, but measurements yielded values below detection limit (b.d.).

2.2 Microscopy

Subsamples of the same gelatinous mats sampled for DNA extraction were analyzed directly after sampling by bright field and phase contrast microscopy. Visualization of subsamples stained with different fluorochromes was achieved by using a Zeiss LSM 510 and the appropriate software (Carl Zeiss MicroImaging GmbH, Göttingen, Germany).

2.3 Staining

Subsamples of the gelatinous mats were preserved in either 2 or 4% formaldehyde/seawater at room temperature, 4°C or -20°C. When applying the protein-targeting fluorochrome SYPRO Red (Molecular Probes, Invitrogen Corporation, Karlsruhe, Germany), staining was conducted at room temperature for at least 45 min (4 h maximum). The dye was diluted beforehand in artificial seawater (salinity 34‰) to a 5 × concentrated solution. Slides were directly subjected to microscopy. The DNA-targeting fluorescent stain 4',6-diamidino-2-phenylindole (DAPI) was also applied to fixed material of the gelatinous spheres. Subsamples of the structures were placed onto individual spots of teflon-coated slides and were dried at 46°C for 1 h. Staining was conducted for 7 min at room temperature with 15 μL of a 2.5 μg mL^{-1} DAPI solution. To remove excess DAPI, the preparations were shortly rinsed with distilled water, followed by rinsing in 96% ethanol and subsequent drying at room temperature. Preparations were finally mounted in a 2:3 mix of Vecta Shield (Vector Laboratories Inc., Burlingame, CA) and Citifluor (Agar Scientific Ltd., Essex, UK), stored at -20°C and subjected to microscopy the following day.

2.4 Microsensor measurements

High resolution geochemical gradients were measured on a *Thiobacterium* mat during the "VICKING" expedition in 2006 (IFREMER) with a laboratory set up. Microsensors for pH, O_2 and H_2S were used, and sensors were calibrated as previously described (Revsbech and Ward, 1983; Jeroschewski *et al.*, 1996; de Beer *et al.*, 1997). The total sulfide ($H_2S+HS^-+S^{2-}$) was calculated from the H_2S concentrations and the local pH using equilibrium constants. Microsensors were mounted on a motor-driven micromanipulator and data acquisition was performed with a DAQ-PAD 6015 (National Instruments Corporation, Austin, TX, USA) and a computer. Relative to the microsensor tips, the surface of the sediment or of the gelatinous sphere was determined with the help of a dissection microscope. During the analyses, the core was kept in an aquarium with water cooled to approx. 1°C to simulate *in situ* temperature conditions. A gentle jet stream, pointing at the water surface of the core, stirred the overlying water and assured the development of a distinct diffusive boundary layer. Flux calculations were performed as in Lichtschlag *et al.* (2010).

2.5 Composition of the gelatinous matrix

Three spheres from the cave mats were separated from sample water and cleaned from attached sediment particles as efficiently as possible. They were then combined and stored in a sterile polypropylene tube at -20°C until further processing. Wet weight of the spheres was determined as 13.35 g. After freeze-drying for 4 days, total dry weight was determined as 0.61 g.

Three replicate subsamples of the dried material, with initial weights between 24 and 25 mg, were used for measuring total carbon, nitrogen and sulfur content of the material with a Thermo Fisher Scientific FlashEA 1112 (Thermo Fisher Scientific Inc., Waltham, MA). No acidification was performed prior to the measurements. Additionally, inorganic carbon was measured with a CM5012 CO_2 Coulometer (UIC Inc., Joliet, IL) for three separate replicate subsamples with initial weights between 21 and 22 mg. Finally, organic carbon, organic nitrogen and organic/elemental sulfur content were calculated accordingly. All values were corrected to a normalized dry weight, which excluded the total sea salt content of approx. 39‰.

Analyses of carbon and nitrogen isotopic composition were conducted with two replicate subsamples of 26 and 27 mg of the freeze-dried material, using a Thermo Fisher Scientific MS DELTA^{plus} XP gas isotope ratio mass spectrometer (Thermo Fisher Scientific Inc.). Isotopic ratios were corrected against air-N_2 and the international VPDB standard for obtaining $\delta^{15}N$ or $\delta^{13}C$, respectively. No acidification was performed prior to the measurements.

2.6 Fluorescence *in situ* hybridization

Subsamples of the cave mats chosen for FISH analyses were fixed in 1 mL of 4% formaldehyde/seawater for 1 h at room temperature. They were then directly transferred to Whatman Nuclepore Track-Etch Membranes (PC MB; 25 mm; 0.2 μm; Whatman, Schleicher & Schuell, Maidstone, UK), the latter being captured in an appropriate filtration apparatus and supported by Whatman ME25 membrane filters (mixed cellulose ester; 25 mm; 0.45 μm; Whatman). After removal of excess fixative, the filters were dried for 2 min at 50°C on a heating plate and were finally stored separately at -20°C in sterile Petri dishes.

FISH was performed as previously described (Snaidr *et al.*, 1997). Mono-labeled probes (Biomers, Ulm, Germany) and hybridization conditions are listed in Table 2. All filter sections were counterstained with 10 µL of a 1 or 2.5 µg mL^{-1} DAPI solution before rinsing with distilled water and air-drying. Finally, the filters were mounted in separate spots of teflon-covered slides with a 2:3 mix of Vecta Shield (Vector Laboratories Inc.) and Citifluor (Agar Scientific Ltd.) and were stored at -20°C prior to microscopy.

Table 2: Oligonucleotide probes and hybridization conditions used for FISH analyses in this study.

Probe	Probe specificity	Probe sequence (5'-3')	Target site within 16S/23S rRNA gene[a]	FA[b] (%)	T_h[c]/T_w[d] (°C)	Positive hybridization with *Thiobacterium* cells	Reference
EUB338(I-III)	Most Bacteria	Equimolar mixture of three probes	16S, 338-355	35	46/48	yes	Daims *et al.* (1999)
EUB338-I	Most Bacteria	GCT GCC TCC CGT AGG AGT	16S, 338-355	35	46/48		Amann *et al.* (1990)
EUB338-II	Planctomycetales	GCA GCC ACC CGT AGG TGT	16S, 338-355	35	46/48		Daims *et al.* (1999)
EUB338-III	Verrucomicrobiales	GCT GCC ACC CGT AGG TGT	16S, 338-355	35	46/48		Daims *et al.* (1999)
NON338	Negative control	ACT CCT ACG GGA GGC AGC	16S, 338-355	10	46/48	no	Wallner *et al.* (1993)
GAM42a[e]	Gammaproteobacteria	GCC TTC CCA CAT CGT TT	23S, 1027-1043	35	46/48	yes	Manz *et al.* (1992)
BET42a[e]	Betaproteobacteria	GCC TTC CCA CTT CGT TT	23S, 1027-1043	35	46/48	no	Manz *et al.* (1992)
GAM660	Free-living or endosymbiotic sulfur-oxidizing bacteria in the Gammaproteobacteria	TCC ACT TCC CTC TAC	16S, 660-674	35	46/48	no	Ravenschlag *et al.* (2001)
Gm705	Methanotrophs in the Gammaproteobacteria except *Methylocaldum*	CTG GTG TTC CTT CAG ATC	16S, 705-722	10	46/48	no	Gulledge *et al.* (2001)
My669	*Methylobacter* spp. and *Methylomonas* spp.	GCT ACA CCT GAA ATT CCA CTC	16S, 669-690	10	46/48	no	Eller *et al.* (2001)
LMU	*Leucothrix mucor*	CCC CTC TCC CAA ACT CTA	16S, 652-669	10	46/48	no	Wagner *et al.* (1994)
G123T[e]	*Thiothrix* spp.	CCT TCC GAT CTC TAT GCA	16S, 697-714	10	46/48	no	Kanagawa *et al.* (2000)
829-Thioploca	Marine *Thioploca* spp. and *Beggiatoa* sp.	GGA TTA ATT TCC CCC AAC ATC	16S, 829-849	10	46/48	no	Teske *et al.* (1995)
Blim575	Marine *Beggiatoa* spp.	CTA GCC GCC TAC ATA CGC	16S, 575-592	10	46/48	no	Mußmann *et al.* (2003)
Blim193	Marine *Beggiatoa* spp.	AAA AGA CGC CCT CTT TCC	16S, 193-210	10	46/48	no	Mußmann *et al.* (2003)
VSO673	Unattached, vacuolate, sulfur-oxidizing spp.	CGC TTC CCT CTA CTG TAC	16S, 656-673	10	46/48	no	Kalanetra *et al.* (2004)
WPF464	Attached, vacuolate, filamentous spp.	AGC TTT AAG TTT TTC TTC CC	16S, 445-464	10	46/48	no	Kalanetra *et al.* (2004)
Thm465	*Thiomargarita* spp. Amon mud volcano	GTC AAG ACT CTA GGG TAT	16S, 465-482	10	46/48	no	Girnth *et al.* (submitted)
Thm482[f]	*Thiomargarita* spp.	CTT CTT CTA TTG CTG ATG	16S, 482-499	10	46/48	no	this study
Biwa829	Freshwater *Thioploca* spp.	AGG TAT ACC CTT CCA ACG TC	16S, 829-849	10	46/48	no	Kojima *et al.* (2003)
ThioBar894[f]	*Thiobacillus halogenesis*	TGA GTT TCA ACT TCC GGC	16S, 894-911	10	46/48	no	this study
ThioBar272[f]	*Thiobacillus halogenesis*	CTA CTG TAT CGT CGC CTT	16S, 272-289	10	46/48	no	this study
THIO1	*Acidithiobacillus* spp.	GCG CTT TCT GGG GTC TGC	16S, 1275-1292	10	46/48	no	González-Toril *et al.* (2003)
Thio820	*Acidithiobacillus* spp.	ACC AAA CAT CTA GTA TTC ATC G	16S, 816-837	10	46/48	no	Peccia *et al.* (2000)
LaSp60	Tubeworm endosymbionts, uncult. Gammaproteobacteria	CCA TCG TTA CCG TTC GAC	16S, 60-77	10	46/48	no	Duperron *et al.* (2009)
RifTO147	*Rif / Tev / Oas* symbiont	GAT TTC TCC GAG TTG TCC	16S, 147-164	10	46/48	no	Nussbaumer *et al.* (2006)
RifTO445	*Rif / Tev / Oas* symbiont	TCC TCA GGC TTT TCT TCC	16S, 445-462	10	46/48	no	Nussbaumer *et al.* (2006)
Ohaa1-65	*O. haakonmosbiensis* symbiont	AGC TCT TGC TGT TAC CGT	16S, 65-82	10	46/48	no	Lösekann *et al.* (2008)
Ohaa1-77	*O. haakonmosbiensis* symbiont	GCC AAG AGC AAG CTC TTG	16S, 77-94	10	46/48	no	Lösekann *et al.* (2008)
Ohaa2-60	*O. haakonmosbiensis* symbiont	GCA TCG TTA CCG TTC GAC	16S, 60-77	10	46/48	no	Lösekann *et al.* (2008)
Ohaa2-77	*O. haakonmosbiensis* symbiont	CCT GCT AGC AAG CTA GCA	16S, 77-94	10	46/48	no	Lösekann *et al.* (2008)

Abbreviations: FISH, fluorescence *in situ* hybridization; rRNA, ribosomal RNA.
[a]*Escherichia coli* positions.
[b]Formamide concentration in the hybridization buffer.
[c]Hybridization temperature.
[d]Washing temperature.
[e]Mixed in a 1:1 ratio with unlabeled competitor oligonucleotides, to assure specific hybridization (Manz *et al.*, 1992; Amann and Fuchs, 2008).
[f]These probes represent newly developed oligonucleotides which have not been tested in corresponding positive control experiments due to inavailability of the respective organisms/16S rRNA gene sequence.
Probe specificity is given for the original design. In addition to the listed probes, 9 other, but so far unpublished probes, were used: 2 designed for a hypersaline *Beggiatoa* sp., 1 designed for sulfur-oxidizing endosymbionts, 1 designed for *Thiomargarita* spp.[f] and 5 designed for smaller subgroups within the Gammaproteobacteria. None of them positively hybridized with the target organisms, why publication of the probe sequences was reserved for the original developers. Specific gammaproteobacterial probes were used at lower stringency than published, i.e. 10% formamide, to create favorable hybridization conditions. For probe Gm705 specificity had been achieved by the original publishers at T_d=51°C (midpoint dissociation temperature).

2.7 Construction of 16S rRNA gene libraries and sequencing

Subsamples for phylogenetic analyses were stored at -20°C, preserved in either PCR-grade water (Sigma-Aldrich Biochemie GmbH, Hamburg, Germany), 1 × TE buffer (Promega Corporation, Madison, WI) or without any fixative. Three different approaches for obtaining total community DNA were applied, including (i) freezing and thawing (FT), (ii) an extraction method developed for marine microorganisms embedded in exopolysaccharides (E1; Narváez-Zapata *et al.*, 2005), and (iii) the UltraClean Soil DNA Isolation Kit (E2; MO BIO Laboratories Inc., Carlsbad, CA).

Universal bacterial primers GM3F (5'-AGA GTT TGA TCM TGG C-3'; Muyzer *et al.*, 1995) and GM4R (5'-TAC CTT GTT ACG ACT T-3'; Muyzer *et al.*, 1995) were used to amplify a nearly complete region of the 16S rRNA gene. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), ligated into pGEM-T Easy (Promega Corporation) and transformed into One Shot TOP10 Chemically Competent *Escherichia coli* (Invitrogen Corporation). Following plasmid preparation (Montage Plasmid Miniprep$_{HTS}$ Kit; Millipore GmbH, Schwalbach, Germany), inserts were subjected to *Taq* cycle sequencing with an ABI Prism 3130x Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequencing was either done with the M13R vector primer, or by using the bacterial primers 907R (5'-CCG TCA ATT CCT TTR AGT TT-3'; Muyzer *et al.*, 1995) and primer2 (5'-ATT ACC GCG GCT GCT GG-3'; Muyzer *et al.*, 1993). Both sequencing approaches yielded good-quality fragments of the 16S rRNA gene with lengths between 400 and 700 bp.

2.8 Sequence analyses

Partial sequences derived from 907R and primer2 were assembled with Sequencher 4.6 software (Gene Codes Corporation, Ann Arbor, MI) and manually checked. In addition, all libraries were screened for putative chimeras with the Mallard program at a cut-off of 99.9% (Ashelford *et al.*, 2006). Anomalous sequences were further investigated by using Pintail (Ashelford *et al.*, 2005), with nearest neighbors of the sequences obtained by using the SILVA-based SINA Webaligner (Pruesse *et al.*, 2007). Sequences with genuine chimeric signals were excluded from further analyses.

To search for a sequence common to either all libraries or all sample sites, gammaproteobacterial sequences, as previously determined with the RDP Classifier

(Wang *et al.*, 2007), and their nearest neighbors (obtained by using the SILVA-based SINA Webaligner and ARB database) were used for tree reconstruction in ARB (Ludwig *et al.*, 2004) by applying maximum likelihood (RAxML).

2.9 Nucleotide sequence accession numbers

All sequences have been submitted to the EMBL database under accession No. FN597289 to FN597418 (Gammaproteobacteria) and FN662907 to FN663128 (all other sequences).

3. Results

3.1 Visual description of the gelatinous mats

At all three sampling sites the gelatinous mats were spherically-shaped masses. A second, less dominant, layer-like mat type was found only in the shallow-water cave. The environmental settings prevailing at these three sites and in previously described habitats are summarized in Table 1.

On the whale bone a yellowish-white gelatinous sphere was observed (Figure 1a). Its diameter was approx. 4 mm. The sphere had evolved within one to two days in an area which was scraped free of bacterial mats of *Beggiatoa* and *Arcobacter* (as indicated by microscopy). During sampling the entire gelatinous sphere was removed and in the next few days nothing alike grew back on the bone.

The mats on top of the deep-sea methane seep sediment had an approximate diameter of 1.5 cm (Figure 1b). Under bright light conditions the gelatinous spheres seemed to be covered by a thin white layer with some thicker white filaments. The white color of the gelatinous spheres is an indication for the presence of elemental sulfur, and microscopic analyses later revealed a high density of light-reflecting granules typical for the sulfur inclusions of giant sulfide-oxidizing bacteria (Teske and Nelson, 2006). The white filaments were also observed surrounding the gelatinous masses on top of the sediment and were morphologically attributed to bacteria of the genus *Beggiatoa*, mat-forming giant sulfide oxidizers known to occur on highly reduced sulfidic sediments

(Ahmad *et al.*, 1999; Mills *et al.*, 2004; de Beer *et al.*, 2006). The underlying sediment was characterized by a beige-brown top layer above a dark gray to blackish horizon.

The largest accumulation of gelatinous mats in this study was found in a shallow-water cave off Paxos in Greece (Figure 1c; Supplementary Figure 1; Supplementary Results and Discussion). The cave walls were almost completely covered by gelatinous mats over an estimated area of 5 to 7.5 m^2, with a sharp boundary below the top of the cave (Dr. Thomas Beer, personal communication). Two morphological types of mats could be observed without a particular zonation in the cave (Figure 1c). The dominating type consisted of spherical structures showing diameters between less than 1 cm and more than 10 cm, the second type was characterized by a rather thin layering of the gelatinous material. Both spherical and layer-like structures of the gelatinous mats appeared whitish similar to the deep-sea spheres. The presence of elemental sulfur and white filamentous bacteria was verified by microscopy. The mats were present in summer 2006 and 2007, but had vanished in January 2007 when they were replaced by a dense filamentous mat on the cave walls (Paul Bowbeer, personal communication).

Figure 1: *Thiobacterium* gelatinous mats analyzed in this study. (a) Gelatinous sphere with an estimated diameter of 4 mm, found on top of a minke whale bone kept in an aquarium. In addition, the bone was covered by dense mats of thiotrophic bacteria (*Beggiatoa* and *Arcobacter*). (b) Gelatinous sphere found on top of a sediment core collected at the Storegga Seeps. (c) Assemblage of spherically shaped and layer-like gelatinous masses coating the walls of a shallow-water cave off Paxos in Greece.

3.2 Microscopic description of *Thiobacterium* microbes

Microscopic examination of the gelatinous spheres from all three sites revealed the presence of high numbers of rod-shaped, sometimes slightly curved chains of sulfur granules. The *Thiobacterium* spp. embedded in the gelatinous material found on the whale

bone had an approximate size of 3-8 μm × 0.9-2 μm (Figure 2a), those from the deep-sea ranged from 3-11 μm × 0.7-2 μm (Figure 2b), and the ones from the shallow-water cave were 2-9 μm × 0.3-2 μm in size (Figure 2c). The number of sulfur granules per cell varied between 3 and 5 (whale bone), 3 and 11 (deep-sea sediment) as well as 3 and 10 (cave). Also, single and pairs of sulfur granules were observed, but it was not clear whether those belonged to living cells or remnants of cell decay. Except for the *Thiobacterium* cells from the whale bone, we found that even within one cell the size of the sulfur inclusions could vary by up to a factor of 5. By phase contrast microscopy cell outlines around the granules could not be observed, but became visible after staining fixed material with the protein-targeting fluorochrome SYPRO Red (Figure 2d). We observed a loss of the internal sulfur deposits in ethanol fixation, because ethanol dissolves elemental sulfur. However, even with formaldehyde/seawater fixation, the sulfur granules vanished with time. At all three sites, immediately after sampling, the *Thiobacterium* cells embedded in the gelatinous mats appeared to be non-motile. However, microscopic examination of gelatinous material obtained from the underside of a red leaf-like structure (cave) revealed a few rod-shaped cells showing directed or tumbling movement outside of the matrix.

In addition to *Thiobacterium*, microscopic analyses of the gelatinous spheres from all three sample sites revealed the presence of many other organisms embedded in and associated with the gelatinous mats. This unexpectedly high microbial diversity is described separately in the Supplementary Results and Discussion and in Supplementary Figure 2.

3.3 Microsensor measurements

Microsensors were used to measure high resolution profiles of oxygen and sulfide concentration, as well as pH, (i) through a gelatinous deep-sea sphere (diameter approx. 1.5 cm) and the first 1.5 cm of the underlying sediment (Figure 3a) and (ii) through the top 4 cm of sediment immediately next to the sphere (Figure 3b). Oxygen penetrated the gelatinous matrix to a depth of approx. 3 to 4 mm. Sulfide from the sediment diffused into the underside of the structure, but no local overlap of oxygen and sulfide was observed in the sphere. The pH profiles showed several minima and maxima, but generally decreased in the sphere, possibly as a consequence of sulfide oxidation. In the

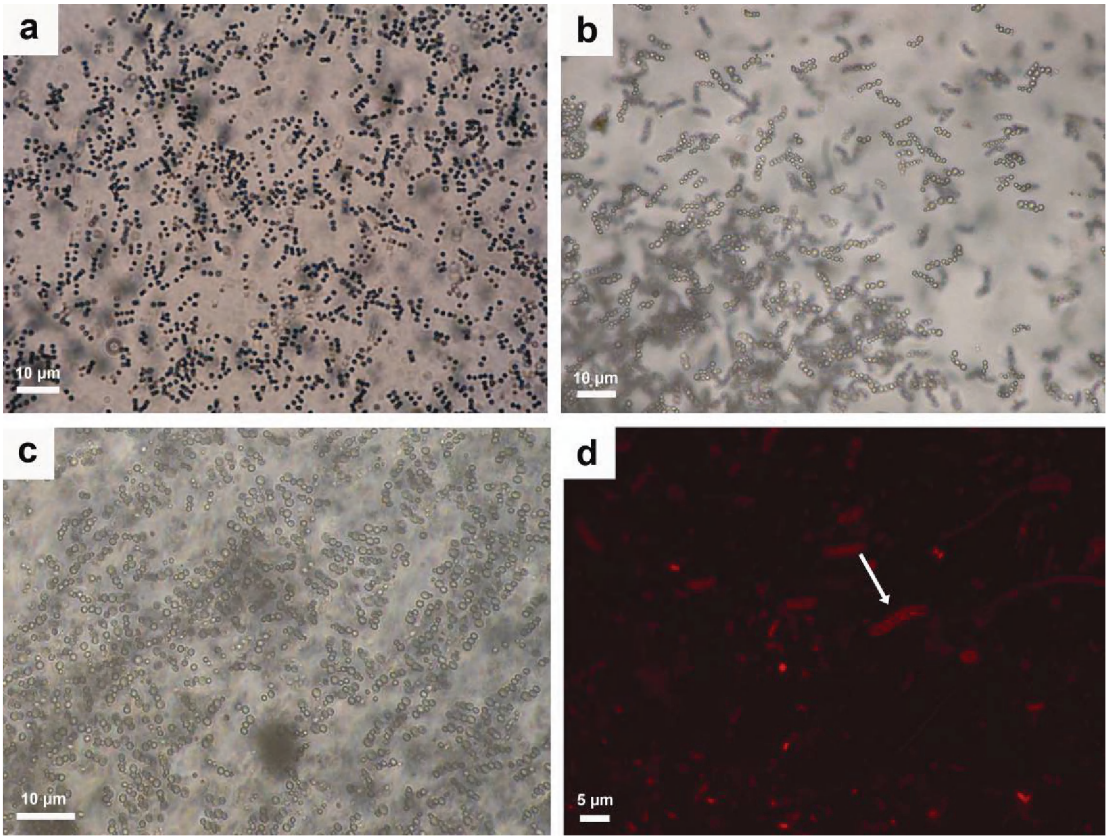

Figure 2: Microscopic observations on *Thiobacterium* cells embedded in the gelatinous spheres. (a) Whale bone sample (bright field), (b) deep-sea sample (phase contrast) and (c) shallow-water cave sample (phase contrast). In all samples, chains of elemental sulfur granules were visible. (d) True cell outlines around the granules were not visible by phase contrast and bright field microscopy, but by staining with the protein-targeting fluorochrome SYPRO Red using epifluorescence microscopy (staining was conducted with fixed material recovered from the cave in 2006).

underlying sediment no oxygen could be detected and sulfide concentrations reached a maximum of 3.2 mM at a depth of 1.5 cm below the sphere. The sulfide and oxygen fluxes into the sphere were 5 and 11 mmol m^{-2} d^{-1}, respectively, matching the stoichiometry of sulfide oxidation with oxygen as electron acceptor. Next to the sphere, oxygen penetrated approx. 2 mm into the sediment. Sulfide was not detectable until 2.7 cm below seafloor (bsf) and then increased linearly with depth. At 2 cm bsf, the pH reached a minimum of 7.6, below which it increased with depth. The oxygen uptake of the sediment next to the sphere was comparable to that in the sphere with 10 mmol m^{-2} d^{-1}.

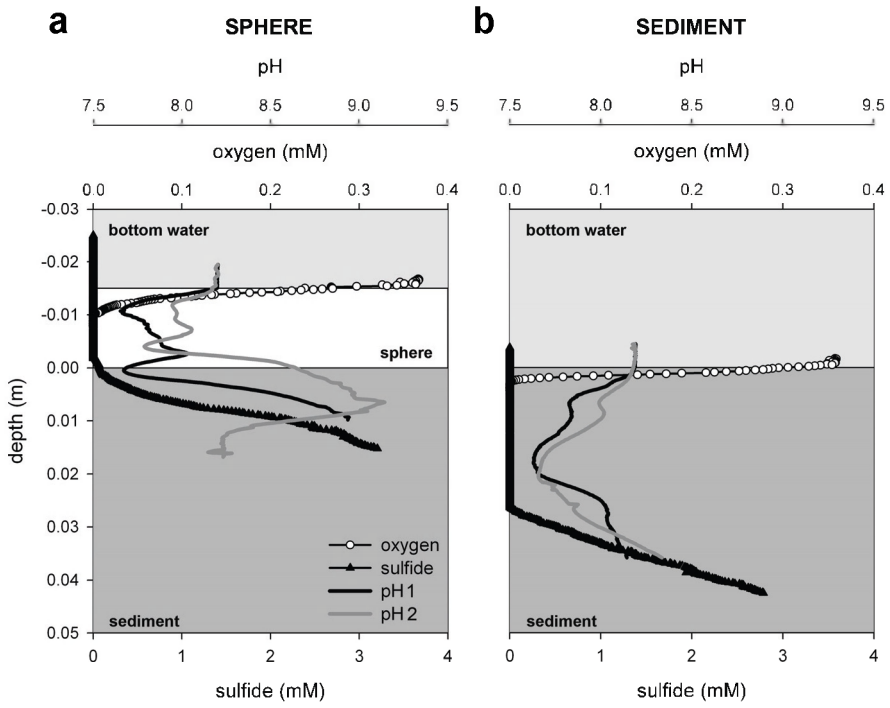

Figure 3: Oxygen, sulfide and pH microprofiles measured (a) through a gelatinous sphere with a diameter of approx. 1.5 cm and (b) within reference sediment next to the sphere (recovered from deep-water seafloor at the Storegga Seeps). Single, *ex situ* measured profiles are shown. Replicate pH profiles 1 (black) and 2 (gray) were retrieved from (a) the same gelatinous sphere and (b) the adjacent sediment. The microprofiles clearly show that the gelatinous spheres had formed in an area where hydrogen sulfide reached the sediment surface. Sulfide and oxygen did not overlap within the sphere, raising the question of how electron transport is mediated within the sphere during sulfide oxidation.

3.4 Elemental and isotopic composition of the matrix

The dried matter comprised 5.48% (w/w) organic carbon, 1.06% (w/w) nitrogen and 3.62% (w/w) organic and elemental sulfur. The molar C : N : S ratio of 6 : 1 : 1 . 5 reflects the enrichment of the cells and matrix with elemental sulfur. The $\delta^{13}C$ measured for two replicates of the freeze-dried cave material resulted in values of -16.1 and -18.3‰. The $\delta^{15}N$ was determined for the same replicates as -6.0 and -6.2‰. More details on the composition of the matrix are given in the Supplementary Results and Discussion.

3.5 Ribosomal RNA gene analyses

Bacterial 16S rRNA gene libraries were constructed with subsamples of the gelatinous spheres from all three sampling sites. Gamma-, Delta- and Epsilonproteobacteria

dominated the mat samples from all three sites. Other abundant classes of bacterial 16S rRNA genes included the Alphaproteobacteria, as well as Flavobacteria, Sphingobacteria, Planctomycetacia and Clostridia (Supplementary Table 1; Supplementary Results and Discussion). Despite the observed abundance of *Thiobacterium* cells in all samples, it was not possible to identify common phylotypes dominating the clone libraries of all three sites (all bacterial libraries per site combined). A substantial diversity of 16S rRNA gene sequences belonging to the Gammaproteobacteria was retrieved from all three mats (Figure 4), including many sequences of sulfur-oxidizing bacteria, such as different types of *Beggiatoa*, sulfide-oxidizing symbionts of tubeworms, and bacteria from hot springs, hydrothermal vents, cold seeps and lava. Subsequent FISH analyses with the GAM42a probe on fixed subsamples of the cave spheres revealed that the *Thiobacterium* cells belong to the Gammaproteobacteria (Figure 5). Unfortunately, further attempts to confine phylogenetic relatedness using more specific oligonucleotide probes for FISH were not successful (Table 2). A probe including all sulfide-oxidizing and sulfur-storing members of the Thiotrichaceae has not been developed so far. Specific gammaproteobacterial probes, if not otherwise indicated, were used at lower stringency than published, i.e. 10% formamide, to create favorable hybridization conditions. However, none of the established FISH probes specific for certain subgroups within the Gammaproteobacteria resulted in positive hybridization with *Thiobacterium* spp.-resembling cells, including those for the Thiotrichaceae-associated genera *Beggiatoa*, *Thioploca*, *Thiomargarita*, *Thiothrix* and *Leucothrix*, as well as probes for other organisms involved in the microbial cycling of sulfur like *Acidithiobacillus,* or for endosymbionts of seep and vent fauna. Further approaches to identify a candidate 16S rRNA gene sequence for *Thiobacterium* by single cell sorting were also not successful (data not shown) and detailed sequence analyses of *Thiobacterium* cells will require special attention in future experiments.

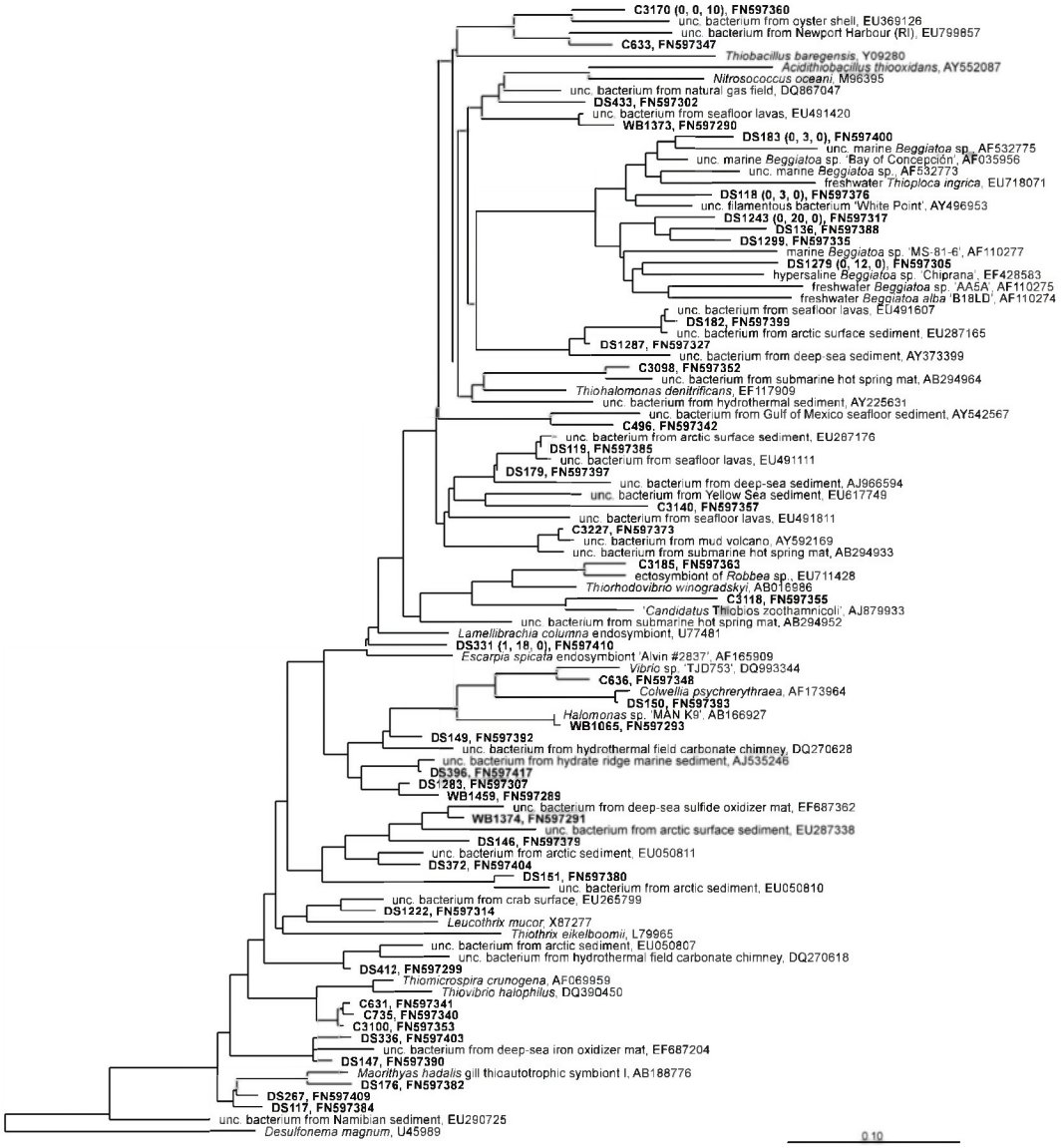

Figure 4: Maximum likelihood tree showing gammaproteobacterial 16S rRNA gene sequences obtained in this study (bold type; WB = whale bone mat, DS = deep-sea sediment mat, C = cave mat) to reference sequences within the Gammaproteobacteria. Only selected sequences are shown. For large clusters of sequences obtained in this study, the number of sequences associated with the given representative is indicated in parentheses, following the order WB, DS and C. Initial calculations were conducted with nearly full length sequences (*Escherichia coli* positions 99-1331). Partial sequences (bold type) were added subsequently to the reconstructed tree by applying parsimony criteria. Deltaproteobacterial sequences were used as outgroup.

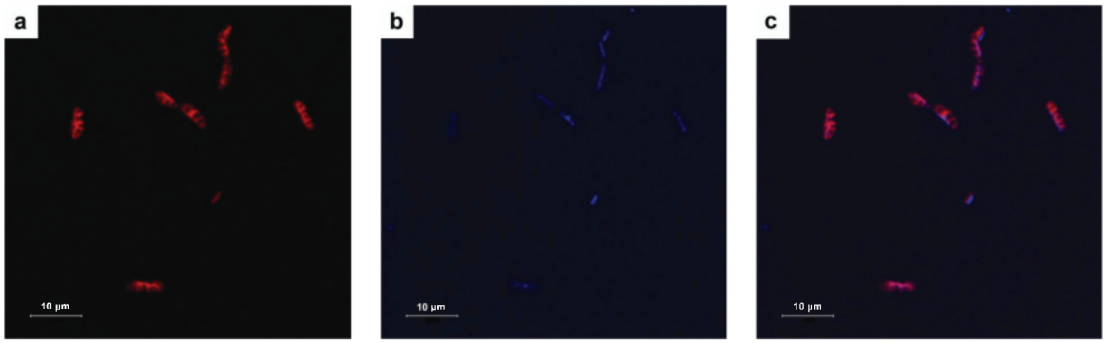

Figure 5: FISH images of *Thiobacterium* cells (cave samples 2007). (a) Positive signal after staining with the Gammaproteobacteria-targeting probe GAM42a (unlabeled competitor BET42a). (b) Corresponding DAPI stain. (c) Overlay of (a, b). In addition, staining of the *Thiobacterium* cells with the probe BET42a (unlabeled competitor GAM42a) did not result in any positive signal. Autofluorescence of the cells was found to be negligible.

4. Discussion

4.1 *Thiobacterium* classification

Since its first description by Molisch in 1912 (Molisch, 1912), no representative of this genus has been cultivated or genomically classified. The visual observation of internally stored sulfur granules and the biogeochemical analyses discussed below suggest that *Thiobacterium* is closely related to other known sulfide-oxidizing and sulfur-storing members of the Thiotrichaceae. Positive hybridization of *Thiobacterium* cells with the probe GAM42a (76% group coverage; Amann and Fuchs, 2008) confirmed their phylogenetic affiliation with the class of Gammaproteobacteria (Figure 5), as previously suggested based on morphological analogies (Kuenen, 2005). However, no common gammaproteobacterial phylotypes (Figure 4) dominating the clone libraries of all three sites could be identified. Reasons might include that one of the universal primers does not have full access to its target site on the *Thiobacterium* spp.-16S rRNA gene, or that the amplification of this gene is underrepresented compared to that of other bacteria with the applied PCR conditions, a problem also known from the giant sulfide oxidizer *Thiomargarita namibiensis* (Schulz, 2006). However, considering the broad range of environmental conditions from which it was described, the genus *Thiobacterium* could be

composed of rather different phylotypes, as it is known for the genus *Beggiatoa* (Figure 4; Ahmad *et al.*, 2006; Teske and Nelson, 2006).

4.2 Niches of *Thiobacterium*

Environmental conditions under which *Thiobacterium* mats have been observed include pH values between 7.2-9.3, temperatures ranging from -1°C to 45.5°C and water depths between 0-2700 m (Table 1). Low $\delta^{15}N$ values of -6.0 and -6.2‰ measured for the cave mats in this study suggest autotrophic growth of the associated organisms (see also Supplementary Results and Discussion). The main environmental factor selecting for *Thiobacterium* seems to be the availability of the electron donor hydrogen sulfide. Their ability to store massive amounts of sulfur, and their preference for sulfidic habitats suggests that *Thiobacterium* could be a sulfide oxidizer, belonging at least functionally into the same group as other mat-forming members of the Thiotrichaceae such as *Beggiatoa*, *Thiomargarita*, *Thioploca* and *Thiothrix*. With microsensor measurements it could be shown that the gelatinous spheres had exclusively formed in an area where hydrogen sulfide reached the sediment surface and that this hydrogen sulfide was depleted within the sphere (Figure 3a). The oxidation of sulfide occurs in several steps, from sulfide to sulfur, and from sulfur to sulfate. Sulfide oxidation can be driven by oxygen, nitrate and Mn- and Fe-oxides. The microsensor profiles (Figure 3a) indicate that the matrix had consumed stoichiometric amounts of oxygen and sulfide (2:1 in aerobic sulfide oxidation), but sulfide and oxygen did not overlap. A pronounced gap between oxygen and sulfide has previously also been observed for *Thioploca* and *Beggiatoa* spp. that are capable of bridging this gap by means of their gliding motility and the use of internally stored nitrate as additional electron acceptor under anoxic conditions (Fossing *et al.*, 1995; Huettel *et al.*, 1996; Mußmann *et al.*, 2003; Sayama *et al.*, 2005; Lichtschlag *et al.*, 2010). However, *Thiobacterium* are neither known to express a gliding behavior within the gelatinous matrix, nor to possess an internal vacuole for nitrate storage. The nature of a potential intermediate electron shuttle therefore remains unknown. The existence of a microoxic and microsulfidic environment within the matrix can be ruled out, as at undetectably low levels of reactants, the diffusive transport will be too low for significant metabolic activity. Also metal cycling, as in the suboxic zone, can be excluded as no solid

phase transport seems possible in these gel-like structures. The potential involvement of electrical currents in the oxidation of sulfide remains a possibility (Nielsen *et al.*, 2008).

It is unknown how and when *Thiobacterium* can outcompete other sulfide-oxidizing bacteria for energy and space. On the whale bone, it grew after *Beggiatoa* and *Arcobacter* mats had been physically removed, indicating that it may only be able to compete with others after specific disturbances. Where it grew, *Beggiatoa* was always observed, either growing thinly on the gelatinous matrix or more densely next to it, indicating that it may compete with *Thiobacterium* for the same energy source and space.

4.3 Conclusion and further questions

Morphological and ecological characteristics of *Thiobacterium* cells and mats suggest that this genus is closely related to other sulfide-oxidizing and sulfur-storing bacteria of the family Thiotrichaceae. Most interestingly, we found a stoichiometrical oxidation of sulfide to sulfate with oxygen without them overlapping. Motile microbes resembling the morphology of *Thiobacterium* have been reported few times (Lackey and Lackey, 1961; Lackey *et al.*, 1965; this study), but so far were never truly accounted to this genus. Molisch described similar motile organisms separately as *Bacillus thiogenus* (Molisch, 1912), hence it remains an important question if they can move inside their gels. It also remains hypothetical which factors trigger the attachment of free cells of *Thiobacterium* in a certain habitat and if mat formation may undergo a succession where other pioneer microbes may be needed. As often reported, the gelatinous masses of *Thiobacterium* were found to be attached to a substratum, e.g. the algae *Lyngbya* (Lackey and Lackey, 1961), rock surface (Vouk *et al.*, 1967), sediment (this study) or a whale bone (this study), suggesting that the production of a gel may not only be used for competing against other microbes, but also functions as an anchor in areas with favorable environmental conditions (Vouk *et al.*, 1967; Seki and Naganuma, 1989). Further it is not clear what role quorum sensing, density-dependent cell-cell communication, may play in the formation, maturation and final disintegration of the gelatinous mats. In this respect, future studies need to assess (i) the mechanisms triggering the appearance of *Thiobacterium* in conspicuous gelatinous mats, (ii) the access to electron donor and acceptor by the cells embedded in the thick matrix, (iii) the 16S rRNA gene based taxonomic affiliation of *Thiobacterium* spp., and (iv)

if and what role other microbes may play in the development of the *Thiobacterium* mats in natural environments.

Supplementary Information accompanies the paper on The ISME Journal website (http://www.nature.com/ismej).

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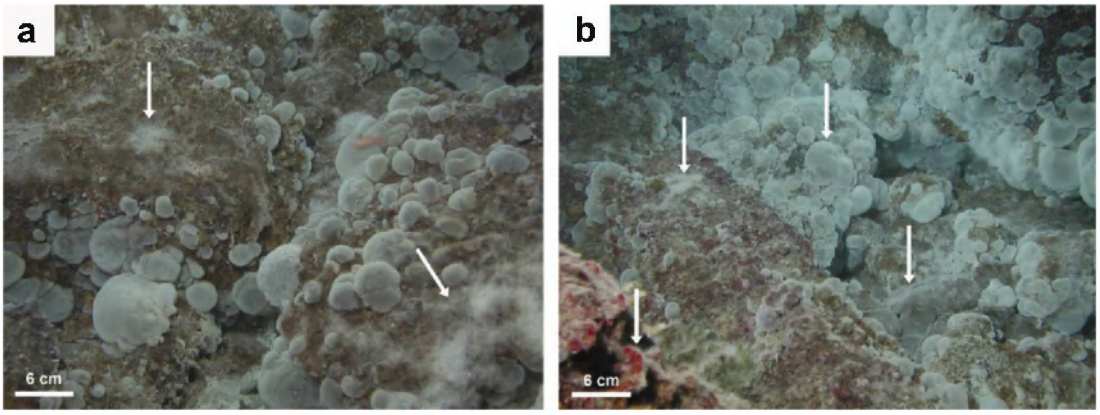
Supplementary Results and Discussion

Chemical characterization of the cave water

Cave water was sampled and stored at -20°C for nutrient measurements, or was fixed with 2.5% ZnAc (ratio 1:4) at 4°C to measure hydrogen sulfide concentrations. Concentrations of hydrogen sulfide, phosphate, nitrite and nitrate in the cave water were all below detection limit. Concentration of silicate and ammonium varied between 1.1 ± 0.0 and 12.7 ± 0.3 µM for silicate, and 3.3 ± 0.4 and 7.6 ± 0.0 µM for ammonium.

Visual appearance of the cave mats

The cave walls were almost completely covered by *Thiobacterium* gelatinous mats. In addition, white filaments were attached to the surface of the gelatinous spheres. Those filaments also occurred outside the gelatinous mats in small patches on the cave walls (Supplementary Figure 1a). In most parts of the cave both, gelatinous mats and filaments, seemed to co-occur and were partly associated with unidentified red leaf-like structures (Supplementary Figure 1b).



Supplementary Figure 1: *Thiobacterium* gelatinous mats observed in the shallow-water cave. (a) Filamentous organisms occurred in close proximity to the gelatinous mats. (b) Most prominent structures in the shallow-water cave: spherically-shaped and layer-like gelatinous mats (upper and lower right arrow), patches with filamentous organisms (upper left arrow) and unidentified leaf-like structures (lower left arrow).

Composition of the gelatinous matrix

The gelatinous material collected in the cave lost approx. 95% of its weight during freeze-drying, indicating that it mostly consisted of water. The remaining dry substance consisted of ca. 80% salt and displayed a grayish-white coloring. Inorganic carbon was determined to account for 0.37 ± 0.02% of the total dry weight. The dried matter comprised 5.48% (w/w) organic carbon, 1.06% (w/w) nitrogen and 3.62% (w/w) organic and elemental sulfur. The molar C:N:S ratio was therefore calculated to be 6:1:1.5. This ratio reflects the enrichment of the cells and matrix with elemental sulfur. In comparison, C:N:S ratios for cultured marine phytoplankton or marine particulate matter were reported as 7.75:1:0.08 (124:16:1.3) and 6.74:1:0.04 (182:27:1), respectively (Giordano *et al.*, 2005).

The $\delta^{13}C$ measured for two replicates resulted in values of -16.1 and -18.3‰. The corrected $\delta^{13}C$ values of -22 to -25‰ for organic carbon of the gelatinous material are not conclusive and match values previously reported for marine organic matter (Peters *et al.*, 1978), or for bulk material from sulfide oxidizer mats (Deming *et al.*, 1997; Zhang *et al.*, 2005). The $\delta^{15}N$ was determined for the same replicates as -6.0 and -6.2‰. These values are characteristic for bacteria from chemosynthetic communities of seep and vent ecosystems (Joye *et al.*, 2004; van Dover and Fry, 1994), possibly indicating autotrophic growth of the associated organisms.

A high microbial diversity is associated with Thiobacterium *gelatinous mats*

Microscopic analyses of the gelatinous spheres from all three sample sites revealed also many other organisms embedded in and associated with the gelatinous mats. The distribution of these associated organisms within the matrix was rather heterogeneous.

At all three sites we found *Beggiatoa* spp.-like filaments attached to the gelatinous mats and surrounding the mats. On the whale bone, filaments attached to the *Thiobacterium* mat had diameters of approx. 2 and 20 μm. In the deep-sea samples filament diameter was 3 to 4 μm. DAPI staining of fixed subsamples supported these observations and gave an indication for the putative presence of one or two additional, morphologically different, filamentous organisms. Furthermore, we observed few rod-shaped cells with an estimated length of 3 to 4 μm (deep-sea). In the shallow-water cave we found numerous morphologically distinct filamentous structures, some of which

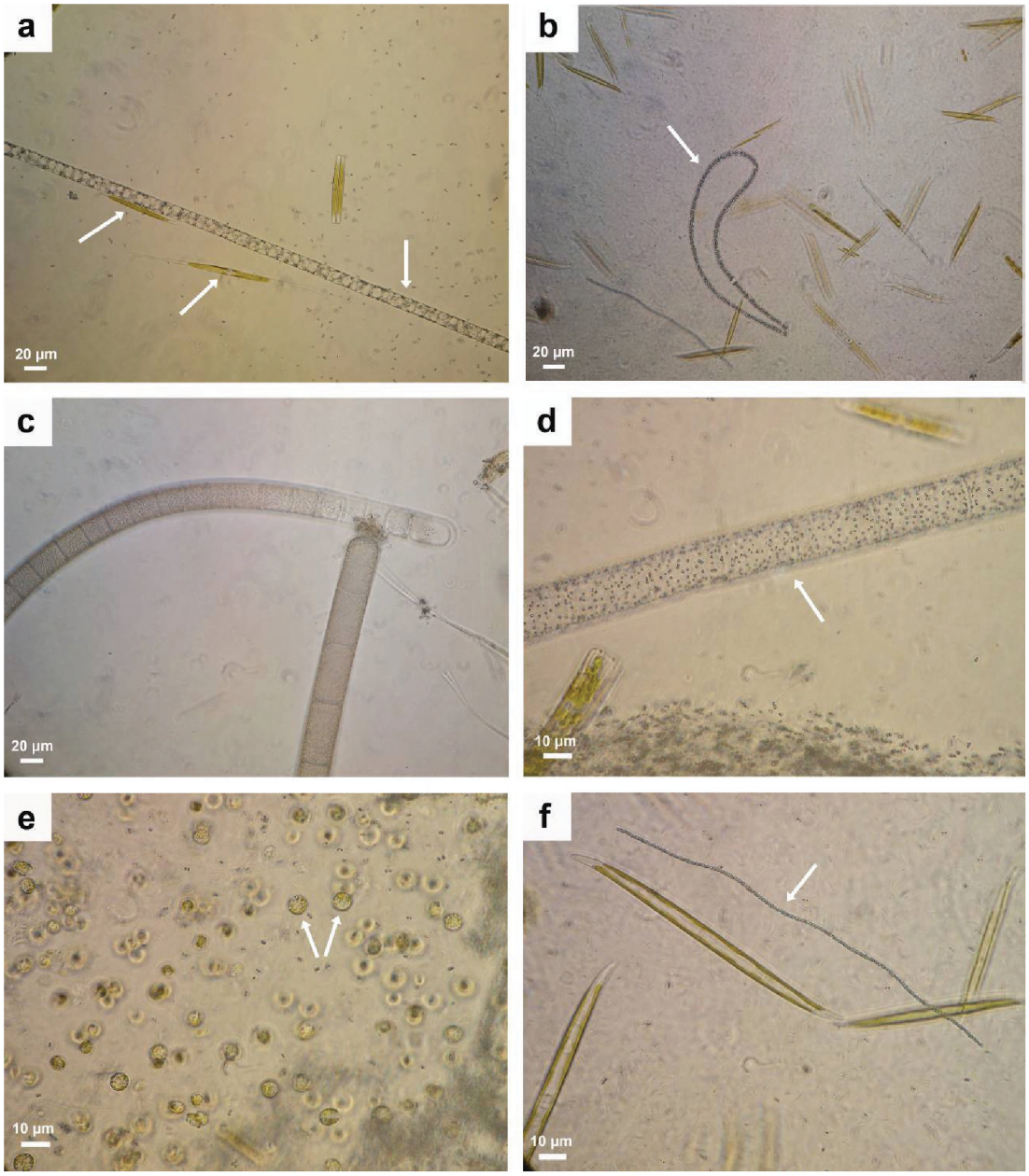
resembled different *Beggiatoa* spp. (diameters between 3 and 50 μm; two examples of 12 and 5 μm are given in Supplementary Figure 2a, b), while others remained unidentified filamentous bacteria (Supplementary Figure 2c, d). Microscopy of the cave mats and its white coverage also revealed the presence of at least three morphologically different diatom species, some of which belong to the family Bacillariophyceae (Supplementary Figure 2a). In one spot we observed high numbers of a spherical, solitary occurring structure, possibly constituting another diatom (Supplementary Figure 2e). Very conspicuous within the group of filamentous structures was the occurrence of long chains of sulfur granules (Supplementary Figure 2f), presumably representing thin, filamentous bacteria. Subsequent analyses on fixed samples of the shallow-water cave spheres, including DAPI staining, as well as FISH with the probe EUB338(I-III), further revealed the presence of rod-shaped bacteria, one spirillum and different filamentous microbes that initially were not visible during bright field microscopy.

In addition to the microscopic studies, bacterial 16S rRNA gene libraries showed that beside the abundant classes (Supplementary Table 1) also sequences assigned to the classes Deferribacteres, SR1 *genera incertae sedis* and Bacteroidetes (whale bone sample), as well as Bacilli, Cyanobacteria, Verrucomicrobiae (cave sample 2006), and OD1 *genera incertae sedis* (whale bone and cave sample 2006) were present in the respective gelatinous mats.

Supplementary Table 1: Main contributors in the different bacterial 16S rRNA gene clone libraries. Taxonomic identification of the sequences was achieved by applying the RDP Naïve Bayesian rRNA Classifier.

Habitat/ Method[a]	Total no.	*Alpha-proteo-bacteria* %	*Gamma-proteo-bacteria* %	*Delta-proteo-bacteria* %	*Epsilon-proteo-bacteria* %	*Flavo-bacteria* %	*Planctomycetacia* %	*Sphingo-bacteria* %	*Clostridia* %	other %	n.a.[b] %
Whale bone											
FT	44	7	9	11	30	11	0	0	0	2	30
E1	31	6	13	32	13	3	0	3	3	10	16
Deep-sea											
FT	86	5	59	3	7	5	1	1	0	0	19
E1	43	0	79	2	2	0	0	2	0	0	14
Cave 2006											
FT	34	3	12	24	12	6	0	3	32	0	9
E1	40	8	20	8	0	20	3	15	13	3	13
Cave 2007											
E2	74	8	34	5	12	4	3	5	0	7	22

[a]DNA-recovery methods: FT = freezing and thawing, E1 = extraction method initially designed for marine biofilms associated with high amounts of exopolysaccharides (Narváez-Zapata *et al.*, 2005), E2 = UltraClean Soil DNA Isolation Kit from the MO BIO Laboratories. The number of freezing and thawing cycles was as follows: whale bone sample 4 times, deep-sea sample 3 and 4 times combined, cave 2006 sample 6 times. Less than 100 mg of gelatinous material were subjected to E1 and E2.
[b]Sequences were not assigned (n.a.), when their classification reliability was lower than 80%.



Supplementary Figure 2: Microbial diversity associated with the gelatinous mats from the shallow-water cave off Paxos. *Thiobacterium* microbes embedded in the structures were already shown in Figure 2c. (a) Two diatom species (arrows on the left), presumably belonging to the family Bacillariophyceae. The right-hand arrow points to a *Beggiatoa* spp.-resembling filamentous organism (diameter approx. 12 µm). (b) *Beggiatoa* spp.-like filament with a diameter of approx. 5 µm. (c) Large, filamentous bacteria collected from the underside of one of the red leaf-like structures. (d) Filamentous bacteria with inclusions resembling elemental sulfur granules. (e) Another suspected diatom species, observed only once. (f) Thin, filamentous bacterium containing sulfur granules.

Statistical evaluation of phylotype diversity associated with the cave Thiobacterium *mats*

Rarefaction analyses and calculation of non-parametric estimators of species richness (Chao1, ACE and Jackknife) were performed with the DOTUR software (Schloss and Handelsman, 2005). Distance matrices needed for DOTUR were generated with the ARB software package (Ludwig *et al.*, 2004). Canonical Correspondence Analysis (CCA; Ramette, 2007; ter Braak, 1986) was used to determine the effects of DNA-recovery versus sample locations on the observed community diversity patterns, as follows. From the taxonomic positioning of all sequences done with the Naïve Bayesian rRNA Classifier of the Ribosomal Database Project (Wang *et al.*, 2007), abundance tables were generated on phylum, class, order, family and genus level. CCA models and their significances (as determined by 1000 permutations of the response matrices) were done with the vegan package implemented in the R statistical environment (http://www.R-project.org).

Rarefaction analysis conducted with phylotypes from the bacterial library of 2007 cave samples (E2) indicated that no saturation of diversity was observed, supported by the calculated richness estimators as determined at a cut-off of 3% genetic divergence for delineating operational taxonomic units (OTUs). Chao1, ACE and Jackknife richness estimators and their associated 95% confidence intervals were 214 [113-475], 210 [116-435], and 197 [135-259] OTUs, respectively. The analyses were performed on two distance matrices, one calculated with all variable regions of the partial 16S rRNA gene, and the other excluding highly variable regions. No major differences in terms of rarefaction analyses or non-parametric estimators were found, indicating that our conclusions on OTU richness were robust.

To determine whether experimental (i.e. DNA-recovery method) or ecological factors (i.e. sample location) mostly explained the observed diversity patterns, the effects of the two factors were assessed by CCA for all sequences (Supplementary Table 2), as well as for two subsets containing either the 5'- or 3'-sequences only (data not shown). The results for all tested sets of sequences indicated that the observed phylotype diversity was best explained by sample location and that the applied DNA-recovery methods had little to no effect on the observed diversity patterns. Those findings were significantly supported within the comprehensive sequence set until the order-level (Supplementary Table 2).

Supplementary Table 2: Effect of DNA-recovery method and sample locations on the variation in microbial diversity patterns for the gelatinous cave spheres at different taxonomic levels

	Phylum	Class	Order	Family	Genus
DNA-recovery method	21%^{ns}	18%^{ns}	28%^{ns}	27%^{ns}	25%^{ns}
Sample location	67%^{a}	71%*	69%*	58%^{ns}	56%^{ns}

CCA of phylotype diversity in the bacterial 16S rRNA gene libraries. Corresponding significances were determined by 1000 permutations of the response matrices and indicated as significant (*, $P \leq 0.05$), marginally significant (a, $P \leq 0.1$) or not significant (ns, $P > 0.1$)

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Chapter 3

A Novel, Mat-forming *Thiomargarita* population associated with a Sulfidic Fluid Flow from a Deep-sea Mud Volcano

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Abstract

A mat-forming population of the giant sulfur bacterium *Thiomargarita* was discovered at the flank of the mud volcano Amon on the Nile Deep Sea Fan in the Eastern Mediterranean Sea. All cells were of a spherical and vacuolated phenotype and internally stored globules of elemental sulfur. With a diameter of 24-65 μm, *Thiomargarita* cells from the Eastern Mediterranean were substantially smaller than cells of previously described populations. A 16S rRNA gene fragment was amplified and could be assigned to the *Thiomargarita*-resembling cells by fluorescence *in situ* hybridization. This sequence is monophyletic with published *Thiomargarita* sequences but sequence similarities are only about 94%, indicating a distinct diversification. In the investigated habitat at the flank of the active Amon mud volcano, highly dynamic conditions favor *Thiomargarita* species over other sulfur-oxidizing bacteria, as previously observed in other marine habitats. In contrast to *Thiomargarita namibiensis* populations, which rely on periodic resuspension from sulfidic sediment into the oxygenated water column, *Thiomargarita* cells at the Amon mud volcano seem to remain stationary at the sediment surface while environmental conditions change around them due to periodic brine flow.

1. Introduction

So far, dense *Thiomargarita* populations have only been described from two marine sulfidic habitats, namely Namibian shelf sediments, where they were first discovered in 1997 at a depth of 100 m (Schulz *et al.*, 1999) and hydrocarbon seeps in the Gulf of Mexico at 640 m (Kalanetra *et al.*, 2005). Despite their remarkable cell size, populations of *Thiomargarita* spp. initially appeared rather inconspicuous in these habitats, as they did not form cohesive bacterial mats. Additionally, single *Thiomargarita*-like cells were sporadically observed in gray microbial mats at the Håkon Mosby mud volcano (Barents Sea) at 1250 m water depth (de Beer *et al.*, 2006).

Cells of the genus *Thiomargarita* show spherical, barrel-like or amorphous shapes and are either organized in chains held together by a mucus sheath, or appear as clusters or individuals (Schulz *et al.*, 1999; Kalanetra *et al.*, 2005; Schulz, 2006). *In situ*, cell diameters between 100-400 µm are most prevalent (Schulz *et al.*, 1999; Kalanetra *et al.*, 2005; de Beer *et al.*, 2006), but single cells were reported to reach a diameter of up to 750 µm (Schulz *et al.*, 1999). In compliance with the defining feature of vacuolated sulfur oxidizers, including marine species of *Beggiatoa* and *Thioploca* (Teske and Nelson, 2006), most of the biovolume of *Thiomargarita* cells is occupied by a large, central vacuole (Schulz *et al.*, 1999; Kalanetra *et al.*, 2005). Highly refractive globules of elemental sulfur, that are internally deposited, confer a whitish appearance to *Thiomargarita* cells (Schulz, 2006). In *Thiomargarita namibiensis*, smaller inclusion bodies of polyphosphate and polyglucose were additionally observed (Schulz and Schulz, 2005). Consistent with these morphological similarities, the genus *Thiomargarita* is phylogenetically most closely related to the large, vacuolated, marine species of *Thioploca* and *Beggiatoa* within the order Thiotrichales of the Gammaproteobacteria (Kalanetra *et al.*, 2005; Schulz, 2006).

Thiomargarita species oxidize sulfide or internally stored sulfur with either oxygen or nitrate to gain energy (Schulz *et al.*, 1999; Schulz and de Beer, 2002; Schulz, 2006). Even though the cells are non-motile, they can populate sediments in which their electron acceptor and energy source do not coexist. Within these habitats, *Thiomargarita* species presumably rely on external transport events such as periodic resuspension of loose, sulfidic sediments possibly caused by fluid and gas eruptions to contact the oxygenated water column (Schulz *et al.*, 1999; Kalanetra *et al.*, 2005; Schulz, 2006). The accumulation

of large internal reservoirs of sulfur as electron donor and nitrate as electron acceptor allows *Thiomargarita* species to overcome the temporal isolation from either reductant or oxidant, and thus to survive periods of starvation (Schulz *et al.*, 1999).

This study reports on a mat-forming *Thiomargarita* population discovered in deep waters of the Mediterranean Sea on highly reduced, sulfidic and briny muds at the southwestern flank of the Amon mud volcano, an active submarine cold seep on the Nile Deep Sea Fan (Dupré *et al.*, 2007). The observed *Thiomargarita* population was studied morphologically as well as phylogenetically. *In situ* observations and biogeochemical measurements allowed inferring the ecological niche occupied by *Thiomargarita* spp. within this habitat.

2. Materials and Methods

2.1 Sample recovery

The sulfur band at the Amon mud volcano was probed and sampled during the BIONIL cruise M70/2 (R/V *Meteor*) in October and November 2006. Sediment cores of 8 cm diameter and approximately 20 cm length were retrieved from stations 765 and 790 during dives 115 and 121 (Table 1) using the remotely operated vehicle (ROV) QUEST4000 (Marum, University of Bremen). After recovery, push cores were immediately processed at *in situ* temperature (14°C) in the cold lab. Subsamples of the cores were used for biodiversity analyses, morphological studies and acridine orange direct cell counts (AODC). Rates of both, sulfate reduction (SR) and anaerobic oxidation of methane (AOM), were determined *ex situ* in subsamples of the cores. Microsensor profiles on the mat were measured *in situ* at stations 790-MICP-1 and 805-PROF-2 during dives 121 and 123 (Table 1). Additionally, one set of profiles was obtained at the source of the sulfur band (805-PROF-1) and another one on adjacent pelagic sediments (reference site, 790-MICP-2)

Table 1: Sampling and measurement sites. All metadata of this study were archived in the PANGAEA database (www.pangaea.de; WDC MARE).

	label (push core / microprofiler)	coordinates	date
biodiversity	M70/2a_765_PUC-8	32°22'04"N, 31°42'16"E	26.10.06
morphology	M70/2a_765_PUC-8, 47, 49, 68	32°22'04"N, 31°42'16"E	26.10.06
AODC	M70/2a_765_PUC-49	32°22'04"N, 31°42'16"E	26.10.06
	M70/2b_790_PUC-68	32°22'03"N, 31°42'15"E	10.11.06
SR	M70/2a_765_PUC-8, 49, 68	32°22'04"N, 31°42'16"E	26.10.06
	M70/2a_763 (reference)	32°21'26"N, 31°32'28"E	26.10.06
AOM	M70/2a_765_PUC-8, 49, 68	32°22'04"N, 31°42'16"E	26.10.06
microprofiling	M70/2b_805_PROF-1 (Fig. 4A, source)	32°22'04"N, 31°42'16"E	12.11.06
	M70/2b_805_PROF-2 (Fig. 4B, mat)	32°22'04"N, 31°42'15"E	13.11.06
	M70/2b_790_MICP-2 (Fig. 4C, reference)	32°22'03"N, 31°42'16"E	10.11.06
	M70/2b_790_MICP-1 (Fig. 4D, mat)	32°22'03"N, 31°42'16"E	10.11.06
video observation	(M70/2a, Station 765)	32°22'02"N, 31°42'17"E	26.10.06

2.2 Cell morphology

Cell diameter measurements were conducted on photographs of fresh mat pieces taken with a digital camera attached to a dissecting microscope at 50 × magnification. Mean diameter and diameter distribution were estimated based on 155 cells. For fluorescein isothiocyanate (FITC) staining, mat pieces were fixed immediately upon retrieval in a 4% formaldehyde/artificial seawater (ASW) solution for 3-4 h at 4°C, washed four times in 1 × phosphate-buffered saline (PBS; pH 7.4) and frozen at -20°C in a PBS/ethanol (1:1) mixture. FITC staining was performed with the following deviations from the protocol of Reinhard *et al.* (2000). Incubation was extended to 5.5 h, cells were washed in PBS and all centrifugation steps were substituted by short spin-downs at low rotational speed in order to preserve the native cell shape. Stained cells were examined and images were acquired using a Zeiss LSM 510 confocal laser scanning microscope (Carl Zeiss MicroImaging GmbH, Göttingen, Germany).

2.3 16S rRNA gene sequencing and phylogenetic reconstruction

Immediately upon retrieval, single cells or mat pieces were frozen in PCR-grade water (Sigma-Aldrich Biochemie GmbH, Hamburg, Germany) or 1 × TE buffer (Promega Corporation, Madison, WI, USA) and preserved for DNA extraction. Chromosomal DNA was obtained using the UltraClean™ Soil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA). The universal bacterial primers GM3F and GM4R (5'-AGAGTTTGATCMTGGC-3', 5'-TACCTTGTTACGACTT-3', Muyzer *et al.*,

1995) were used for amplification of nearly full length 16S rRNA gene sequences in a polymerase chain reaction (PCR). Primers VSO233F (5'-CCTATGCCGGATTAGCTT-3', Salman *et al.*, in preparation) and VSO673R (5'-CGCTTCCCTCTACTGTAC-3', Kalanetra *et al.*, 2004) were combined to specifically amplify a 441 base pair 16S rRNA gene fragment of vacuolated sulfur-oxidizing bacteria out of the bulk environmental DNA. Reaction mixtures were set up with the 5Prime MasterTaq Kit (5Prime GmbH, Hamburg, Germany) in a total volume of 25 µL (0.5 µM of each primer, 250 µM of each desoxyribonucleoside triphosphate, 1 × MasterTaq Kit PCR buffer, 0.3 mg mL^{-1} bovine serum albumin, 0.625 U Taq DNA polymerase; final concentrations in PCR-grade water). In case of universal bacterial PCRs an initial denaturation step at 95°C for 15 min was followed by 30 cycles of 95°C for 1 min, 42°C for 1 min, 72°C for 3 min and a single, final elongation at 72°C for 10 min. Amplification with VSO233F/VSO673R comprised an initial denaturation at 96°C for 5 min, 25 cycles of 96°C for 30 sec, 55°C for 30 sec, 72°C for 3 min preceding a final elongation at 72°C for 10 min. Pooled and purified products of replicate PCRs were cloned and libraries were screened for presence of vacuolated sulfur oxidizer-affiliated 16S rRNA gene sequences by partial sequencing (ABI Prism 3130x Genetic Analyzer; Applied Biosystems, Foster City, CA). The phylogenetic affiliation was inferred with the ARB software package (Ludwig *et al.*, 2004) based on release 95 of the SILVA SSURef database (Pruesse *et al.*, 2007). Inserts of interest were then resequenced from plasmid preparations in both directions resulting in double strand coverage.

Phylogenetic trees with representatives of Gamma- and Deltaproteobacteria were calculated in ARB using neighbor-joining, maximum likelihood (RAxML), and maximum parsimony methods. The different reconstruction methods were applied in order to test the stability of tree topology. A total of 318 full-length sequences (≥ 1200 bp) were used for initial calculation of trees in order to stabilize tree topology. Terminal nucleotide positions that were not covered by all sequences were excluded with an appropriate filter. Different positional conservatory filters (30%, 50%) excluding highly variable positions were applied. Partial sequences of the vacuolated sulfur oxidizer group, including the *Thiomargarita* sequences retrieved in this study, were added subsequently to each of the inferred trees without allowing changes in the overall tree topology. Finally, a consensus

tree of the different reconstruction approaches was built based on the neighbor-joining tree calculated without any conservatory filter, thus including 1292 nucleotide positions.

Similarity values were calculated in ARB for pairwise comparisons of *Thiomargarita* sequences AF129012, AY632420 and the representative (AMV865) of all affiliated sequences retrieved from the Amon mud volcano. Calculations were based on *E. coli* positions 252-656, which were covered by all sequences, but excluded binding sites of primers used for initial amplification.

2.4 Nucleotide sequence accession number

The sequence AMV865 has been submitted to the EMBL database under accession number FN597050.

2.5 Fluorescence *in situ* hybridization

The probe Thm465 (5'-GTCAAGACTCTAGGGTAT-3') was designed using the ARB ProbeDesign tool to specifically match *Thiomargarita* sequences retrieved from the Amon mud volcano. The probe's specificity was further tested with the online Probe Match tool provided by the Ribosomal Database Project (Cole *et al.*, 2005). Clone-FISH (Schramm *et al.*, 2002) with the monolabeled probe Thm465 was employed to test the probe's binding specificity at formamide concentrations of 0-60% in *E. coli* clones expressing full-match and single-mismatch 16S rRNA gene fragments. Successful expression was tested with probe VSO673 (Kalanetra *et al.*, 2004). Negative control experiments were further carried out with *Thiomargarita namibiensis* cells, which show three mismatches to probe Thm465. Sediment samples with *T. namibiensis* cells had been collected off the coast of Namibia (24°03'S, 14°15'E) in 2008 (R/V *Meteor* cruise M76/1a,b) and since were kept at 4°C as described by Schulz (2006). Horseradish peroxidase (HRP) labeled probes Thm465, EUB338 (I-III) (Daims *et al.*, 1999) and NON338 (Wallner *et al.*, 1993) as well as Cy3-labeled probes Thm465 and VSO673 were purchased from Biomers (Ulm, Germany).

Mat samples from the Amon sulfur band were fixed as described for FITC staining. Five microliters of the fixed cell suspension were spotted onto microscopic slides. Cell chains of *T. namibiensis* were manually removed from stored sediment samples. The chains were fixed for 1 h in a solution of 4% formaldehyde in ASW at room temperature,

washed for 2 h in ASW, incubated in an ASW/ethanol (1:1) mixture for 1 h, washed again in ASW for 40 min and then were transferred onto microscopic slides. Slides were dried at 46°C for 30 min, cells were embedded in 0.1% low gelling point agarose (United States Biochemical Corp., Cleveland, Ohio), which then was dried at 46°C for 1 h. Cells were dehydrated in ethanol (50, 80, 96%, each for 1 min) and slides were air-dried at room temperature.

For CARD-FISH experiments, an online available protocol (SILVA-web-protocol 2; http://www.arb-silva.de/fileadmin/graphics_fish/SILVA_FISH_protocols_card_080702.pdf, accessed 09.07.2009) was used with the following modifications. Permeabilization of cells was achieved by treatment with a 10 mg mL^{-1} lysozyme solution for 1 h, followed by inactivation of endogenous peroxidases through incubation in 0.15% H_2O_2 in methanol. Hybridization with HRP-labeled probes in humid chambers was performed in a two-step process. Fifteen microliters hybridization buffer amended with HRP-labeled probe were spotted onto the cells and slides were incubated over night. Additional 0.5 µL of probe solution (50 ng μL^{-1}) were subsequently added followed by further incubation for 2 h. Slides were washed in washing buffer and 1 × PBS (pH 7.6), for 15 minutes each. For probe Thm465, hybridization stringencies were adjusted via modulation of formamide concentration (30, 35, 40, 50 and 60%; vol/vol) in hybridization buffers. Control hybridizations were carried out with HRP labeled probes EUB338 (I-III) and NON338. Cells were embedded in a 4:1 mixture of mounting media Citifluor AF1 (Citiflour Ltd., Leicester, UK) and Vectashield (Vector laboratories, Burlingame, CA, USA). Fluorescent signals were examined with a Zeiss Axio Imager.M1 epifluorescence microscope (Carl Zeiss MicroImaging GmbH, Göttingen, Germany).

2.6 Biogeochemical measurements

Rates for sulfate reduction and anaerobic oxidation of methane were determined *ex situ* as described in detail elsewhere (Treude *et al.*, 2003) by whole core injection (Jørgensen, 1978) and short term incubation for 12 h with $^{35}SO_4^{2-}$ (5-10 µL carrier-free $^{35}SO_4^{2-}$, dissolved in water, 50 kBq) and $^{14}CH_4$ (25 µL $^{14}CH_4$, dissolved in water, 2.5 kBq) tracers, respectively. Depth profiles of total sediment-embedded cells were obtained by AODC according to a modified protocol (Boetius and Lochte, 1996) of Meyer-Reil (1983). High resolution depth profiles were measured with an *in situ* microprofiler unit (Boetius and

Wenzhöfer, 2009) that was operated by an ROV. On the electronic unit of the microprofiler sensors for H_2S, pH, O_2, and temperature (Pt100) measurements were mounted (Revsbech *et al.*, 1983; Revsbech and Ward, 1983; Jeroschewski *et al.*, 1996). Microsensor profiles started in the bottom water 5 cm above the sulfur band and proceeded until 4-11 cm into the sediment directly beneath the *Thiomargarita* mat. Microsensor calibration and measurements as well as profile analyses were performed as reported and summarized in de Beer *et al.* (2006). A recording current meter (RCM 11 equipped with a CTD; Aanderaa, Norway) was deployed in close vicinity of the sulfur band, which recorded average bottom water oxygen concentrations every 10 minutes for a period of 33 hours approximately 0.5 m above the seafloor.

3. Results and Discussion

3.1 Site description

The Nile Deep Sea Fan features intense gas seepage from a variety of submarine structures, including mud volcanoes (Loncke *et al.*, 2004; Dupré *et al.*, 2007). The Amon mud volcano is located offshore Egypt (Fig. 1A) in the transition zone between the eastern and central provinces of the Nile Deep Sea Fan at the margin of the Messinian platform (Dupré *et al.*, 2007). An up to 10 m deep fault was discovered around the southwestern flank of the mud volcano (Fig. 1B) by high-resolution bathymetry mapping using the AUV Aster^x (IFREMER; Dupré *et al.*, 2008). East of the fault at the mud volcano rise, a lateral outflow of blackish, liquid sediment was discovered (hereafter named 'sulfur band'). It had a length of approximately 60 m and an average width of 2-3 m, in some sections reaching over 6 m. The source of the outflow was covered with pelagic sediments and carbonate crusts (Fig. 1C). At a distance of about 10 m away from the source a thin and patchy *Thiomargarita* mat began to cover the blackish muds, became increasingly denser downstream and was spread almost over the entire width of the structure (Fig. 1D). Finally, the sulfur band ended abruptly as a thick mat surrounded by pelagic sediments. Push coring in the middle section of the sulfur band revealed that the whitish bacterial mat was only about 1 mm thick and was lying on top of a fluidic, dark gray to black sediment horizon of approximately 6-8 cm thickness. This upper horizon

was followed by a more consolidated, light-gray sediment layer (Fig. 1E).

A high-density fluid, presumably brine, was observed to episodically cover parts of the microbial mat on the sulfur band (Fig. 1F) and appeared to migrate along the structure. On the Nile Deep Sea Fan, brine is produced when subsurface fluids dissolve Messinian evaporite deposits and become highly enriched in salt while ascending towards the seafloor. Even though evaporites are believed to be absent on the Messinian platform (Loncke *et al.*, 2004), and brine pools have not been observed there, isolated salt deposits might be located in the vicinity of the Amon mud volcano (Dupré *et al.*, 2007). The high-density fluid observed on the sulfur band could not be sampled, but brines previously probed at other sites in the Mediterranean Sea (Sass *et al.*, 2001; Charlou *et al.*, 2003) including mud volcanoes on the Nile Deep Sea Fan (Omoregie *et al.*, 2008; Huguen *et al.*, 2009) were found to carry sulfide and methane in high concentrations of several millimoles per liter.

3.2 Morphology of *Thiomargarita*-resembling cells

Morphological examination of the microbial mat covering the sulfur band revealed the presence of numerous conspicuously whitish, spherical cells (Fig. 2A). These cells were tightly attached to sediment particles and occurred as individuals, bearing a strong resemblance to *Thiomargarita* cells described from the Gulf of Mexico (Kalanetra *et al.*, 2005).

Thiomargarita-resembling cells collected at the Amon mud volcano had a mean diameter of 47 μm ± 8 μm (n = 155). Assuming a spherical shape, this corresponds to an average cellular biovolume of 5.2×10^4 μm^3. Minimum and maximum diameter were recorded as 24 and 65 μm. Microscopy further revealed that the cells were non-motile and contained internal sulfur deposits in the peripheral layer (Fig. 2B). The presence of a vacuole was shown indirectly by absence of fluorescence signals within the center of cells stained with fluorescein isothiocyanate (FITC), a dye used for labeling proteins via their amine groups (Fig. 2C). FITC signals were restricted to a peripheral, cytoplasmic shell of approximately 2-3 μm, which equals 23-34% of the average cellular biovolume.

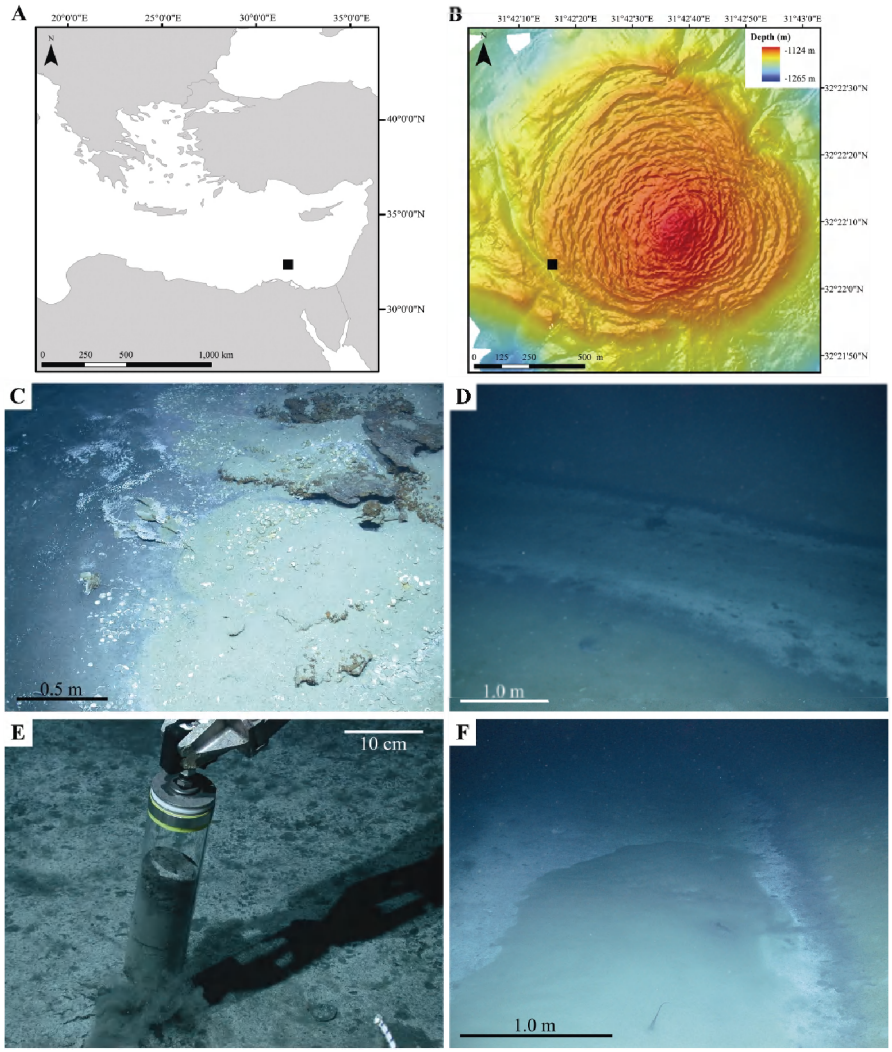

Fig. 1: Location and visual appearance of the sulfur band and the associated *Thiomargarita* mat at the flank of the Amon mud volcano. A) The Amon Mud volcano (■) is situated on the Nile Deep Sea Fan in the Eastern Mediterranean Sea. The map was generated in ArcMap (ArcGIS Desktop 9.2) with continental margin data provided by ESRI (Kranzberg, Germany). B) The bathymetry map of the Amon mud volcano obtained during *Meteor* expedition M70/2 (BIONIL) using the AUV Aster^{x} equipped with the EM120 multibeam (IFREMER/Geosciences Azur) shows the volcano's radial symmetry distorted by the tectonically highly disturbed surrounding area (Dupré *et al.*, 2008). At the eastern margin of a V-shaped depression situated at the southwestern rim of the mud volcano the sulfur band was discovered and sampled (■). C) Source region of the sulfur band in an area of carbonate crust-covered seafloor. D) Section of the sulfur band covered with the white *Thiomargarita* mat. The blackish mudflow is visible only in narrow stripes along the mat. E) Close up and push coring of the sulfur band mat. F) Section of the sulfur band mat covered with a puddle of brine fluid flowing along the sulfur band. On the bottom of the picture a fish diving into the dense fluid can be seen. Photographs C-F were acquired with the ROV QUEST4000 (Marum, University of Bremen).

The presence of a large, central vacuole surrounded by internal deposits of elemental sulfur is a feature shared with *Thiomargarita* spp. described so far (Schulz *et al.*, 1999; Kalanetra *et al.*, 2005). However, uniformly spherical, single cells with a small diameter and a narrow diameter range as discovered at the Amon mud volcano are clearly different from previously studied populations.

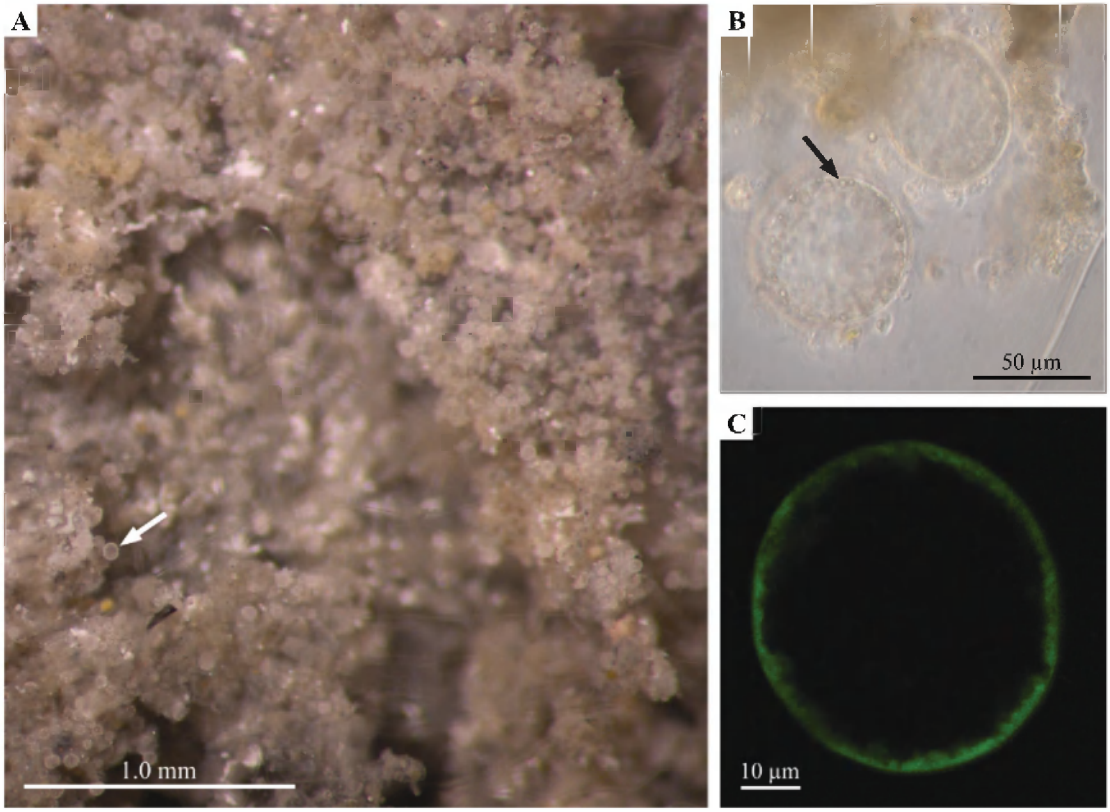

Fig. 2: Morphology of *Thiomargarita* cells from the Amon mud volcano. A) Top of a push core from the sulfur band microbial mat presenting sediment-embedded spherical *Thiomargarita* cells (→). B) Phase contrast micrograph of spherical *Thiomargarita* cells exhibiting highly refractive sulfur inclusions (→). C) CLSM image of an individual, FITC-stained *Thiomargarita* cell featuring a large, central vacuole. Sulfur globules are not visible because FITC staining was performed with cells fixed in ethanol, which is known to dissolve sulfur.

3.3 16S rRNA gene sequencing and phylogenetic inference

The phylogenetic studies on spherical cells collected from the sulfur band aimed to investigate whether the morphological resemblance to *Thiomargarita* species could be confirmed by 16S rRNA gene analyses. Initial attempts to amplify a nearly complete 16S

rRNA gene of *Thiomargarita* with general bacterial primers were not successful, as no sequence phylogenetically associated with the two published sequences of this genus, AF129012 (Schulz *et al.*, 1999) and AY632420 (Kalanetra *et al.*, 2005), could be retrieved. Difficulties in amplifying *Thiomargarita* 16S rRNA gene sequences had previously been encountered and were ascribed to epibiont contamination (Kalanetra *et al.*, 2005; Schulz, 2006). Here, sediment-associated microbes are a more likely source of contamination, since no epibiont-carrying mucus sheath was observed and the cells were directly pipetted from the thin mat overlaying the mud. Furthermore, we suggest that general 3'-specific traits or modifications of the *Thiomargarita* 16S rRNA gene might hinder a successful amplification, because none of the published sequences exceeds *E. coli* position 848.

A total of 101 *Thiomargarita*-affiliated 16S rRNA gene sequences, with a length of 441 base pairs (*E. coli* positions 233-673) each, were amplified by using a set of primers specifically targeting vacuolated sulfur oxidizers, including *Thiomargarita* spp. These sequences were retrieved from two different kinds of samples that contained either whole mat pieces or only few picked spherical cells. Removal of duplicates left 19 unique sequences featuring similarity values of 99.0-99.8%. Based on the high similarity, a single representative clone sequence (AMV865) was chosen. Phylogenetic inference included AMV865 in the monophyletic group comprising both published *Thiomargarita* sequences (Fig. 3A).

Sequences retrieved from *Thiomargarita namibiensis* and *Thiomargarita* spp. from the Gulf of Mexico share a similarity of 98.3%. A comparison of these with AMV865 resulted in substantially lower similarity values of 94.5 and 94.3%. Hence, the phylogenetic results support the morphological findings, suggesting that the *Thiomargarita* population from the Amon mud volcano is divergent from previously identified populations of the Namibian continental shelf (Schulz *et al.*, 1999) and the Gulf of Mexico (Kalanetra *et al.*, 2005).

3.4 *In situ* identification of *Thiomargarita*-related cells

To test whether the *Thiomargarita*-affiliated 16S rRNA gene sequence AMV865 is associated with the mat-forming spherical cells from the Amon mud volcano, catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH) with probe Thm465

was performed. The specificity of this probe for sequence AMV865 was proven in Clone-FISH experiments. The subsequent application of CARD-FISH was necessary to better deal with autofluorescence of *Thiomargarita* cells and sediment. Using this technique, spherical cells from the sulfur band as well as *Thiomargarita namibiensis* control organisms gave strong hybridization signals with probes EUB338 (I-III). At 35% formamide spherical cells from the Amon mud volcano bound probe Thm465 efficiently (Fig. 3B), confirming the origin of AMV865 from the sulfur band-associated *Thiomargarita*-resembling cells. Signal intensities of *T. namibiensis* cells hybridized with probe Thm465 at 35% formamide were comparable to background autofluorescence (Fig. 3C), indicating that the sequence AMV865 does not occur in *T. namibiensis* (100-300 µm diameter). Thus, both populations are phylogenetically different.

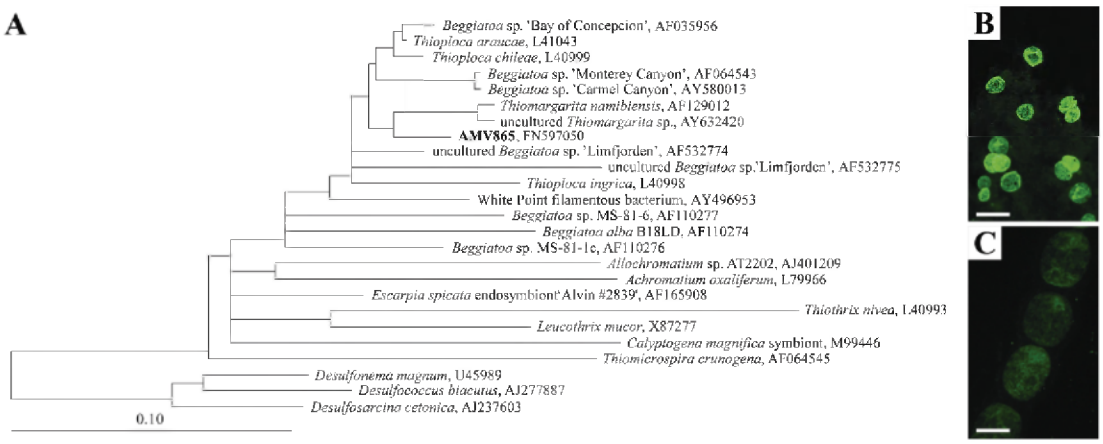

Fig. 3: A) Phylogenetic 16S rRNA gene based tree showing the affiliation of *Thiomargarita* sequence AMV865 obtained from the Amon mud volcano to selected reference sequences within the Gammaproteobacteria. Trees were calculated using maximum likelihood, neighbor-joining and maximum parsimony analyses based on full-length sequences (1229-1292 nucleotide positions, depending on the applied conservatory filters). Partial sequences L41043, L40999, AF129012, AY632420 and FN597050 (AMV865) were subsequently inserted into the reconstructed trees by using parsimony criteria with global optimization, without allowing changes in the overall tree topology. Here, a consensus tree based on nine trees is shown and unstable branching orders are visualized by multifurcation. B) CARD-FISH signal of *Thiomargarita* cells from the Amon mud volcano stained by probe Thm465. Scale bar is 100 µm. C) In contrast, *Thiomargarita namibiensis* cells display only autofluorescence signals when hybridized with probe Thm465. Scale bar is 100 µm.

3.5 Ecological niche of the novel *Thiomargarita* population

At the Amon mud volcano, the distinct spatial restriction of the *Thiomargarita* mat to the dark gray, reduced sediment of the sulfur band is striking (Fig. 1D and F). No *Thiomargarita* mats were found associated with other reduced sediment patches in the center of Amon, or at any other Nile Deep Sea Fan mud volcano studied to date (Omoregie *et al.*, 2009). Different biogeochemical analyses were carried out at the sulfur band in order to identify parameters and processes creating the ecological niche for the genus *Thiomargarita* at this site.

Total cell counts revealed maximum densities of 2.9×10^9 to 4.1×10^9 cells cm^{-3} (two replicates) in the upper 2-3 cm of sediment underlying the *Thiomargarita* mat. Values of this range are relatively high for cold seep sediments in the Eastern Mediterranean (Omoregie *et al.*, 2009). Deeper than 8 cm, cell numbers dropped below 0.8×10^9 cells cm^{-3} in the underlying light-gray sediment layer.

Sulfate reduction (SR) was restricted to the top 3 cm beneath the *Thiomargarita* mat. In this horizon, averaged SR rates ranged between 7 and 30 nmol cm^{-3} day^{-1} with an integrated SR rate of 0.5 mmol m^{-2} day^{-1} (0-15 cm; three replicates). Anaerobic oxidation of methane (AOM) was very low within the top 3 cm with a maximum of 3 nmol cm^{-3} day^{-1}. Below 8 cm depth, i.e. outside the blackish mud layer, no AOM could be detected. The integrated AOM rate (0-15 cm; three replicates) was calculated as 0.1 mmol m^{-2} day^{-1}. The sediment contained only little methane (>0.01 mM) and AOM rates were less than 25% of SR rates, suggesting the oxidation of other hydrocarbons with sulfate. Higher hydrocarbons are enriched in the subsurface fluids of Amon and other mud volcanoes of this region (Mastalerz *et al.*, 2007). Degradation of organic matter can considered being insignificant for driving sulfate reduction, since no sulfate reduction could be detected in a reference core taken close to the sulfur band. At the sulfur band, microbial production of sulfide by sulfate reduction was 30-150 times lower than reported for *Thiomargarita* habitats in Namibian shelf sediments (14-76 mmol m^{-2} day^{-1}, Schulz *et al.*, 1999; Brüchert *et al.*, 2003).

Continuous stationary oxygen *in situ* measurements of the bottom water close to the source of the sulfur band were performed over 33 hours at a height of 0.5 m above seafloor. Decreases in oxygen concentration by 23 and 10% were recorded over a period of 5 and 2 hours, respectively. *In situ* microprofiling of oxygen, sulfide and pH also

demonstrated a notable variability in time and space, indicating non-steady state conditions at the sediment-water interface. *In situ* microsensor profiles were recorded at three stations on the sulfur band during two different dives; additionally, one set was recorded at an adjacent reference site ca. 20 meters away (Fig. 4). At the reference site, no sulfide was detected in the sediment and oxygen penetrated about 4 cm deep (Fig. 4C). At the source of the sulfur band, close to where the first patches of the whitish *Thiomargarita* mat were visible, both the surface mud and the overlying bottom water were highly sulfidic (Fig. 4A). The sulfidic muds of the sulfur band showed ambient temperatures (13.5-13.6°C), which remained constant with depth. The absence of a temperature gradient indicates that there were no warm fluids advected upward from beneath the mat, or laterally from the mud volcano. Also, the stationary CTD did not record temperature anomalies.

Two replicate sets of profiles were obtained from the middle section of the *Thiomargarita* mat with a difference of 2.5 days (Fig. 4B, D). These profiles showed the presence of sulfide in the sediment (up to 0.7 mM) and a depletion of oxygen already within the first two millimeters of the mat and the underlying sediments. Measurements of pH revealed a decrease from pH 8.2 to 7.8 in both mat profiles, presumably caused by sulfide oxidation. An apparently temporal difference in the sulfide profile was observed in the boundary layer just above the sediments. One set of profiles (Fig. 4B) showed a complete depletion of sulfide and oxygen within the mat, as previously observed in other sulfide-oxidizer mats (Jørgensen and Revsbech, 1983; Nelson *et al.*, 1986). The second set of mat profiles obtained two days earlier (Fig. 4D) showed a different situation: sulfide (0.4 mM) was present in the bottom water boundary layer up to 1.5 cm above the mat, and oxygen concentrations were low but highly variable. This measurement has most likely recorded the chemical signature of a thin lateral outflow of sulfidic brine from the flank of the mud volcano over the mat. A large temporal overflow of the sulfur band by brine was observed visually during the first dive to this site (Fig. 1F; Station 765, 26.10.2006; Tab. 1), but this structure had vanished within 14 days when the remotely operated vehicle returned to the site for the *in situ* measurements.

Sulfide concentrations measured in sediments of the sulfur band underlying the *Thiomargarita* mat (0.45-0.7 mM at ca. 3 cm depth) were at the lower end of the range reported for *Thiomargarita* habitats off Namibia (0.1-0.8 mM, Schulz *et al.*, 1999; 22 mM,

Brüchert *et al.*, 2003) and in the Gulf of Mexico (up to 19 mM, Kalanetra *et al.*, 2005). Similar to other vacuolated sulfur oxidizers such as *Beggiatoa* and *Thioploca*, *Thiomargarita* species are thought to convert sulfide in a complete oxidation reaction via elemental sulfur to sulfate (Teske and Nelson, 2006), using nitrate or, preferably, oxygen as electron acceptor (Schulz and de Beer, 2002; Schulz, 2006). Concluding from the net equation of aerobic sulfide oxidation, 2 mol of oxygen are needed to convert 1 mol of sulfide to sulfate ($HS^- + 2O_2 \rightarrow SO_4^{2-} + H^+$). The ranges of sulfide and oxygen fluxes associated with the *Thiomargarita* mat were 1-20 mmol m^{-2} day^{-1} and 10-46 mmol m^{-2} day^{-1}, respectively. However, a budget calculation based on these measurements is not possible as the system was not in steady state.

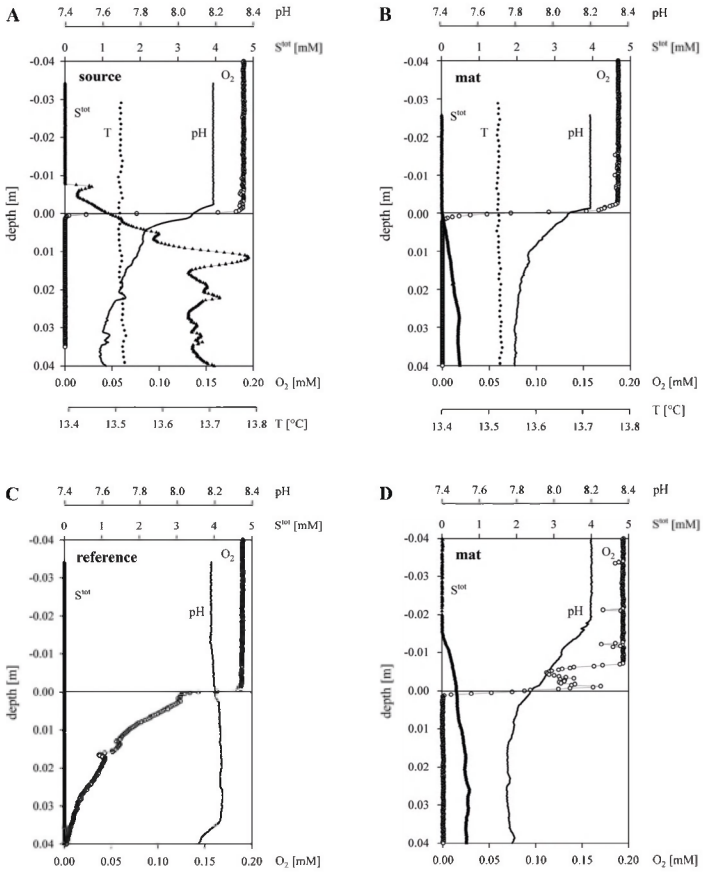

Fig. 4: *In situ* microprofiles from the sulfur band and a near-by reference site. Profiles of oxygen (O_2), total sulfide (S^{tot}), pH and temperature (T) are shown. When available, averaged data of two replicate profiles are presented. Single profiles are shown for oxygen (B), pH (C) and temperature (A, D). The pH profile in subfigure B displays averaged data of two profiles deeper than -0.016 m.
A) Profiles from the source of the sulfur band, where first patches of the white *Thiomargarita* mat were visible (Dive 123). B) Profiles from the middle section of the sulfur band, recorded 2.5 days later than D (Dive 123). C) Profiles from a reference site ca. 20 m off the sulfur band (Dive 121). D) Profiles from the middle section of the sulfur band (Dive 121).

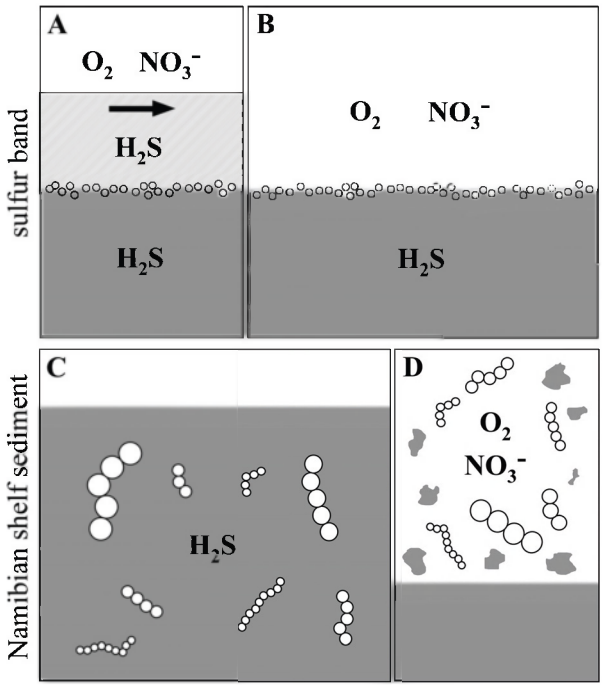

Fig. 5: Proposed ecological niche for the *Thiomargarita* population of the Amon sulfur band in comparison with *Thiomargarita namibiensis* from Namibian shelf sediments. At the Amon mud volcano a short period of exposure to sulfidic brine (A) contrasts a prolonged phase in which cells are in contact with oxygenated bottom water (B). *T. namibiensis* cells are buried in sulfidic shelf sediment most of the time (C) and only briefly contact oxygenated bottom water during externally triggered resuspension events (D). Panel C and D are adapted from Schulz (2006).

Altogether, these observations suggest that some sulfide is produced in the sediments by the anaerobic oxidation of hydrocarbons, but that lateral transport of sulfide with muds and brines flowing from the flank of the mud volcano may be more important for shaping this microbial habitat. It remains an interesting question, which environmental factors are providing a selective advantage for the *Thiomargarita* population at the sulfur band. Brine flows could temporarily isolate the sediment from direct contact with the overlying, oxygenated bottom water and cause high sulfide concentrations (Fig. 5A). In this situation, sulfide could be oxidized by nitrate storing vacuolated sulfide oxidizers such as *Thiomargarita* (Schulz, 2006). Nitrate reservoirs could be replenished and sulfide or stored sulfur could be oxidized with oxygen when the brine flow ceases (Fig. 5B). Although measurements of internal nitrate concentrations could not be carried out with *Thiomargarita* cells from the sulfur band, there is substantial evidence for nitrate accumulation in vacuoles of other *Thiomargarita* spp. (Schulz *et al.*, 1999; Kalanetra *et al.*, 2005) and closely related vacuolated sulfur oxidizers of the genera *Beggiatoa* and *Thioploca* (Fossing *et al.*, 1995; McHatton *et al.*, 1996; Sayama *et al.*, 2001;

Mußmann *et al.*, 2003; Kalanetra *et al.*, 2004; Hinck *et al.*, 2007). The ability to store enormous amounts of nitrate likely is one of the most important features allowing the giant *Thiomargarita namibiensis* cells to populate the sulfidic sediments off Namibia (Schulz *et al.*, 1999). *Thiomargarita* cells from the sulfur band, however, feature a rather narrow diameter and a low vacuole-to-biovolume ratio that is even inferior to some wide *Beggiatoa* spp. Accordingly, *Thiomargarita* cells at the sulfur band may survive only short periods of anoxia, even if nitrate would be stored in concentrations similar to other *Thiomargarita* spp. This would fit the scenario of short overflows of sulfidic brine causing transient anoxic conditions. Previous investigations have shown that among the sulfur-oxidizing microbes *Thiomargarita* species have an exceptional ability to deal with disturbances such as sediment resuspension and exposure to high sulfide concentrations (Schulz *et al.*, 1999; Kalanetra *et al.*, 2005; Schulz, 2006). Accordingly, populations of *T. namibiensis* are alternately exposed to sulfidic and oxic conditions by resuspension from the sediment into the water column (Fig. 5C, D). Strong mechanical disturbance is unlikely to occur in cases of the mat-forming *Thiomargarita* population of the sulfur band as well as in *Thiomargarita* habitats in the Gulf of Mexico, where cells populate the sediment surface (Kalanetra *et al.*, 2005). In contrast, temporarily high sulfide concentrations resulting from overflow of sulfidic brine would strongly select against *Beggiatoa* or *Thioploca* spp. at the sulfur band. At Amon, the *Thiomargarita* population remains stationary at the sediment surface, while environmental conditions are fluctuating (Fig. 5A, B). It remains to be explored which mechanism selects for the *Thiomargarita* populations associated with a brine pool, a mud volcano and methane hydrate sites the Gulf of Mexico (Kalanetra *et al.*, 2005). Regardless of the type of disturbance, *Thiomargarita* spp. seem to be particularly successful in dynamic environments where their non-motile behavior is an advantage over gliding, vacuolated sulfur oxidizers of the genera *Beggiatoa* and *Thioploca*. These motile organisms orient themselves in gradients by chemotactic responses, to finely adjust their position with respect to the oxygen-sulfide interface (Møller *et al.*, 1985; Hüttel *et al.*, 1996; Preisler *et al.*, 2007). In highly dynamic environments, where the position of this interface is drastically changing and might be even located in the water column, motile organisms would expend energy while not reaching their desired niche. In such cases, the strategy of *Thiomargarita* spp. to remain stationary and to live temporarily on stored resources

while tolerating high oxygen and sulfide concentrations might be a competitive feature of this genus.

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Chapter 4

Niche Differentiation among Mat-forming, Sulfide-oxidizing Bacteria at Cold Seeps of the Nile Deep Sea Fan (Eastern Mediterranean Sea)

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Abstract

Sulfidic mud flows of the Nile Deep Sea Fan are populated by different types of mat-forming sulfide-oxidizing bacteria. The predominant sulfide oxidizers were identified by microscopic and phylogenetic analyses as (i) filamentous sulfur bacteria resembling *Beggiatoa* species and vacuolated attached filaments, (ii) *Thiomargarita* species or (iii) *Arcobacter* species. High resolution *in situ* microprofiles revealed different geochemical gradients for the three types of mats. The decisive factor in selecting for the different mat-forming bacteria are spatial or temporal variations in the supply of oxygen and sulfide. Filamentous sulfide oxidizers were associated with non-overlapping steep gradients of oxygen and sulfide. A dense population of *Thiomargarita* was favored by temporarily changing supplies of oxygen and sulfide. *Arcobacter* mats occurred when oxygen and sulfide overlapped in the bottom-water interface. The occupation of different niches by mat-forming sulfide-oxidizing bacteria is analyzed with regard to the biology of the organisms (e.g. gliding motility, nitrate and sulfur storage) and the ecology of the habitats (microenvironments, geochemistry, geology).

1. Introduction

The Nile Deep Sea Fan hosts numerous active seepage structures, including mud volcanoes, debris flows, carbonate cements, pockmarks and brine pools in water depths of 500 to 3500 m (Dupré *et al.*, 2007; Gontharet *et al.*, 2007; Huguen *et al.*, 2009; Loncke *et al.*, 2004; Omoregie *et al.*, 2008; Omoregie *et al.*, 2009). At these structures, venting of hydrocarbon-enriched water, brine or gas supports the growth of chemosynthetic communities by supplying them with energy-rich substrates such as methane and sulfide (Sibuet and Olu, 1998). In reduced deep-sea habitats, sulfide-oxidizing bacteria like the filamentous *Beggiatoa* (Ahmad *et al.*, 1999; Nelson *et al.*, 1989), the spherical 'sulfur pearl' *Thiomargarita* (Girnth *et al.*, in review) or the vibrio-shaped *Arcobacter* (Omoregie *et al.*, 2008; Taylor *et al.*, 1999) can form dense and conspicuous mats on the seafloor. These bacteria thrive at the oxic-anoxic interface and oxidize sulfide to elemental sulfur, and mostly further to sulfate, by using either oxygen or nitrate (Gevertz *et al.*, 2000; Schulz, 2006; Sievert *et al.*, 2007; Teske and Nelson, 2006). In addition, unknown vacuolated attached filaments (VAF) have been discovered that form mats on various biotic and abiotic surfaces near shallow hydrothermal vents (Jacq *et al.*, 1989; Kalanetra *et al.*, 2004), offering yet another example of conspicuous microbes thriving on energy-rich sulfide.

At cold seeps, sulfide is either produced by microbial anaerobic oxidation of methane and higher hydrocarbons (Knittel and Boetius, 2009) or advected by sulfidic subsurface muds, fluids or brines (Kopf, 2002; Omoregie *et al.*, 2008). Mats of sulfide-oxidizing bacteria at cold seeps are important primary producers of biomass supporting a heterotrophic food web (van Gaever *et al.*, 2006). For example, Lichtschlag *et al.* (2010) estimated at the deep-sea mud volcano Håkon Mosby (Norwegian Sea) an annual biomass production of up to 8.4×10^4 mol C based on sulfide oxidation by *Beggiatoa* spp. Secondly, mat-forming sulfide oxidizers act as benthic filters against the toxic gas hydrogen sulfide (Lavik *et al.*, 2009). During aerobic sulfide oxidation, though, this may happen at the expense of high local oxygen consumption at the sediment surface. Regarding anaerobic sulfide oxidation, it is not yet completely clear if and how alternative electron acceptors are used by VAF or mat-forming *Arcobacter* spp., and whether ammonium or dinitrogen is the final end product of nitrate reduction in giant vacuolated sulfur bacteria like *Beggiatoa* and *Thiomargarita* spp. (Jørgensen and Nelson,

2004; Mußmann *et al.*, 2007; Sayama *et al.*, 2005; Schulz, 2006). In addition to their role in linking the carbon, sulfur and nitrogen cycles in sediments, some sulfide-oxidizing bacteria such as *Thiomargarita namibiensis* are also suspected to be involved in the marine phosphorus cycle, i.e. in the formation of phosphorites through the release of phosphate under anoxic conditions (Schulz and Schulz, 2005). Therefore, considering their different ecological functions in nutrient and energy cycling, microbial mats constitute important components of marine chemosynthetic ecosystems. This study combines visual observations from ROV dives with *in situ* biogeochemical measurements and analyses of the morphology and phylogeny of mat-forming sulfide oxidizers to characterize and compare their ecological niches. In the central province of the Nile Deep Sea Fan, three different types of bacterial mats associated with sulfidic mud flows were observed that formed distinct patches with a diameter ranging from 20 cm to 50 m. The main question of the study was as to the role of energy fluxes and their spatial gradients in defining niches for mat-forming sulfide-oxidizing bacteria in cold seep ecosystems.

2. Materials and Methods

2.1 Sampling sites

The Amon mud volcano (AMV) is located in the transition zone between the central and eastern province of the Nile Deep Sea Fan (NDSF) at a water depth of 1118 m (Figure 1a, b). It has a conical shape with a height of 90 m and a diameter of 2.6 to 2.8 km (Dupré *et al.,* 2007). Here we sampled the tip of the central mud flow characterized by abundant patches of white mats. As a second sampling site, we chose the southwestern flank of the AMV associated with subsurface flows of briny mud ('sulfur band'). A detailed description of the AMV and its habitats is given by Dupré *et al.* (2007, 2008), Foucher *et al.* (2009) and Felden (2009). The third sampling site, 'Pockmark area' (Figure 1a, c), was 130 km west of the AMV on a flat zone of the NDSF at a water depth of 1700 m dominated by debris flow, carbonate cements and pockmarks (Dupré *et al.*, in press). All *in situ* measurements and samples in this study were performed during the BIONIL expedition, leg M70/2a and b (October/November 2006), on RV *Meteor* and with the help of the remotely operated vehicle (ROV) QUEST4000 (Marum, University

of Bremen, Germany). The exact coordinates of measurements and sample sites are listed in Supplementary Table 1.

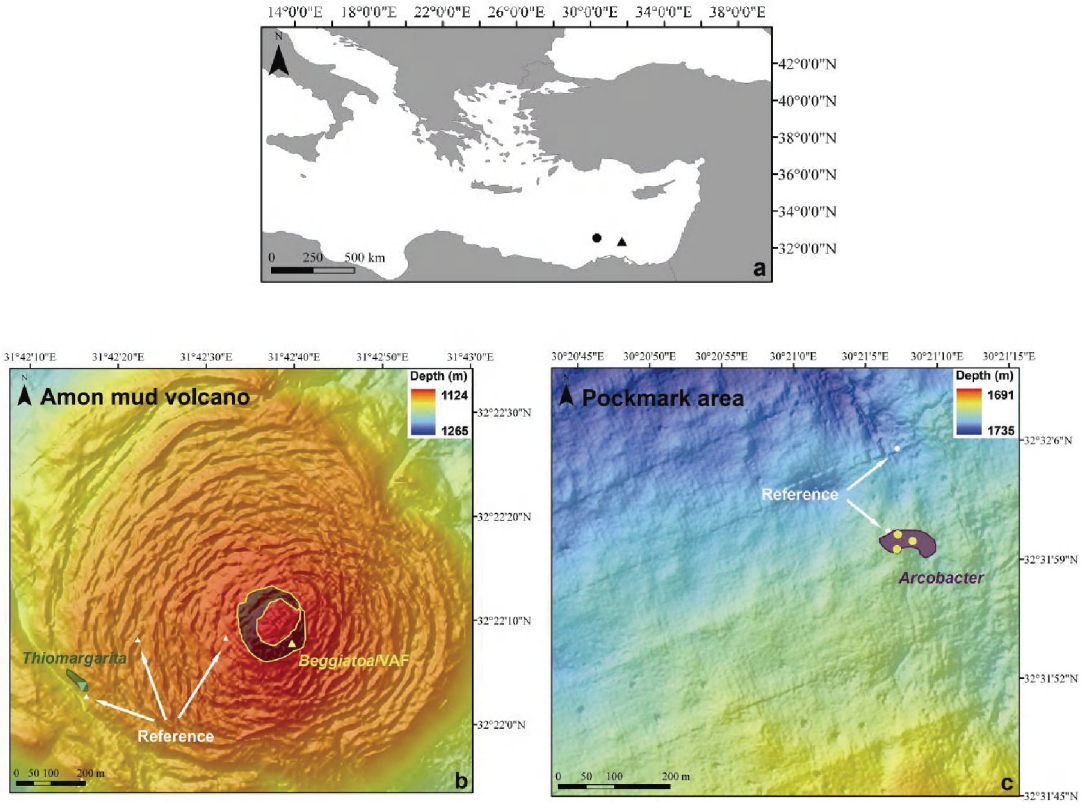

Figure 1: Eastern Mediterranean target sites. (a) Two sites in the central province of the Nile Deep Sea Fan were visited during this study, including the Amon mud volcano (black triangle) and a pockmark-dominated area (black circle). The map was generated in ArcMap (ArcGIS Desktop 9.3; ESRI, Kranzberg, Germany). (b) Bacterial mats formed by filamentous sulfur bacteria resembling *Beggiatoa* spp. and vacuolated attached filaments (VAF; yellow) were observed near the active center of the Amon mud volcano. Dense populations of *Thiomargarita* spp. (green) were found at the southwestern flank of the mud volcano. A detailed description of the *Beggiatoa*/VAF and *Thiomargarita* habitats can also be found in Felden (2009) and Girnth *et al.* (in review). The map was modified from Dupré *et al.* (2008). (c) Cotton-ball-like sulfur precipitates indicating the presence of *Arcobacter* spp. (purple) were discovered in an area characterized by flat carbonate cements covering the seafloor, mud flows and pockmarks. The map was kindly provided by the Ifremer (France) and was recorded during the BIONIL expedition (2006). For panel (b) and (c): Push core samples analyzed in this study were taken in close proximity to each other at the marked locations (triangles and circles). 'Reference' measurements and sampling were conducted nearby.

2.2 Microsensor measurements

High resolution geochemical gradients were measured *in situ* with a ROV-operated microprofiler (Boetius and Wenzhöfer, 2009). Microsensors for sulfide, pH, oxygen (Jeroschewski *et al.*, 1996; Revsbech *et al.*, 1983; Revsbech and Ward, 1983) and temperature (Pt100; UST Umweltsensortechnik GmbH, Geschwenda, Germany) were mounted on the profiler. Profiles started in the bottom water directly above the sediment surface and proceeded until a depth of 6 to 7 cm below seafloor (bsf). Microsensor calibration, measurements and profile analyses were performed as previously described (de Beer *et al.*, 2006).

2.3 Benthic chamber measurements

Total benthic oxygen uptake (TOU) was measured *in situ* with a ROV-operated benthic chamber module (Boetius and Wenzhöfer, 2009). A detailed description of the instrument as well as information on calibration and calculation of the TOU is given by Felden (2009).

2.4 Sampling

Push cores of 8 cm diameter were used to recover bacterial mat samples together with the upper 20 to 30 cm of sediment underlying the mats. Onboard, subsampling of the mat-forming bacteria and dissection of the sediment for further analyses were performed at *in situ* temperature (14°C) in a cold room immediately after recovery, or after reestablishment of mats on cores disturbed by degassing during recovery (within 24 hours). Core numbers and respective analyses are listed in Supplementary Table 1.

Subsamples of the bacterial mats were analyzed by bright field and phase contrast microscopy immediately after sampling and documented by digital photography. A calibrated eyepiece micrometer or a standardized object micrometer were used for estimating cell dimensions.

2.5 Geochemical analyses

Rates for sulfate reduction (SR) were determined *ex situ* by whole core injection (Jørgensen, 1978). Incubation at *in situ* temperature (14°C) proceeded for 12 h with $^{35}SO_4^{2-}$ (5-10 µL carrier-free $^{35}SO_4^{2-}$, dissolved in water, 50 kBq) tracer. Following

incubation, sediment samples were preserved in 20 mL of 20% (w/v) ZnAc solution. Finally, rates for SR were measured and calculated in the home laboratories of the MPI Bremen as previously described (Kallmeyer *et al.*, 2004; Treude *et al.*, 2003).

Pore-water analyses for concentrations of chloride, sulfate, total sulfide and dissolved inorganic carbon (DIC) were conducted as previously described by Felden (2009). Elemental sulfur concentrations were measured by high-performance liquid chromatography (Zopfi *et al.*, 2004) from sediment sections extracted for 12 h in a 0.1% ZnAc/methanol solution.

2.6 Fluorescence *in situ* hybridization (FISH) experiments

Sediment sections were fixed in 4% formaldehyde/seawater, washed two times with 1 × phosphate-buffered saline (PBS; pH 7.2 to 7.4), and were finally stored at -20°C in a 1:1 mixture of PBS and ethanol until further processing. Catalyzed reporter deposition FISH (CARD-FISH) was then carried out with suitable dilutions of the fixed samples as previously described (Ishii *et al.*, 2004; Pernthaler *et al.*, 2002). Horseradish peroxidase (HRP)-labeled probe sequences (Biomers, Ulm, Germany) and probe hybridization details are given in Table 1. All cells were counterstained with the DNA-targeting fluorescent stain 4',6-diamidino-2-phenylindole (DAPI). For each sample, a minimum of 30 grids was randomly counted.

Table 1: Oligonucleotide probes and hybridization conditions used for CARD-FISH analyses in this study.

Probe	Probe specificity	Probe sequence (5'-3')	Target Site within 16S rRNA gene[a]	FA[b] (%)	T_h[c]/T_w[d] (°C)	Reference
EUB338(I-III)	Most Bacteria	Equimolar mixture of the following three probes	338-355	30	46/46	Daims *et al.* (1999)
EUB338	Most Bacteria	GCT GCC TCC CGT AGG AGT	338-355	30	46/46	Amann *et al.* (1990)
EUB338-II	Planctomycetales	GCA GCC ACC CGT AGG TGT	338-355	30	46/46	Daims *et al.* (1999)
EUB338-III	Verrucomicrobiales	GCT GCC ACC CGT AGG TGT	338-355	30	46/46	Daims *et al.* (1999)
NON338	Negative control	ACT CCT ACG GGA GGC AGC	338-355	20	46/46	Wallner *et al.* (1993)
DSS658	Desulfosarcinales	TCC ACT TCC CTC TCC CAT	658-685	60	46/46	Manz *et al.* (1998)
ARCH915	Most Archaea	GTG CTC CCC CGC CAA TTC CT	915-935	30	46/46	Raskin *et al.* (1994)
ANME1-350	ANME-1 archaea	AGT TTT CGC GCC TGA TGC	350-367	40	46/46	Boetius *et al.* (2000)
ANME2a-647	ANME-2 archaea	TCT TCC GGT CCC AAG CCT	647-664	55	46/46	Knittel *et al.* (2005)
ANME3-1249	ANME-3 archaea	TCG GAG TAG GGA CCC ATT	1250-1267	40	46/46	Niemann *et al.* (2006)
ANME3-1249H3	helper probe for ANME3-1249	GTC CCA ATC ATT GTA GCC GGC	1229-1249	40	46/46	Lösekann *et al.* (2007)
ANME3-1249H5	helper probe for ANME3-1249	TTA TGA GAT TAC CAT CTC CTT	1268-1288	40	46/46	Lösekann *et al.* (2007)
ARC94	*Arcobacter* spp.	TGC GCC ACT TAG CTG ACA	94-111	20	46/48	Snaidr *et al.* (1997)

[a] *E.coli* positions.

[b] Formamide concentration in the hybridization buffer.

[c] Hybridization temperature.

[d] Washing temperature.

2.7 Phylogenetic analyses

Subsamples of the different mats were preserved in either PCR-grade water (Sigma-Aldrich Biochemie GmbH, Hamburg, Germany) or 1 × TE buffer (Promega Corporation, Madison, WI) and stored at -20°C. Universal bacterial primers GM3F and GM4R (5'-AGAGTTTGATCMTGGC-3', 5'-TACCTTGTTACGACTT-3'; Muyzer *et al.*, 1995) were used for amplification of nearly full length 16S rRNA gene sequences in polymerase chain reactions (PCR). Based on previous experiences, template was derived from the original sample solutions by freeze-thawing. Replicate amplifications were conducted as described in Girnth *et al.* (in review). Pooled and purified PCR products were cloned, and representative clones were selected for plasmid preparation after partial sequencing. Purified plasmids were subjected to *Taq* cycle sequencing with an ABI Prism 3130x Genetic Analyzer (Applied Biosystems, Foster City, CA). Nearly full length 16S rRNA gene sequences were assembled with the Sequencher 4.6 software (Gene Codes Corporation, Ann Arbor, MI) and manually checked for ambiguities. The sequences were examined for chimeric signals by using the Pintail program (Ashelford *et al.*, 2005) and trusted nearest neighbors obtained with the SILVA-based SINA Webaligner (http://www.arb-silva.de/aligner/; Pruesse *et al.*, 2007). Sequences with genuine chimeric signals were excluded from further analyses. Phylogenetic trees were reconstructed in the ARB software package (Ludwig *et al.*, 2004).

2.8 Nucleotide sequence accession numbers

Sequence data will be submitted to the EMBL database.

3. Results

Three different types of white bacterial mats (Figure 2) from the central province of the Nile Deep Sea Fan (NDSF) were compared with regard to the identity of the mat-forming bacteria, and to their niche differentiation. By ROV observation, the mats were not clearly distinct from one another, and all of them were associated with patchy flows

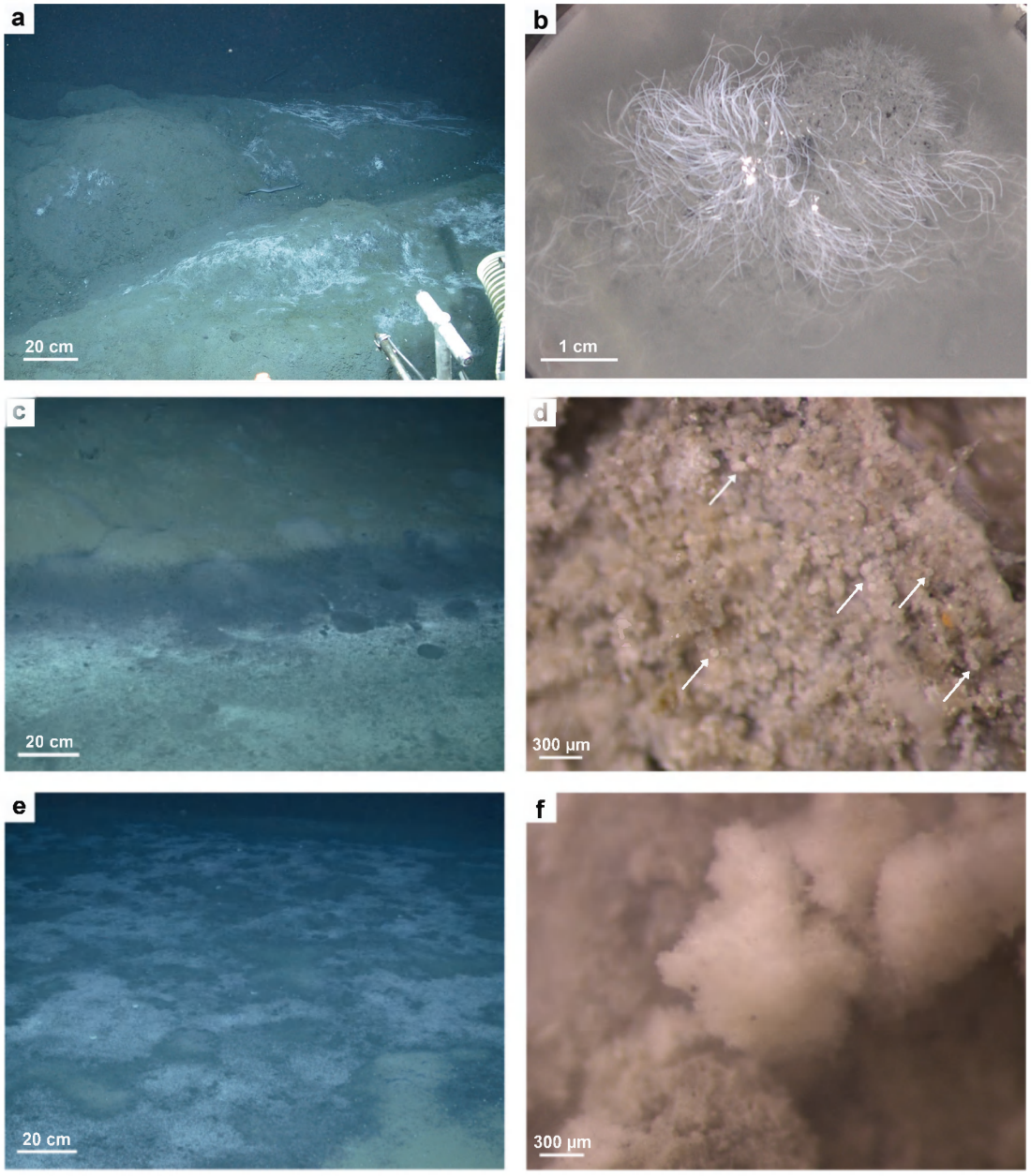

Figure 2: Three morphologically different types of sulfide oxidizer mats were observed in the central province of the Nile Deep Sea Fan. (a) Bacterial mats near the center of the Amon mud volcano were dominated by filaments resembling *Beggiatoa* spp. and vacuolated attached filaments. Picture Source: Marum, QUEST4000. (b) Single white filaments were visible on the sediment surface upon recovery of push cores taken from the bacterial mat patches. (c) Sulfidic muds flowing from the southwestern flank of the Amon mud volcano were covered by a thin white bacterial mat. Picture Source: Marum, QUEST4000. (d) Microscopy revealed a dense population of single, spherical *Thiomargarita* cells (arrows; Girnth *et al.*, in review). (e) *Arcobacter* mat in a pockmark-dominated area of the central Nile Deep Sea Fan. White precipitates resembling cotton balls were found on top of blackish, highly reduced sediments. Picture Source: Marum, QUEST4000. (f) At higher magnification, the cotton balls consisted of dense assemblages of filamentous sulfur, which are known as metabolic products of marine *Arcobacter* spp. (picture taken aboard the ship; Omoregie *et al.*, 2008; Taylor *et al.*, 1999).

of highly reduced, blackish subsurface mud (Figure 2a, c, e). Their white color resulted from the presence of intracellular or extracellular elemental sulfur, a product of bacterial sulfide oxidation. Push core retrieval of the mats and the subsequent visual or microscopic examination on board revealed that each mat type was dominated by different sulfide oxidizers: (i) different sulfur-storing filaments (Figure 2b), (ii) sulfur-storing spheres resembling *Thiomargarita* (Figure 2d; Girnth *et al.*, in review), or (iii) *Arcobacter* as indicated by the presence of filamentous sulfur scaffolds (Figure 2f).

3.1 Filamentous sulfide oxidizer mats at the deep-sea mud volcano Amon

At the border of the elevated, central dome of the Amon mud volcano (AMV; Figure 1b), patchy white mats (Figure 2a) were observed to occur irregularly within an area of approx. 0.027 km^2, covering 10 to 20% of this zone. The mats showed diameters of approx. 20 cm to 5 m and were mostly associated with the top of hummocks created from former mud flows and gas dilation (Dupré *et al.*, 2007; Dupré *et al.*, 2008). Push coring of the mats led to sporadic gas release. Upon push core recovery, single white filaments on the sediment surface could be recognized by the naked eye (Figure 2b).

Microscopic analyses of these mats revealed the presence of four morphologically different groups of sulfur-storing, filamentous bacteria. The narrowest AMV filaments (<20 μm) appeared as long threads of sulfur granules and could not be clearly identified. AMV filaments of 20 to 30 μm in diameter (Figure 3a) combined morphological traits characteristic for giant sulfide oxidizers of the genera *Beggiatoa* and *Thioploca* (Teske and Nelson, 2006). *Beggiatoa* and *Thioploca* species are both giant, gliding, multicellular, sulfur-storing filaments that are distinguished from each other based on their occurrence as either individuals (*Beggiatoa*) or in bundles surrounded by a common sheath (*Thioploca*). In addition, *Beggiatoa* spp. have rather rounded terminal cells, while those of *Thioploca* spp. appear tapered (Teske and Nelson, 2006). AMV filaments occurred as individual filaments composed of rather cylindrical cells (*Beggiatoa*-like), but the terminal cells appeared tapered (*Thioploca*-like). Hence, a clear identification seems difficult. Though, taking into account the most discriminating feature, i.e. occurrence as individuals or in bundles, these filaments are likely *Beggiatoa*. AMV filaments of 135 μm in diameter were clearly reminiscent of the genus *Beggiatoa*, as they occurred as individual filaments that were composed of disk-shaped cells and had rounded, only slightly tapered filament tips

(Figure 3b). None of the *Beggiatoa* spp.-resembling filaments was observed to glide, but the sample recovery process may have impaired physiological functions. Individual AMV filaments of 40 to 60 µm in diameter seemed morphologically deviant from filaments attributed to the genus *Beggiatoa* (see above), as they had rather elongated cylindrical cells and almost semicircular filament tips (Figure 3c). Presence of a central vacuole was indicated by sulfur globules appearing to be positioned around a hollow space when focusing on different planes throughout the filament. No gliding was observed.

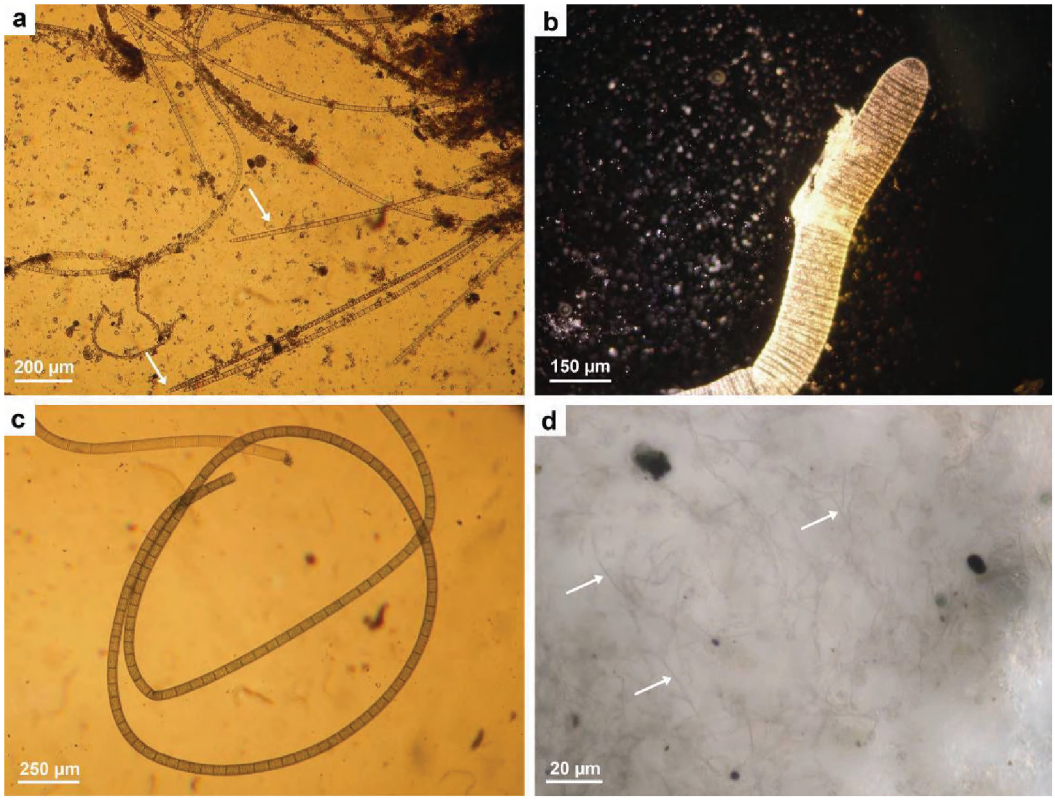

Figure 3: Microscopic analyses of *Beggiatoa*/VAF and *Arcobacter* mats from the Nile Deep Sea Fan. (a) Bright field image of *Beggiatoa*-type filaments with a diameter of approx. 20 µm (arrows). (b) Dark field microscopic image of a 135 µm wide *Beggiatoa*-type filament. The bright color of the filament is caused by the refractive nature of the internally stored sulfur globules. (c) Bright field image of a sulfur-storing filament that, based on subsequent phylogenetic analyses, potentially represents a type of vacuolated attached filament. (d) Phase contrast image of a white, cotton-ball-like aggregate, revealing thin filaments of elemental sulfur (arrows). These aggregates are typical for *Arcobacter* spp.-type mats (Omoregie *et al.*, 2008; Taylor *et al.*, 1999).

The distribution of the different filament types within the mat patches was heterogeneous and varied among the analyzed samples. Most of the observed filaments belonged to the three smaller size classes, and only few filaments of the largest filament type could be recovered. Subsequent 16S rRNA gene sequencing resulted in two distinct groups of sequences within the gammaproteobacterial cluster of giant, sulfur-storing bacteria within the Thiotrichales. A representative sequence was chosen from each group for further phylogenetic analyses (AMV1058 and AMV346). The sequence AMV1058 was obtained from a sample of a single picked *Beggiatoa* spp.-resembling filament of 20 to 30 μm diameter. Phylogenetically, this sequence was found to be closely related to the two published sequences of marine *Thioploca* spp. (L41043 and L40999) and a sequence of a large marine *Beggiatoa* sp. from the Bay of Concepción (AF035956, 99.1% similarity; Figure 4). AMV346 was obtained from a sample of three picked filaments of 40 to 60 μm diameter and clustered with a monophyletic group of VAF (vacuolated attached filaments) sequences derived from shallow hydrothermal vents at White Point, California, (AY496953, 98.0% similarity; Kalanetra *et al.,* 2004), from the Escanaba Trough along the Gorda Ridge (AY883934, 98.4% similarity; Kalanetra *et al.*, unpublished) and from deep-sea hydrothermal vents on the Juan de Fuca Ridge (AY883933, 98.4% similarity; Kalanetra *et al.*, unpublished; Figure 4). AMV346 was most closely related to an unidentified filamentous bacterium associated with a white microbial mat found at the deep-sea mud volcano Milano (Olympi field) in the Eastern Mediterranean Sea (AY592917, 99.7% similarity; Heijs *et al.,* 2005). Representative sequences for the smallest and largest filaments from the AMV center mats could not be obtained so far.

3.2 *Thiomargarita* mats on a mud flow of the Amon mud volcano

Location and structure of the *Thiomargarita* mat as well as morphological and phylogenetic analyses were previously described in detail by Girnth *et al.* (in review). Briefly, a dense population of *Thiomargarita* cells was found on top of a sulfidic mud outflow at the southwestern flank of the AMV (Figure 1b). The mat appeared as a coherent thin white layer (Figure 2c) over an estimated area of 3 × 50 m and was composed of single, spherical cells (Figure 2d). With a diameter of 24 to 65 μm, *Thiomargarita* cells from the AMV were substantially smaller than cells of previously described populations (Kalanetra *et al.*, 2005; Schulz *et al.*, 1999). A 16S rRNA gene

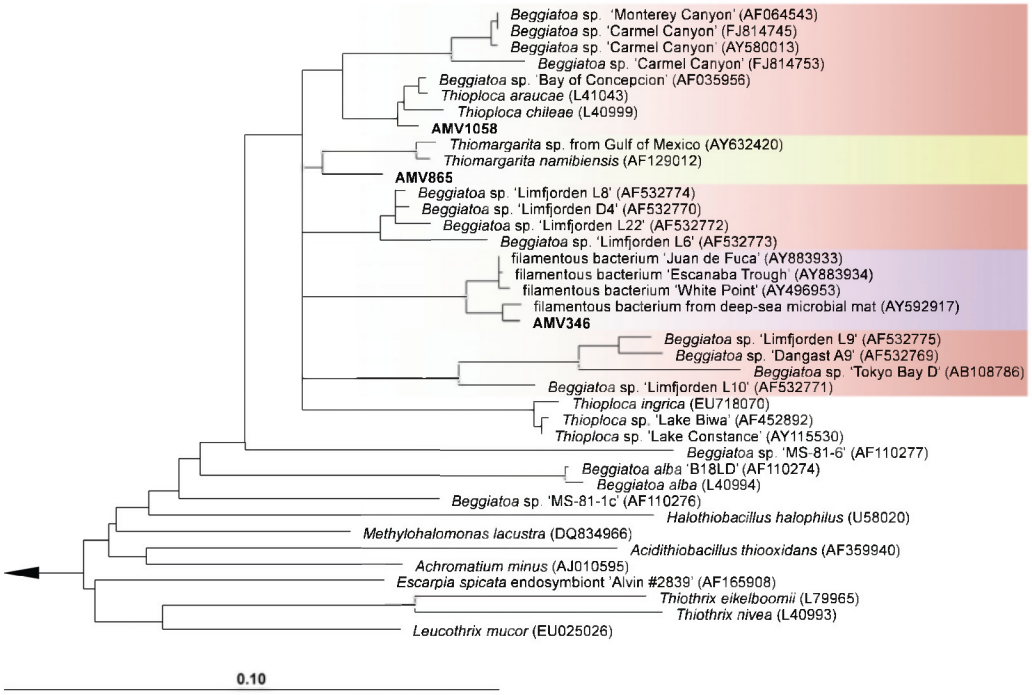

Figure 4: Phylogenetic 16S rRNA gene based tree showing the affiliations of the two filamentous-mat-associated sequences AMV1058 and AMV346 to reference sequences within the Gammaproteobacteria. The previously published *Thiomargarita* sequence AMV865 (Girnth *et al.*, in review) is also shown. Initial tree reconstruction was conducted with nearly full length sequences by applying neighbour joining, maximum likelihood (RAxML) and maximum parsimony methods as well as different conservatory filters. The final tree is based on 1108 valid columns (50% conservatory filter; *E. coli* positions 126 to 1307) with neighbour joining. Short sequences L41043, L40999, AY632420, AF129012, AY592917 and AMV865 were added subsequently by applying parsimony criteria. Only selected sequences are shown. Unstable branching orders in the group of interest were visualized by multifurcation. Deltaproteobacterial sequences were used as outgroup. The bar indicates 10% estimated phylogenetic divergence. Color coding is as follows: red = marine, vacuolated filaments; yellow = *Thiomargarita* spp.; purple = marine, vacuolated, attached filaments.

fragment (AMV865) was amplified and assigned to *Thiomargarita*-resembling cells by fluorescence *in situ* hybridization. This sequence is monophyletic with published *Thiomargarita* sequences (Figure 4), but sequence similarities are only about 94%, indicating a distinct diversification from other shallow-water or deep-sea types. This was confirmed by CARD-FISH analyses, showing that AMV865 does not occur in *Thiomargarita namibiensis* (Girnth *et al.*, in review).

3.3 Mat-forming *Arcobacter* spp. on mud flows in the pockmark area

The central 'Pockmark area' approx. 130 km west of the AMV (Figure 1a, c) is characterized by many different signs of active seepage, including abundant pockmarks of 3 to 10 m diameter, gassy and sulfidic mud flows, authigenic carbonate cements, and assemblages of chemosynthetic tubeworms and bivalves (Duperron *et al.*, 2007; Gontharet *et al.*, 2007; Loncke *et al.*, 2004). On the mud flows, whitish mats were observed consisting of cotton-ball-like precipitates of sulfur (Figure 2e, f). Here, a large mat covering an area of approx. 60 × 100 m was studied. Light microscopy of the white precipitates on replicate push core samples confirmed that these consisted of thin sulfur filaments (Figure 3d) resembling structures formed by *Arcobacter* spp. (Omoregie *et al.*, 2008; Taylor *et al.*, 1999).

Light microscopy did not reveal cells associated with the sulfur precipitates (Figure 3d). However, PCR of mat samples obtained from two replicate mat positions retrieved two related 16S rRNA gene sequences (POCKM3418 and POCKM3495). Phylogenetically, both sequences were closely related to an epsilonproteobacterial group comprising 16S rRNA gene sequences of *Arcobacter nitrofigilis* (L14627) and '*Candidatus* Arcobacter sulfidicus' (AY035822; Figure 5). POCKM3418 and POCKM3495 were only 94.5% similar to each other. Similarity to the published sequences of *A. nitrofigilis* and '*Ca.* Arcobacter sulfidicus' was 92.0 and 92.8% for POCKM3418, and 91.8 and 92.1% for POCKM3495, respectively.

CARD-FISH was applied to quantify *Arcobacter* cells in the mat and upper 3 cm of sediment using the *Arcobacter* spp.-specific probe ARC94 on each of the three NDSF mat types. Indeed, high cell numbers were detected with this probe in the sediment underlying the pockmark-associated *Arcobacter* mat (3.0 to 7.8 × 10^8 cells cm^{-3} sediment; Table 2).

In addition to the *Arcobacter* mat samples, several *Arcobacter* spp.-related 16S rRNA gene sequences were obtained from clone libraries constructed with samples of the *Beggiatoa*/VAF and *Thiomargarita* mats. These sequences (AMV208, AMV279, AMV284, AMV514, AMV652, AMV658, AMV700, AMV716, AMV729 and AMV3269) were associated with the same epsilonproteobacterial group as POCKM3418 and POCKM3495 (Figure 5). Similarities between all *Arcobacter* spp.-related sequences from the NDSF varied between 92.9 and 99.9%. Using the specific probe ARC94, *Arcobacter*

cells were detected beneath the *Beggiatoa*/VAF and *Thiomargarita* mats in abundances of $<0.6 \times 10^8$ cells cm^{-3}, i.e. 10 to 100 fold less than associated with the *Arcobacter* mats (Table 2).

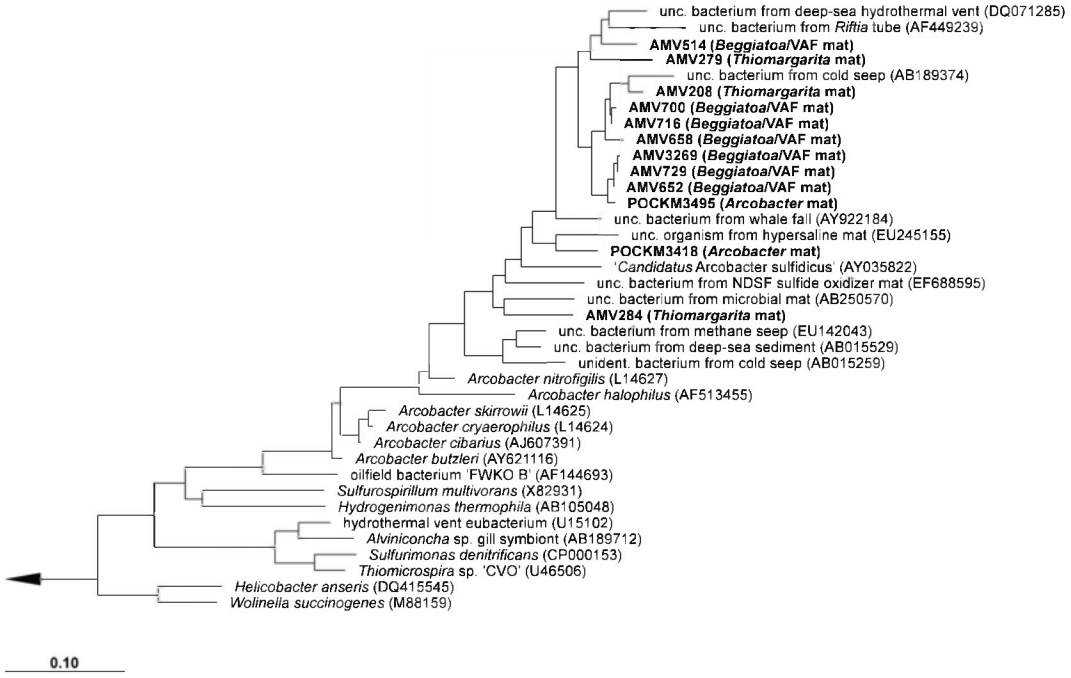

Figure 5: Maximum likelihood tree showing the affiliations of all Nile Deep Sea Fan *Arcobacter* spp.-related 16S rRNA gene sequences obtained in this study (bold type) to reference sequences within the Epsilonproteobacteria. Tree reconstruction was conducted with nearly full length sequences (*E. coli* positions 88 to 1349; 1167 valid columns at 50% conservatory filtering). Only selected sequences are shown. Deltaproteobacterial sequences were used as outgroup. The bar indicates 10% estimated phylogenetic divergence.

3.4 Biogeochemistry of the mat habitats

All three mat habitats were characterized by high average sulfide fluxes (~1 to 88 mmol m^{-2} d^{-1}; Table 3), but methane fluxes varied between 881 mmol m^{-2} d^{-1} in the *Arcobacter* mat, 72 mmol m^{-2} d^{-1} in the *Beggiatoa*/VAF mats and 0 mmol m^{-2} d^{-1} in the *Thiomargarita* mat (F. Wenzhöfer, unpublished data). All mat habitats showed highly elevated

Table 2: CARD-FISH analyses.

Sampling site	Depth (cm)		ARCH915		ANME1		ANME2		ANME3		EUB338(I-III)		DSS658		ARC94	
		Total free cells	Free cells	Percent	Free cells	Percent	Free cells	Percent	Free cells	Percent	Free cells	Percent	Free cells	Percent	Free cells	Percent
		1×10^9	1×10^9	Total	1×10^9	ARCH915	1×10^9	ARCH915	1×10^9	ARCH915	1×10^9	Total	1×10^9	EUB338(I-III)	1×10^9	EUB338(I-III)
Beggiatoa/VAF mats	0-1	4.35 ± 0.25	0.51	12	0.02	5	0.03	6	0	0	3.87	89	0.01	<	0.06	1
(Amon mud volcano)	1-2	1.38	ND	ND	0.01	ND	0.01	ND	0.003	ND	0.89	64	0.001	<	0.04	5
	2-3	0.64 ± 0.02	0.17	27	0.01	8	0	0	0	0	0.31	49	0.01	4	0.02	6
	3-4	0.89 ± 0.17	0.23	26	0.01	3	0	0	0	0	0.39	44	0	0	ND	ND
	4-5	0.93 ± 0.08	0.38	41	0.13	34	0.004	1	0	0	0.46	50	0	0	ND	ND
	5-6	0.91 ± 0.26	0.41	45	0.05	12	0.01	2	0	0	0.30	33	0.004	1	ND	ND
Thiomargarita mat	0-1	3.00 ± 0.29	0.39	13	0.004	1	0	0	0	0	1.12	37	0	0	0.01	1
(Amon mud volcano)	1-2	3.17 ± 0.84	0.34	11	0	0	0.01	4	0	0	0.94	30	0	0	0.01	1
	2-3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.005	ND
	3-4	1.49 ± 0.17	0.18	12	0.01	8	0.001	1	0.01	4	0.66	44	0.004	1	ND	ND
	4-5	0.68 ± 0.05	0.07	10	0.03	35	0.01	12	0.01	16	0.13	19	0.004	3	ND	ND
	9-10	0.51 ± 0.14	0.08	16	0.01	8	0	0	0.01	14	0.12	23	0.003	2	ND	ND
	14-16	0.44 ± 0.02	0.11	25	0.003	2	0	0	0.004	4	0.07	17	0.004	5	ND	ND
Arcobacter mat	0-1	5.16 ± 1.48	0.42	8	0.05	11	0.01	2	0	0	2.97	58	0.01	<	0.78	26
(Pockmarks)	1-2	5.13 ± 1.10	0.75	15	0.18	24	0.01	1	0	0	3.47	68	0.003	<	0.77	22
	2-3	3.08 ± 0.39	0.49	16	0.20	40	0.004	1	0	0	1.85	60	0.001	<	0.30	16
	3-4	2.40 ± 0.73	0.45	19	0.11	25	0.01	3	0	0	1.32	55	0.003	<	ND	ND
	4-5	1.98 ± 0.10	0.57	29	0.11	19	0.01	1	0	0	0.61	31	0	0	ND	ND
	9-10	0.84 ± 0.47	0.11	13	0.05	46	0	0	0	0	0.10	12	0	0	ND	ND

Cell numbers are per cm^{-3} of sediment. Total cell numbers were determined by DAPI-staining. '<' refers to percentages between 0 and 1%. For total free cells at 1-2 cm of the *Beggiatoa*/VAF mats no standard deviation is available, as this value is based on a single measurement (n = 1). All other total free cell numbers are based on n = 2. Specific cell counts are based on n = 1 for ARCH915, all ANME, EUB338(I-III) and DSS658, and n = 2 for ARC94. ND, no data.

Table 3: Biogeochemical characterization of the Nile Deep Sea Fan *Beggiatoa*/VAF, *Thiomargarita* and *Arcobacter* dominated habitats.

Sampling site	Measurement/Analysis								Cell properties[g]							
	Microprofiles					TOU ($mmol\ m^{-2}\ d^{-1}$)	SR[a] ($mmol\ m^{-2}\ d^{-1}$)	elemental sulfur in the upper 1 cm ($mol\ m^{-3}$)								
	oxygen penetration depth (mm)	overlap oxygen/sulfide	DOU ($mmol\ m^{-2}\ d^{-1}$)	sulfide flux ($mmol\ m^{-2}\ d^{-1}$)	ratio DOU:sulfide flux				cell size	motility	electron donor	electron acceptor	elemental sulfur	vacuole	nitrate accumulation	
Beggiatoa/VAF mat[b]	< 1	no	42.1 (±2.8)	40	1:1	50	18.5-138.3 (average: 54.1)	0.44 (±0.22)	10-200 µm (width)	gliding	H_2S / S^0	O_2 / NO_3^-	stored	yes	yes	*Beggiatoa*
(Amon mud volcano)									4-112 µm (width)	no	H_2S / S^0	O_2	stored	yes	no	VAF
Thiomargarita mat[c]	1.5-2.5	temporarily	10-46	1-20	not determined	35	0.3-0.7 (average: 0.5)	5.03 (±2.18)	100-750 µm	no	H_2S / S^0	O_2 / NO_3^-	stored	yes	yes	*Thiomargarita*
(Amon mud volcano)																
Arcobacter mat[d]	1.75	~ 1 mm	27.4 (±16.2)	88.2 (±64.1)	1:3	71	9.1-112.1 (average: 40.7)	1.44 (±0.92)	0.5 × 2.0 µm	yes	H_2S	O_2	excreted	no	no	*Arcobacter*
(Pockmarks)	1	~ 1 mm	39.5 (±4.8)	35.1 (±13.3)	1:1											
Reference Amon mud volcano[e]	> 10	no	11.9 (±5.0)	0	not determined	5	0-0.5 (average: 0.2)	0.09 (±0.02)	Detailed information can be found in Teske and Nelson (2006), Kalanetra *et al.* (2004), Schulz (2006), Sievert *et al.* (2007) and Wirsen *et al.* (2002).							
(Amon biogenic mounds)																
Reference Pockmarks[f]	> 10	no	0.5 (±0.4)	0	not determined	1.4	0.14 (±0.04)	0.09								

Abbreviations are as follows: VAF (vacuolated attached filaments), DOU (diffusive oxygen uptake), TOU (total benthic oxygen uptake), SR (sulfate reduction), S^0 (elemental sulfur), ND (no data).

[a] Integrated rates were calculated for the upper 15 cm of sediment.

[b] Microprofiles, TOU, and SR are from Felden (2009). Average values and standard deviations were calculated from replicate measurements (n) conducted during the BIONIL cruise 2006: DOU (n = 2), sulfide flux (n = 1), TOU (n = 1), SR (n = 5), elemental sulfur (n = 2).

[c] Microprofiles, TOU, and SR are from Girnth *et al.* (in review) and Felden (2009). Average values and standard deviations were calculated from replicate measurements (n) conducted during the BIONIL cruise 2006: DOU (n = 6), sulfide flux (n = 6), TOU (n = 1), SR (n = 3), elemental sulfur (n = 2).

[d] Three sets of microprofiles were recorded for the *Arcobacter* mat system: on the mat (upper values), at the border of the mat (lower values) and on adjacent, non-sulfidic seafloor (next to mat). Average values and standard deviations were calculated from replicate measurements (n) conducted during the BIONIL cruise 2006: DOU (n = 2), sulfide flux (n = 2), TOU (n = 1), SR (n = 9), elemental sulfur (n = 3).

[e] Reference microprofiles, TOU, and SR are from Girnth *et al.* (in review) and Felden (2009). Average values and standard deviations were calculated from replicate measurements (n) conducted during the BIONIL cruise 2006: DOU (n = 2), sulfide flux (n = 2), TOU (n = 1), SR (n = 4), elemental sulfur (n = 2).

[f] Average values and standard deviations were calculated from replicate measurements (n) conducted during the BIONIL cruise 2006: DOU (n = 2), sulfide flux (n = 2), TOU (n = 1), SR (n = 3), elemental sulfur (n = 1).

[g] *Beggiatoa*: Values are given for wide, vacuolated marine species only (Teske and Nelson, 2006). VAF: This is based on one study where VAF were observed at shallow hydrothermal vents (Kalanetra *et al.*, 2004). *Thiomargarita*: Values refer to described populations off Namibia (Schulz *et al.*, 1999) and in the Gulf of Mexico (Kalanetra *et al.*, 2005). *Arcobacter*: Values refer to the coastal strain '*Candidatus* Arcobacter sulfidicus', for which filamentous sulfur formation has been recorded (Sievert *et al.*, 2007). Elemental sulfur is derived from sulfide oxidation.

elemental sulfur concentrations in the top sediment horizon compared to the reference sites outside the mat (Table 3). On average, 1.44 mol m^{-2} were contained in the *Arcobacter* mat, and 0.44 and 5.03 mol m^{-3} in mats of *Beggiatoa*/VAF and *Thiomargarita*, respectively. Total microbial cell numbers as determined by DAPI-staining were in the same range for all three mat types, with maximum values of 4.35×10^9 cells cm^{-3} (*Beggiatoa*/VAF mats), 3.17×10^9 cells cm^{-3} (*Thiomargarita* mat) and 5.16×10^9 cells cm^{-3} (*Arcobacter* mat) found in the upper sediment layers (Table 2). CARD-FISH analyses showed relatively high total archaeal cell numbers between 8 to 45% of total cell numbers. Anaerobic methanotrophs (ANME) of the ANME-1 clade were found in the sediment below all mat types. ANME-2 cells were only found in very low numbers beneath all three mat types. ANME-3 cells were present below the *Beggiatoa*/VAF and *Thiomargarita* mats. Cells hybridizing with the *Desulfosarcina/Desulfococcus*-specific probe DSS658 identifying sulfate-reducing bacteria associated with anaerobic oxidation of methane were found in all investigated mat habitats.

Biogeochemical data characterizing the AMV mats of the central dome and the mud flow on the southwestern flank are discussed in detail by Felden (2009) and Girnth *et al.* (in review), and are briefly summarized in Table 3 for comparison. This study has obtained the first *in situ* measurements on an *Arcobacter* mat associated with a deep-sea cold seep (Figure 6a-c). Three sets of profiles were recorded during the same dive, in the middle of the large *Arcobacter* mat (Figure 6a, 'mat'), in the transition zone from mat to non-sulfidic seafloor (Figure 6b, 'mat border') and on adjacent non-sulfidic seafloor (Figure 6c, 'reference', less than 5 m away from the mat). On the mat, oxygen penetrated approx. 1.75 mm into the sediment. Sulfide reached the sediment surface, in a zone where oxygen and sulfide overlapped (Figure 6a). Microprofiles recorded at the border of the mat showed comparable conditions in that oxygen penetrated approx. 1 mm into the sediment and overlapped with sulfide (Figure 6b). Average oxygen and sulfide fluxes on the mat and at the mat border were calculated as 27 and 88 mmol m^{-2} d^{-1} (mat) and 39 and 35 mmol m^{-2} d^{-1} (mat border), yielding ratios of 1:3 and 1:1 (oxygen:sulfide). The pH decreased slightly beneath the sediment surface at both locations (Figure 6a, b), likely due to ongoing sulfide oxidation. At the adjacent reference site outside the mat, average oxygen consumption in the sediment was much lower (flux 0.5 mmol m^{-2} d^{-1}) than on the *Arcobacter* mat and at its border. Sulfide could not be detected and pH decreased

more gradually (Figure 6c). Temperature was constant at approx. 13.6°C throughout all profiles at all sites (Figure 6a-c), suggesting that in this area transport of gassy and sulfidic muds was disconnected from elevated geothermal heat flux typical for mud and fluid expulsion from deep subsurface reservoirs. The measured pore-water chloride concentrations remained as well constant (approx. 595 mM) over the analyzed depth (Figure 7a).

Decreases in sulfate as well as increases in total sulfide and dissolved inorganic carbon (DIC) concentrations (Figure 7a) indicated two main zones (3 to 6 cm bsf and 10 to 16 cm bsf) of sulfide production and generation of inorganic carbon compounds. In contrast, at the adjacent reference site not covered by the *Arcobacter* mat, pore-water sulfate, sulfide and DIC concentrations remained constant over the analyzed depth (Figure 7b). High sulfate reduction rates (Figure 7c) peaking at approx. 8 cm bsf were recorded beneath the mat. Integrated rates of SR were calculated as 41 mmol m^{-2} d^{-1} (0 to 15 cm; average values). Sulfate reduction rates matched total sulfide flux, indicating that microbial hydrocarbon oxidation was its main source. Total benthic oxygen uptake for the *Arcobacter* mat was measured *in situ* as 71 mmol m^{-2} d^{-1} (Table 3). This suggests that most of the sulfide efflux was consumed by aerobic respiration.

4. Discussion

This study focused on comparing the ecological niches of different thiotrophic mats in the central province of the Nile Deep Sea Fan, occupied by (i) filamentous sulfur bacteria resembling *Beggiatoa* and VAF, (ii) *Thiomargarita* species and (iii) *Arcobacter* species. Niche differentiation is discussed in view of the biology of the organisms (e.g. gliding motility, nitrate storage and use) and the characteristics of the habitats (microenvironments and dynamics, geochemistry). Interestingly, *Arcobacter* cells were found in relatively high numbers in all three sulfidic habitats, but outcompeted other sulfide oxidizers only under specific conditions.

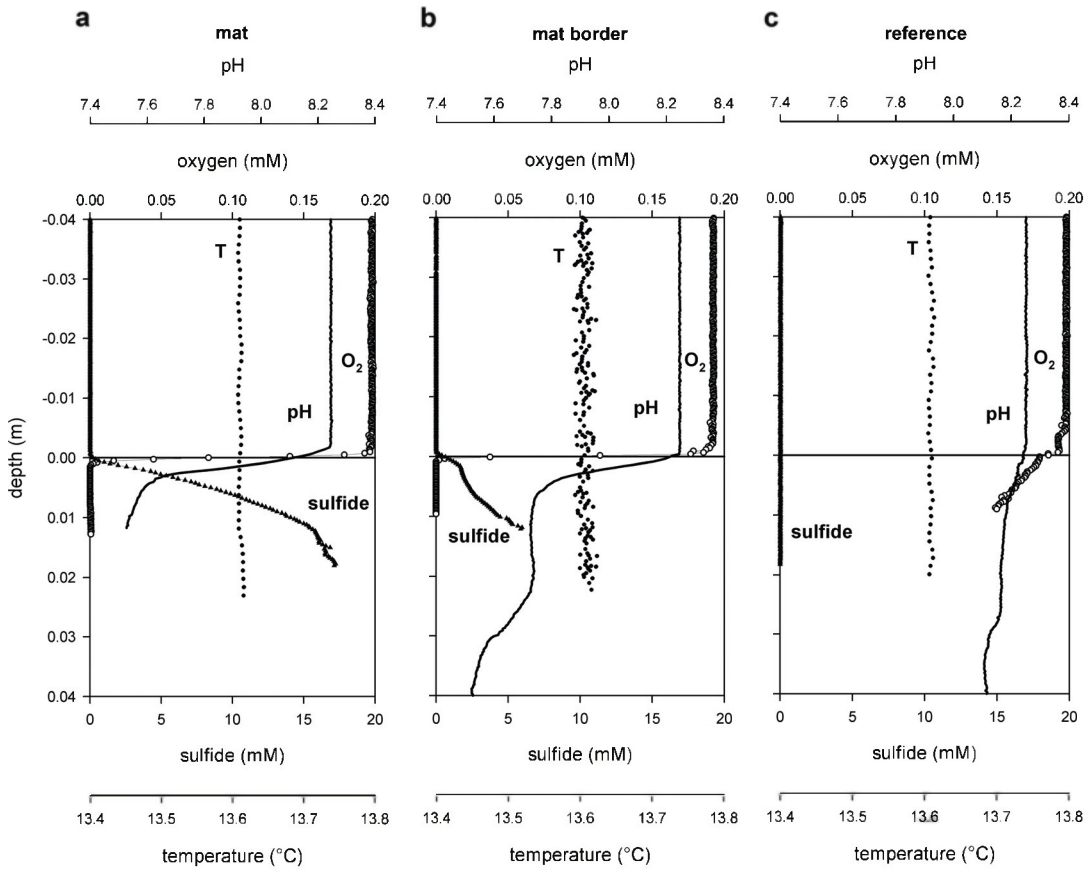

Figure 6: High resolution *in situ* microprofiles of oxygen (O_2), sulfide, pH and temperature (T) recorded (a) on the *Arcobacter* mat, (b) at the border of the *Arcobacter* mat and (c) next to the *Arcobacter* mat ('reference').

4.1 *Arcobacter* mats are associated with oxygen-sulfide interfaces in the benthic boundary layer

In situ microprofiling revealed that *Arcobacter* spp. dominate thiotrophic mats when gradients of oxygen and sulfide overlap in the benthic boundary layer (Figure 6a, b and 8c). Filamentous sulfur formation by '*Ca.* Arcobacter sulfidicus', one of the next relatives of the mat-forming *Arcobacter* phylotypes POCKM3418 and POCKM3495 retrieved in this study (Figure 5), had been observed under similar conditions (Sievert *et al.*, 2007). '*Ca.* Arcobacter sulfidicus' is a highly motile organism, aggregating within the oxic-anoxic interface and oxidizing sulfide mainly to elemental sulfur (Sievert *et al.*, 2007; Taylor and Wirsen, 1997). Aerobic oxidation of sulfide to sulfur is a slightly alkaline

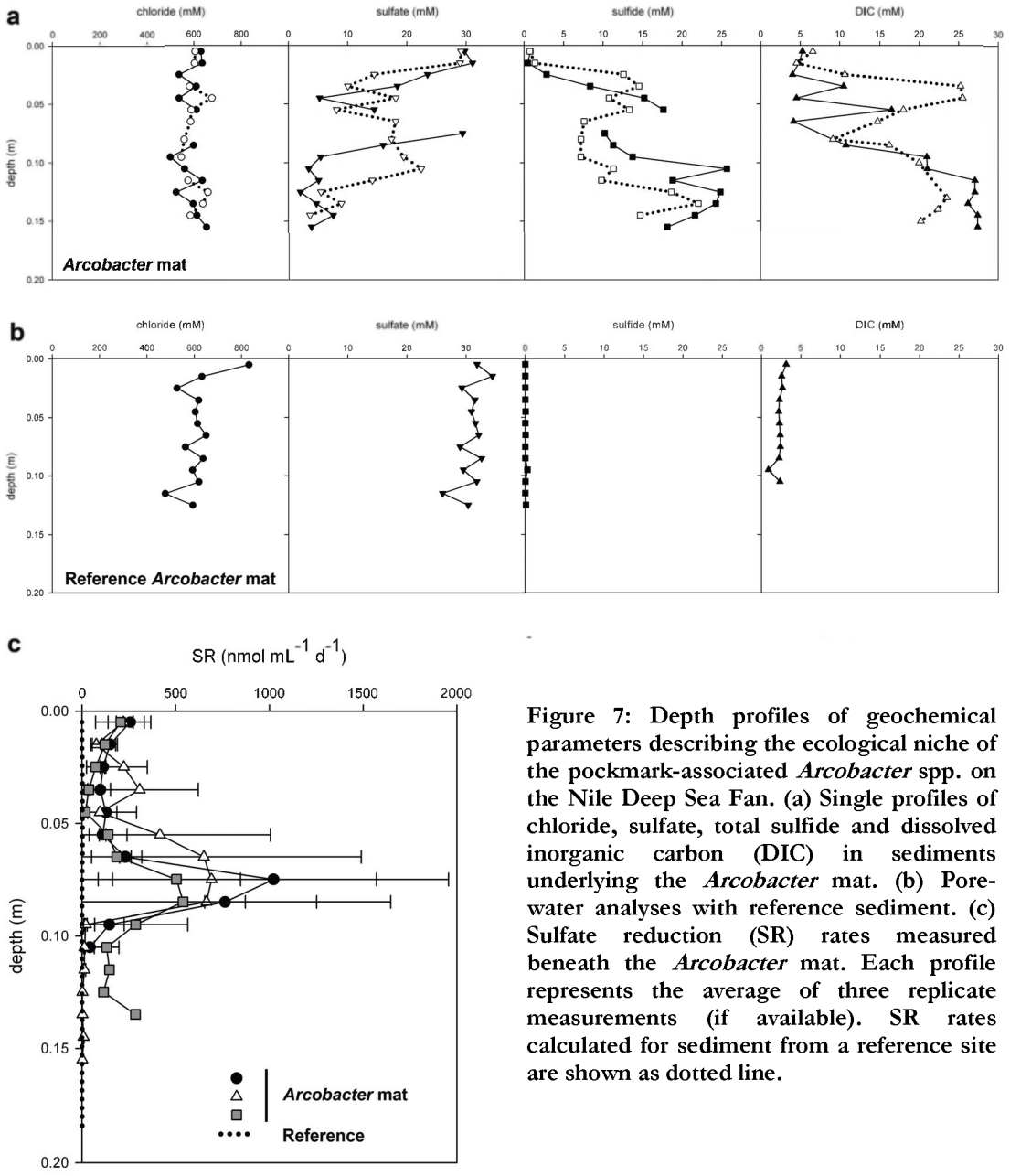

Figure 7: Depth profiles of geochemical parameters describing the ecological niche of the pockmark-associated *Arcobacter* spp. on the Nile Deep Sea Fan. (a) Single profiles of chloride, sulfate, total sulfide and dissolved inorganic carbon (DIC) in sediments underlying the *Arcobacter* mat. (b) Pore-water analyses with reference sediment. (c) Sulfate reduction (SR) rates measured beneath the *Arcobacter* mat. Each profile represents the average of three replicate measurements (if available). SR rates calculated for sediment from a reference site are shown as dotted line.

reaction ($2HS^- + O_2 \rightarrow 2S^0 + 2OH^-$), but an increase in pH remained undetected during measurements in the pockmark-associated mat (Figure 6a, b). Instead, a pH decrease was detected in the sediments below the *Arcobacter* mat, and chemical sulfide oxidation can not be excluded. Sievert and colleagues (2007) observed a similar phenomenon during

gradient experiments with '*Ca.* Arcobacter sulfidicus' conducted in microslide capillaries. They attributed the decrease in pH to the slightly acidic behavior of hydrogen sulfide when dissociating in aqueous solution and to the assumption that '*Ca.* Arcobacter sulfidicus' may also be capable of oxidizing sulfide to sulfate. On the NDSF *Arcobacter* mat, the oxygen:sulfide flux ratio of 1:3 (Table 3) indicated that an alternative electron acceptor might have been involved in sulfide oxidation. Recent studies in African shelf waters suggested *Arcobacter* spp. to anaerobically oxidize sulfide with nitrate (Lavik *et al.*, 2009).

It has been hypothesized before that *Arcobacter*, based on their small cell size and high motility, might act as pioneer colonizers when habitat conditions are dynamic (Bernard and Fenchel, 1995; Sievert *et al.*, 2007). Later, *Arcobacter* might be replaced by other sulfide oxidizers as these establish in the respective habitats and compete for sulfide more efficiently (Bernard and Fenchel, 1995). In addition, *Arcobacter* mats were also found in the deep Eastern Mediterranean associated with fluidic habitats at brine flows (Omoregie *et al.*, 2008) and floating above large brine pools (Huguen *et al.*, 2009), indicating that they are not restricted to benthic habitats.

4.2 Selective advantage for filamentous sulfide oxidizers in non-overlapping oxygen-sulfide gradients

Microscopic and phylogenetic analyses of AMV filamentous sulfide oxidizer mats indicated the presence of *Beggiatoa* spp. and VAF (Figure 3a-c and 4). *Beggiatoa* are known to thrive in microoxic (Jørgensen and Revsbech, 1983) or suboxic niches (Mußmann *et al.*, 2003), where they oxidize sulfide or internally stored elemental sulfur by using either oxygen or nitrate (Teske and Nelson, 2006). Intracellular storage of nitrate in vacuolated species enables the gliding filamentous organisms to reach deeper sulfide reservoirs (McHatton *et al.*, 1996; Teske and Nelson, 2006). In the Eastern Mediterranean deep sea, *Beggiatoa* spp.-resembling filaments were found to be associated with a cold seep habitat characterized by high sulfide and oxygen fluxes (Table 3; Felden, 2009). *In situ* microsensor profiles showed a spatial gap of 1 to 2 mm between oxygen and sulfide (Figure 8a). Under these conditions, only motile and nitrate-accumulating sulfide oxidizers such as the gliding filamentous bacteria *Beggiatoa* can access both the electron

acceptor and the electron donor, and non-motile *Thiomargarita* and non-vacuolated *Arcobacter* are outcompeted. The ratio of 1:1 between sulfide flux and sulfide oxidation (Table 3) indicated that not enough oxygen was available to allow for the complete aerobic conversion of sulfide to sulfate ($HS^- + 2O_2 \rightarrow SO_4^{2-} + H^+$) and that most likely nitrate was used as electron acceptor. In addition to *Beggiatoa* filaments, a potentially novel type of VAF was found to contribute to the AMV filamentous sulfide oxidizer mats (based on 16S rRNA gene analyses). The ecological preferences of VAF have not been described in detail so far, but VAF were found to not accumulate nitrate above ambient seawater concentration within their vacuoles, and they are suspected to withstand only brief temporal separation of sulfide and oxygen (Kalanetra *et al.,* 2004). At the AMV, VAF would have been associated with a habitat where no sulfide emanated into the bottom water (Figure 8a), raising the question how these organisms could gain access to their energy source sulfide under the measured conditions. A combination of both aerobic and anaerobic chemotrophy occurring within the same microbial mat has previously been postulated for a white, filamentous microbial mat found at the Mediterranean deep-sea mud volcano Milano (Heijs *et al.,* 2005). There, filamentous bacteria phylogenetically closely related to VAF and AMV346 (Figure 4) had been observed as part of a potentially methane- and sulfide-driven bacterial community.

4.3 *Thiomargarita* at the Amon mud volcano thrive on sulfidic fluid flow

As previously described by Girnth *et al.* (in review), a dense population of *Thiomargarita* occurred on top of a sulfidic mud outflow at the southwestern flank of the AMV. Single, spherical cells were observed on the sediment surface and further microscopic analyses revealed that all cells were of a vacuolated phenotype (Girnth *et al.*, in review). Sulfidic brine periodically covering the *Thiomargarita* population could supply the cells with energy-rich sulfide and concurrently separate them from the oxygenated bottom water. If anoxic conditions were resulting, *Thiomargarita* could rely on internally stored nitrate for sulfide oxidation (Schulz, 2006). Once a brine flow has passed, residual sulfide in the upper sediment layer beneath the mat as well as stored elemental sulfur could be oxidized aerobically and internal nitrate reservoirs could be replenished by *Thiomargarita* (Girnth *et al.*, in review). *In situ* recorded microprofiles of oxygen and sulfide (Figure 8b)

as well as microscopic imaging of a central vacuole within the AMV *Thiomargarita* cells supported this assumption (Girnth *et al.*, in review).

In conclusion, *Thiomargarita* spp. can dominate dynamic habitats where their electron donor and acceptor only temporarily overlap and the position of their interface may drastically change (Girnth *et al.*, in review; Schulz, 2006). In such habitats, gliding filamentous sulfur oxidizers, such as *Beggiatoa* spp., that rely on defined chemical gradients (Preisler *et al.*, 2007) can be outcompeted (Girnth *et al.*, in review). In addition, *Thiomargarita* spp. have been found to endure peaks of oxygen (Schulz *et al.*, 1999; Schulz, 2006) and exposure to high sulfide concentrations (200-19 000 μM in the upper 3 cm bsf, Kalanetra *et al.*, 2005; 100-800 μM in the upper 3 cm bsf, Schulz *et al.*, 1999). In contrast, *Beggiatoa* spp. avoid such high sulfide concentrations (Preisler *et al.*, 2007) and are also known to feature a phobic response to elevated oxygen concentrations (Møller *et al.*, 1985). Mat-forming *Arcobacter* spp. can also proliferate in dynamic environments and have been found to tolerate high sulfide concentrations (1-2 mM, Sievert *et al.*, 2007), and to grow at low oxygen concentrations (1-10 μM, Sievert *et al.*, 2007). However, due to the lack of an internal vacuole in which they could store an additional electron acceptor, *Arcobacter* cells may not be able to survive or form mats if long periods of anoxia occur.

4.4 Conclusion and Outlook

During the BIONIL cruise (2006), three different sulfide oxidizer mats were investigated and compared with each other regarding niche differentiation between the different mat-forming bacteria. Analyses of geochemical data including *in situ* microprofiles of oxygen and sulfide, as well as special properties of each of the organisms (e.g. cell size, motility, internal nitrate accumulation) helped to explain the dominance of either sulfide oxidizer in the respective NDSF mats. Filamentous sulfide oxidizers resembling *Beggiatoa* spp. and VAF were associated with non-overlapping oxygen-sulfide gradients, *Thiomargarita* spp. populated a habitat with temporarily changing supplies of oxygen and sulfide, and *Arcobacter* mats occurred when oxygen and sulfide overlapped in the bottom water. In addition, *Arcobacter* spp. were found to occur in the *Beggiatoa*/VAF and *Thiomargarita* mats, suggesting that species succession could play an important role in these habitats.

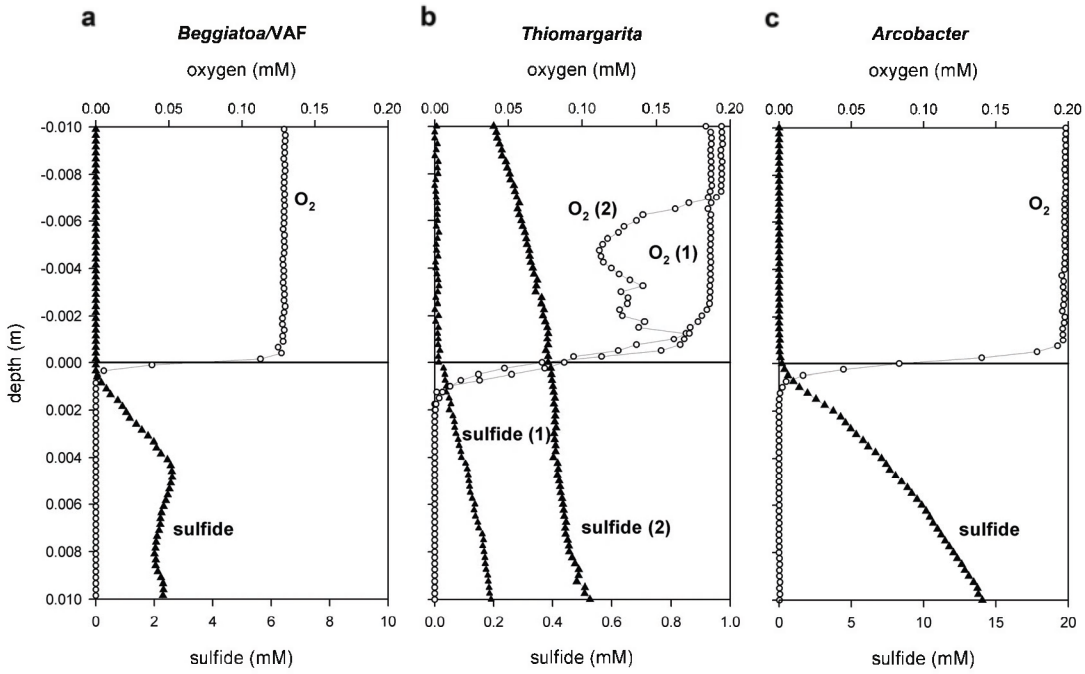

Figure 8: High resolution *in situ* microprofiles of oxygen (O_2) and sulfide recorded (a) at the Amon *Beggiatoa*/VAF mats (modified from Felden, 2009), (b) at two positions on the Amon *Thiomargarita* mat (modified from Girnth *et al.*, in review) and (c) on the *Arcobacter* mat (close up on Figure 6a; this study).

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Supplementary Information

Supplementary Table 1: Measurements and analyses conducted during this study.

Sample site	Measurement/Analysis[a]	Station/Dive[b]	Position[c]	Depth[c]	PANGAEA event label[c]
Beggiatoa / VAF mats[d]	Elemental sulfur (S^0)	760/113	32.368833° N 31.711048° E	1122 m	M70/2a_760_PUC-39 and -41
(Amon mud volcano)	CARD-FISH	760/113	32.368832° N 31.711038° E	1122 m	M70/2a_760_PUC-40
	16S rRNA clone libraries	760/113	32.368832° N 31.711038° E	1122 m	M70/2a_760_PUC-40, -44, and -55
Thiomargarita mat[e]	Elemental sulfur (S^0)	765/115	32.367685° N 31.704393° E	1158 m	M70/2a_765_PUC-46 and -47
(Amon mud volcano)	CARD-FISH	765/115	32.367695° N 31.704423° E	1158 m	M70/2a_765_PUC-49
Arcobacter mat	Microprofiler (O_2, pH, sulfide, T, DOU, sulfide flux)	841/127	32.533403° N 30.352263° E	1697 m	M70/2b_841_PROF-1 ('mat')
(Pockmarks)		841/127	32.53346° N 30.351922° E	1697 m	M70/2b_841_PROF-3 ('mat border')
	Benthic chamber (TOU)	833/126	32.533363° N 30.352310° E	1694 m	M70/2b_833_CALMAR-1
	SR	770/116	32.533282° N 30.352092° E	1697 m	M70/2a_770_PUC-12
		773/117	32.533167° N 30.352163° E	1698 m	M70/2a_773_PUC-14 and -68
		841/127	32.533470° N 30.352047° E	1697 m	M70/2b_841_PUC-12, -41 and -68
	Porewater analyses (SO_4^{2-}, Cl, total sulfide, DIC)	773/117	32.533158° N 30.352152° E	1698 m	M70/2a_773_PUC-52
		784/120	32.533213° N 30.352007° E	1697 m	M70/2a_784_PUC-9
	Elemental sulfur (S^0)	770/116	32.533255° N 30.352113° E	1697 m	M70/2a_770_PUC-51
		773/117	32.533158° N 30.352152° E	1698 m	M70/2a_773_PUC-52
		784/120	32.533213° N 30.352007° E	1697 m	M70/2a_784_PUC-9
	CARD-FISH	773/117	32.533152° N 30.352172° E	1698 m	M70/2a_773_PUC-38
	16S rRNA clone libraries	773/117	32.533152° N 30.352172° E	1698 m	M70/2a_773_PUC-38
		784/120	32.533188° N 30.352052° E	1697 m	M70/2a_784_PUC-44
Reference Amon mud volcano	Elemental sulfur (S^0)	765/115	32.368915° N 31.706165° E	1140 m	M70/2a_765_PUC-14 and -52
Reference Pockmarks	Microprofiler (O_2, pH, sulfide, T, DOU, sulfide flux)	841/127	32.533518° N 30.351830° E	1697 m	M70/2b_841_PROF-2 ('reference')
	Benthic chamber (TOU)	841/127	32.533488° N 30.351755 ° E	1696 m	M70/2b_841_CALMAR-4
	SR, Porewater analyses, Elemental sulfur (S^0)	773/117	32.534872° N 30.351975° E	1702 m	M70/2a_773_PUC-15, -36, -39 and -48

[a]Abbreviations are as follows: CARD-FISH (catalyzed reporter deposition fluorescence *in situ* hybridization), 16S rRNA (16S ribosomal RNA), T (temperature), DOU (diffusive oxygen uptake), TOU (total benthic oxygen uptake), SR (sulfate reduction), DIC (dissolved inorganic carbon), PUC (push core).

[b]Station and dive numbers refer to events during the BIONIL expedition, leg M70/2a and b. Dive 113 (24.-25.10.2006), Dive 115 (26.-27.10.2006), Dive 116 (28.-29.10.2006), Dive 117 (29.-30.10.2006), Dive 120 (03.-04.11.2006), Dive 126 (17.11.-18.11.2006), Dive 127 (19.-20.11.2006).

[c]Position and depth of the according measurements or sampling events were acquired from the PANGAEA database (www.pangaea.de). Position and depth are given for the first event only if push cores were recovered adjacent to each other.

[d]Detailed biogeochemical analyses and *in situ* measurements were previously discussed by Felden (2009).

[e]A detailed description of the habitat where dense populations of *Thiomargarita* were found has been given by Girnth *et al.* (in review) and Felden (2009).

Chapter 5

Diversity of Mat-forming Sulfide Oxidizers in Deep-sea Thiotrophic Bacterial Mats associated with Cold Seeps of the Norwegian Continental Margin

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Abstract

Mats of sulfide-oxidizing bacteria are a typical feature of active cold seep ecosystems at continental margins and are usually associated with relatively high rates of fluid flow, gas emission and oxygen consumption. Thiotrophic mats at cold seeps are often dominated by one type of sulfide oxidizer, but recent investigations showed a high diversity of Gamma- and Epsilonproteobacteria capable of mat formation by efficient exploitation of sulfide and oxygen fluxes. Here, we investigated different cold seep habitats of the deep continental margin off Norway, which is characterized by bottom water temperatures close to the freezing point. The seeps investigated include subsurface gas chimneys, complex pockmarks, and the deep-sea mud volcano Håkon Mosby (HMMV). Macroscopically different types of mats were observed associated with hydrocarbon seepage, ranging from small grayish mat patches of 20 cm to 5 m in diameter to huge white mats covering up to 850 m^2 of the seafloor. Microscopic and 16S rRNA gene based phylogenetic analyses revealed a high diversity of sulfur-storing and non-sulfur storing thiotrophs between and within gray mats. In contrast, the largest mats found at the HMMV were composed only of two types of *Beggiatoa* filaments. Based on these observations and geochemical data, we hypothesize that large continuous mats develop in stable biogeochemical conditions selecting for only 1 to 2 types of cells, whereas small patchy mats indicate rather dynamic habitats hosting a variety of competing thiotrophic bacteria.

1. Introduction

At active cold seep systems such as mud volcanoes, pockmarks and gas chimneys, hydrocarbon-rich muds, fluids, brines or gases are transported from the subsurface seabed to the seafloor and into the hydrosphere (Cathles *et al.*, 2010; Dimitrov, 2002; Hovland *et al.*, 2005; Judd *et al.*, 2002; Milkov, 2000). Cold seeps are often referred to as benthic hot-spot ecosystems and can support enormous microbial biomasses (Jørgensen and Boetius, 2007). This is due to the high energy availability in the form of reduced chemical substances such as methane, sulfide, iron and other compounds which are utilized for chemosynthetic production. Among the variety of chemosynthetic microbial processes, anaerobic oxidation of methane (AOM) coupled to sulfate reduction (SR) in subsurface sediments is a key process at cold seeps. This process controls the release of the potential greenhouse gas methane to the water column and produces bicarbonate and energy-rich sulfide (Boetius *et al.*, 2000). AOM-derived bicarbonate can precipitate as authigenic carbonate, thereby forming a hard substratum for the development of diverse benthic communities (Boetius *et al.,* 2000; Foucher *et al.,* 2009). Sulfide serves as electron donor for free-living sulfide oxidizers and symbiotic bacteria of bivalves and tubeworms (Boetius *et al.,* 2000; Dubilier *et al.,* 2008; Niemann *et al.,* 2006). These organisms form typical dense assemblages of high biomass at active cold seeps, with distinct patterns which are usually related to fluid flow and gas venting (Niemann *et al.,* 2006; Sahling *et al.,* 2002).

Most conspicuous are the mat-forming free-living sulfide oxidizers at cold seeps, which produce whitish, grayish or orange patches covering the seafloor (de Beer *et al.*, 2006; Larkin and Henk, 1996; Niemann, 2006; Omoregie *et al.*, 2008). The wet biomasses can be as high as 500 g m^{-2} (estimated for the giant *Beggiatoa*; Boetius and Suess, 2004). Previous observations found the cold seep mats to be composed of bacteria like the giant filamentous *Beggiatoa* (Ahmad *et al.*, 1999; Mills *et al.*, 2004; Teske and Nelson, 2006), the giant spherical *Thiomargarita* (Girnth *et al.,* in review; Kalanetra *et al.,* 2005), *Thiobacterium* spp. forming gelatinous mats (Grünke *et al.*, 2010) or the small vibrio-shaped *Arcobacter* (Grünke *et al.*, in preparation; Omoregie *et al.,* 2008; Sievert *et al.,* 1999). These diverse bacteria have in common that they thrive at oxic-anoxic interfaces and oxidize sulfide to elemental sulfur or sulfate by using either oxygen or nitrate. Previous

investigations revealed several morphological and physiological traits that allow these organisms to adapt to different regimes of their electron donor (sulfide) and acceptor (oxygen or nitrate). For example, *Beggiatoa* spp. can exploit defined chemical gradients based on chemotactic responses to elevated sulfide and oxygen concentrations (Møller *et al.,* 1985; Preisler *et al.,* 2007). Internal storage of elemental sulfur as well as nitrate accumulation in vacuolated species enables some *Beggiatoa* filaments to bridge non-overlapping gradients of their electron donor and acceptor by means of their gliding motility (McHatton *et al.*, 1996; Mußmann *et al.*, 2003; Teske and Nelson, 2006). Sulfur- and nitrate-storing *Thiomargarita* spp. seem to be especially successful in dynamic habitats with changing chemical gradients (Girnth *et al.*, in review), and are thought to rely on external transport events like periodic sediment resuspension or episodic brine overflow to contact their electron donor or acceptors (Girnth *et al.*, in review; Kalanetra *et al.*, 2005; Schulz *et al.*, 1999; Schulz, 2006). Marine, mat-forming *Arcobacter* spp. form external cotton-ball-like sulfur precipitates that are believed to retain these highly motile organisms at their preferred oxygen-sulfide interface (Moussard *et al.,* 2006; Sievert *et al.,* 2007; Taylor *et al.,* 1999). Further, internal nitrate accumulation by these organisms has not been investigated, why they are thought to rely on constantly overlapping gradients of sulfide and oxygen (Grünke *et al.*, in preparation). Finally, complementing these adaptations and special requirements, temporal dynamics of fluid flow and oxygen availability at cold seeps may induce competition and succession of mat-forming sulfide oxidizers, which could be another important factor influencing niche differentiation between different mat-forming thiotrophic bacteria (Bernard and Fenchel, 1995). The ecological importance of thiotrophic microbial mats at cold seeps lies in their function as benthic filters (e.g. removal of toxic sulfide) and the production of biomass (Lichtschlag *et al.*, 2010). Furthermore, these microorganisms cause substantial fluxes of carbon, sulfur, nitrogen and phosphate at the seafloor, making them important biogeochemical drivers in cold seep ecosystems (Lichtschlag *et al.*, 2010; Sayama *et al.*, 2005; Schulz and Schulz, 2005; Teske and Nelson, 2006).

This study aimed at revealing the diversity of mat-forming thiotrophs of cold seeps at the deep Nordic margin, and investigated if prevailing temperatures close to the freezing point (-1.5 to 0°C) select for different seep thiotrophs as previously identified in temperate or warm ocean realms. At the Norwegian continental margin several active

deep-sea seeps hosting chemosynthetic communities are known, including the Håkon Mosby mud volcano (HMMV) on the southwest Barents Sea slope (de Beer *et al.*, 2006; Hovland *et al.*, 2005; Lichtschlag *et al.*, 2010; Niemann *et al.*, 2006), the pockmark fields in the Nyegga area (Hovland *et al.*, 2005), and the gas chimneys in the northeastern area of the Storegga Slide (Vicking cruise report, 2006). At these systems, macroscopically different thiotrophic mats have been observed, but the identity and diversity of the dominating sulfide-oxidizing bacteria remains unknown. Other questions targeted by this study are as to the link between macroscopic and microscopic differences in the mat shape and composition, and as to the biogeochemical gradients associated with these variations. This was investigated by combining microscopy of cells with the construction of 16S rRNA gene based clone libraries, and with *in situ* and *ex situ* measurements of gradients in oxygen and sulfide as well as other pore-water components.

2. Materials and Methods

2.1 Sampling sites

Samples for this study were recovered in 2006 during the Vicking cruise aboard R/V *Pourquoi Pas?* (ROV *Victor 6000*; Ifremer, France) to the Storegga Slide, the Nyegga region and the HMMV (Figure 1A). In 2007 and 2009, additional samples were recovered at the HMMV during the ARK-XXII/1b expedition aboard R/V *Polarstern* (ROV *Quest 4000*; Marum, University Bremen, Germany), and during the ARK-XXIV/2b expedition on R/V *Polarstern* (ROV *Quest 4000*; Marum). A general overview of all target sites is given in Table 1. Detailed information on sample sites and measurements are available in Supplementary Table 1.

The Storegga Slide is located south of the Vøring Plateau off Norway and is one of the largest submarine slides (Bryn *et al.*, 2005). Clay diapirs and fluid escape structures, e.g. pockmarks, are known from the northern edge of the slide (Bouriak *et al.*, 2000). During the Vicking cruise an area associated with subsurface gas chimneys (CNE5.7 and CNE03; Foucher *et al.*, 2009) was investigated (Figure 1B).

The Nyegga area is located at the northeastern flank of the Storegga Slide, and previous studies revealed the presence of complex pockmarks associated with authigenic

carbonates and submarine hydrate pingoes (Hovland *et al.*, 2005; Hovland and Svensen, 2006). In addition, seep-associated organisms like tubeworms and bacterial mats had been observed (Hovland and Svensen, 2006). During the Vicking cruise two complex pockmarks, G11 and G12, were investigated (Figure 1C).

The Håkon Mosby mud volcano (Figure 1D) is located ~ 900 km north of the Storegga and Nyegga regions on the southwest Barents Sea slope (Figure 1A). It appears nearly concentric and has been subdivided into three main habitats (Niemann *et al.*, 2006): (i) the active flat center of the mud volcano that appears devoid of chemosynthetic communities, (ii) a narrow zone surrounding the center covered by white thiotrophic bacterial mats, and (iii) dense populations of siboglinid tubeworms in the outer hummocky rim of the mud volcano above thick layers of gas hydrate. The transition zone between the bacterial mats and the pogonophora fields is characterized by small patchy grayish bacterial mats (de Beer *et al.*, 2006).

Table 1: Major target sites visited within this study included gas chimneys CNE5.7 and CNE03 at the Storegga Slide, pockmarks G11 and G12 in the Nyegga area, and various sites at the Håkon Mosby mud volcano (HMMV).

Mat type	Cruise	Date	Site[a]	Target site Latitude	Target site Longitude	Depth
Storegga mats	Vicking	31.05.-01.06.2006	VKGD275	64°45'15"N (CNE5.7) 64°45'15"N (CNE03)	4°58'52"E (CNE5.7) 5°04'09"E (CNE03)	~ 720 m (CNE03)
Nyegga mats	Vicking	24.05.-27.05.2006	VKGD272	64°40'00"N (G11) 64°39'47"N (G12)	05°17'30"E (G11) 05°17'18"E (G12)	~ 740 m
HMMV white mats	Vicking	03.06.-05.06.2006	VKGD276			
	ARK-XXII/1b	02.02.2007 05.07.2007	PS70/112-1 PS70/132-1	72 °00'15"N (HMMV)	14°43'30"E (HMMV)	~ 1250 m
HMMV gray mats	Vicking	05.06.-07.06.2006	VKGD277			
	ARK-XXIV/2b	25.07.2009	PS74/176-1			

[a]PANGAEA-labeling (www.pangaea.de).

2.2 Sampling

Bacterial mat samples and the upper 20 to 30 cm of sediment underlying the mats were recovered with push cores (Supplementary Table 1). The highly gassy sediment samples of the HMMV were incubated close to *in situ* temperature (0 to 1°C) for 1 to 2 days

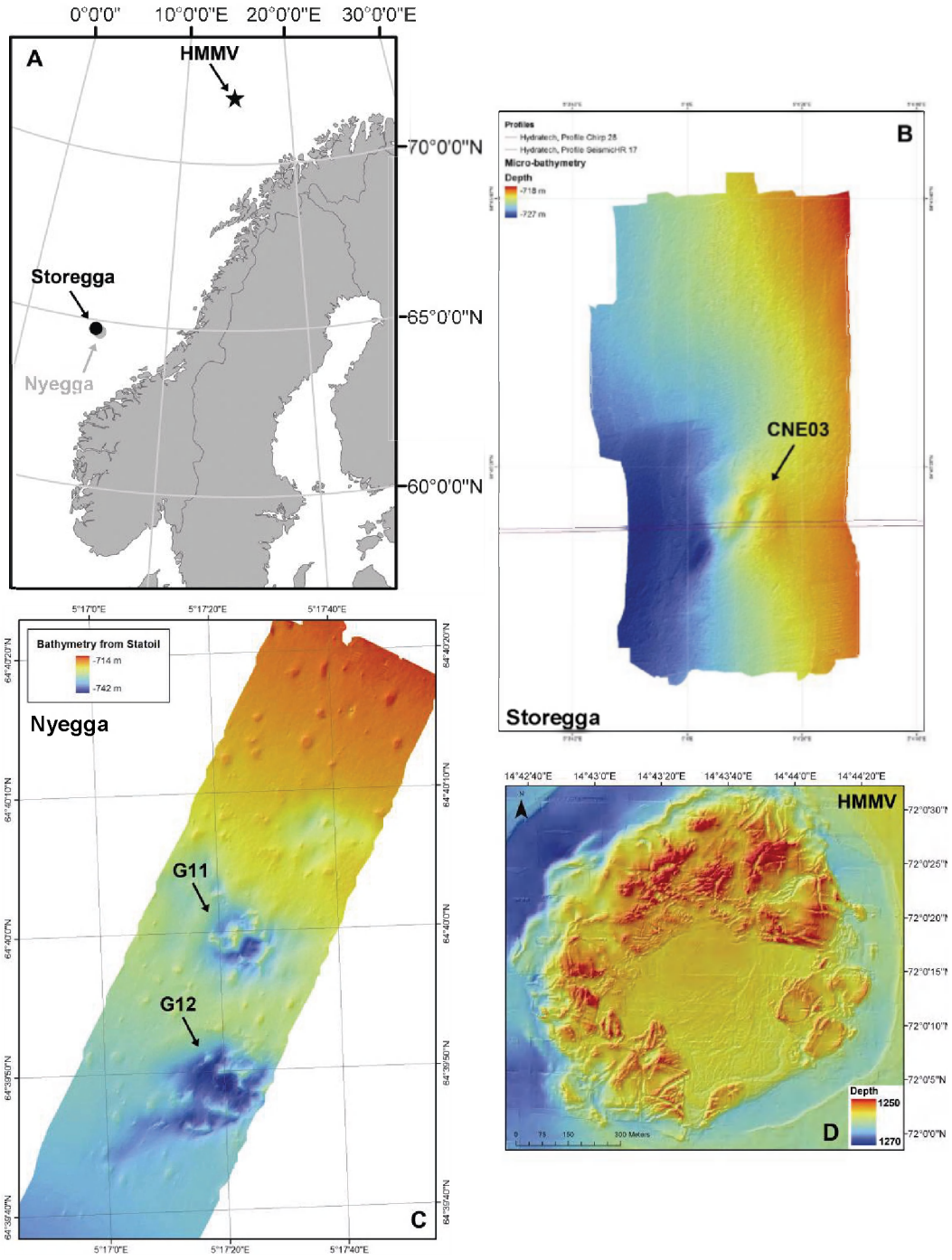

Figure 1: Target sites at the Norwegian Margin (A) included gas seeps at the Storegga Slide, complex pockmarks in the Nyegga area and the deep-sea mud volcano Håkon Mosby (HMMV). The map was generated in ArcMap (ArcGIS Desktop 9.3) with continental margin data provided by ESRI (Kranzberg, Germany). (B) Microbathymetry showing the seafloor above the subsurface gas chimney CNE03 (Catherine Satra and Stéphanie Dupré, unpublished data of Vicking cruise 2006, chief scientist Herve Nouzé, Ifremer). (C) Microbathymetry of two complex pockmarks G11 and G12 (Hovland *et al.*, in press). (D) Microbathymetric map of the HMMV recorded in 2003 (Edy *et al.*, 2004).

before subsampling, to allow bacterial mats and geochemical gradients to reestablish. The subsampling of the mats as well as pore-water extraction and dissection of the sediment for further analyses were also performed in the cold room.

Bacterial mats were analyzed by bright field and phase contrast microscopy aboard the ship and documented by digital photography. A calibrated eyepiece micrometer or a standardized object micrometer were used for estimating cell dimensions.

2.3 Microsensor measurements

High resolution geochemical gradients were measured *in situ* with a ROV-operated microprofiler unit (Treude *et al.*, 2009) in a blackish spot in the Nyegga G12 pockmark area and on a reference site between the Nyegga pockmarks G11 and G12. Microsensors for sulfide, pH, oxygen (de Beer *et al.*, 1997; Jeroschewski *et al.*, 1996; Revsbech and Ward, 1983) and temperature (Pt100; UST Umweltsensortechnik GmbH, Geschwenda, Germany) were mounted on the profiler. In addition, *ex situ* microprofiles were determined in retrieved push cores from the Nyegga and Storegga areas with a laboratory set up. Here, microsensors for sulfide, oxygen and pH were mounted on a motor-driven micromanipulator and data acquisition was performed with a DAQ-PAD 6015 (National Instruments Corporation, Austin, TX, USA) and a computer. The surface of the sediment was determined with the help of a dissection microscope relative to the microsensor tips. During the analyses, the cores were kept in an aquarium with water cooled to approximately 1°C to simulate *in situ* temperature conditions. A gentle jet stream stirred the overlying water and assured the development of a distinct diffusive boundary layer. Microsensor calibration, measurements, profile analyses and calculation of fluxes (*in situ* and *ex situ*) were performed as previously described by de Beer *et al.* (2006) and Lichtschlag *et al.* (2010).

2.4 Geochemical analyses

Rates for sulfate reduction (SR) of the Storegga and Nyegga mats were determined *ex situ* by whole core injection (Jørgensen, 1978). Onboard, incubation with 5 to 10 µL carrier-free $^{35}SO_4^{2-}$ (dissolved in water, 50 kBq) was carried out in the dark and at *in situ* temperature for approximately 12 to 24 h. Following incubation, sediment samples were

preserved in 20 mL of 20% (w/v) ZnAc solution. Rates for SR were measured and calculated as previously described (Kallmeyer *et al.*, 2004; Treude *et al.*, 2003). More details can be found in Felden *et al.* (in review).

Pore-water extraction as well as geochemical pore-water analyses (chloride, sulfate, total dissolved sulfide ($H_2S+HS^-+S^{2-}$) and dissolved inorganic carbon (DIC)) for Nyegga mat sediments and one reference site were conducted as previously described by Lichtschlag *et al.* (2010). Elemental sulfur concentrations were measured by high-performance liquid chromatography (Zopfi *et al.*, 2004) from sediment sections extracted for 12 h in a ZnAc/methanol solution.

Nitrate loss from the overlying water with time was measured in two different cores recovered from Nyegga mats (VKGD272/PC-11 and VKGD272/PC-32) as well as from one reference site (VKGMTB2). Background nitrate concentrations were determined in the overlying water as 11 μM (VKGD272/PC-11), 7 μM (VKGD272/PC-32) and 10 μM (VKGMTB2). To these cores, defined concentrations of nitrate were added to the overlying water, namely 37 μM (VKGD272/PC-11), 33 μM (VKGD272/PC-32) and 22 μM (VKGMTB2). To assure mixing, the overlying water was gently stirred by air flow. Water samples were taken at different time intervals, acidified and stored at 4°C before measuring with a NOx analyzer (Thermo Environmental Instruments, Franklin, MA, USA).

2.6 Cell counts (AODC)

Sediment sections intended for direct cell counts were fixed onboard in 4% formaldehyde/seawater at 4°C. Total sediment-embedded cells were counted after staining with acridine orange according to a modified protocol (Boetius and Lochte, 1996) of Meyer-Reil (Meyer-Reil, 1983). For each sample, at least 2 replicate filters and a minimum of 30 grids per filter were randomly counted.

2.7 Fluorescence *in situ* hybridization (FISH) experiments

Sediment samples recovered during the Vicking cruise were fixed in 4% formaldehyde/seawater, washed two times with 1 × phosphate-buffered saline (PBS; pH 7.2 to 7.4), and were finally stored at -20°C in a 1:1 mixture of PBS and ethanol until further processing. Catalyzed reporter deposition FISH (CARD-FISH) was then carried

out with suitable dilutions of the fixed samples as previously described (Ishii *et al.*, 2004; Pernthaler *et al.*, 2002). Horseradish peroxidase (HRP)-labeled probe sequences (Biomers, Ulm, Germany) and probe hybridization details are given in Supplementary Table 2. All cells were counterstained with the DNA-targeting fluorescent stain 4',6-diamidino-2-phenylindole (DAPI). For each sample, a minimum of 30 grids were counted randomly distributed across the filter.

2.8 Clone library construction and sequencing

Subsamples of all mats recovered during the Vicking cruise were preserved in either PCR-grade water (Sigma-Aldrich Biochemie GmbH, Hamburg, Germany) or 1 × TE buffer (Promega Corporation, Madison, WI) and stored at -20°C. Mat samples taken from the first three positions of a transect along a white mat at the HMMV (ARK-XXII/1b) were directly subjected to replicate polymerase chain reactions (PCR) onboard; only samples from the fourth position were preserved as during the Vicking cruise. Universal bacterial primers GM3F (5'-AGAGTTTGATCMTGGC-3'; Muyzer *et al.*, 1995) and GM4R (5'-TACCTTGTTACGACTT-3'; Muyzer *et al.*, 1995) were used for amplification of nearly full length 16S rRNA gene sequences in all cases. Replicate amplification reactions for the preserved mat samples were set up as follows: 0.5 μM of each primer (Biomers), 250 μM of each dNTP (Roche, Mannheim, Germany), 0.3 mg mL^{-1} BSA (Sigma-Aldrich Biochemie GmbH), 1 × MasterTaq Buffer with 1.5 mM Mg^{2+} and 0.025 U μL^{-1} MasterTaq (5Prime, Hamburg, Germany) in a total volume of 25 μL. Replicate PCR reactions onboard were set up with 25 μL of PCR Master Mix (Promega Corporation) and 0.5 μM of each primer (Biomers) in a final volume of 50 μL. PCR conditions for all samples included an initial denaturation step of 15 min at 95°C, 30 cycles of 95°C/1 min, 42°C/1 min and 72°C/3 min, prior to a final elongation at 72°C for 10 min. Onboard conducted PCR reactions were subsequently stored at -20°C until further processing in the home laboratories. Replicate PCR products were pooled and purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), ligated into pGEM-T Easy (Promega Corporation) and transformed into One Shot TOP10 Chemically Competent *E. coli* (Invitrogen Corporation, Karlsruhe, Germany) following the given specifications.

2.9 Sequence analyses

After partial sequencing with vector primers, clone libraries were screened for putative chimeras with the Mallard program at a cut-off of 99.9% (Ashelford *et al.*, 2006). Anomalous sequences were excluded from further analyses.

Representative clones associated with clusters containing giant filamentous bacteria of the genus *Beggiatoa* were selected for plasmid preparation after partial sequencing. Purified plasmids were subjected to *Taq* cycle sequencing with an ABI Prism 3130x Genetic Analyzer (Applied Biosystems, Foster City, CA). Nearly full length 16S rRNA gene sequences were assembled with the Sequencher 4.6 software (Gene Codes Corporation, Ann Arbor, MI) and manually checked for ambiguities. The sequences were examined for chimeric signals by using the Pintail program (Ashelford *et al.*, 2005) and trusted nearest neighbors obtained with the SILVA-based SINA Webaligner (Pruesse *et al.*, 2007). Sequences with genuine chimeric signals were excluded from further analyses. Finally, representative 16S rRNA gene sequences were used for further phylogenetic analyses.

2.10 Phylogenetic analyses

To obtain insight into the 16S rRNA gene based bacterial diversity, partial sequences were analyzed with the RDP Classifier (Wang *et al.*, 2007) and the ARB software package (Ludwig *et al.*, 2004). As the focus of this study was on characterizing giant mat-forming sulfide oxidizers, phylogenetic trees were reconstructed with almost full length 16S rRNA gene sequences closely associated to this group by applying neighbour joining, maximum likelihood (RAxML) and maximum parsimony methods as included in the ARB software package (Ludwig *et al.*, 2004). Initial calculations were conducted with nearly full length sequences (*E. coli* positions 127 to 1307) and by applying different conservatory filters. Short sequences were subsequently added to the inferred trees without allowing changes in the overall tree topology. Deltaproteobacterial sequences were used as outgroup. A consensus tree based on the different reconstruction approaches was built wherein unstable branching orders were visualized by multifurcation.

2.11 Nucleotide sequence accession numbers

Sequence data will be submitted to the EMBL database.

3. Results

3.1 Storegga mats

The seafloor above the gas chimneys in the northeastern area of the Storegga Slide resembled a rather flat pockmark (Figure 1B) of approximately 180 m diameter. The gas chimney was associated with carbonate crusts hosting filter-feeding communities, and with patches of reduced sediments. Above some of the blackish sediment patches we identified 20 to 50 cm large gray mats, surrounded by tubeworms and other megafauna (Figure 2A-C). The microscopic appearance of the mats was highly variable. In one push core we discovered spherical gelatinous mats (Figure 2D) that are known to be formed by *Thiobacterium* spp., small rod-shaped bacteria that store several grains of elemental sulfur internally (Figure 2E). A detailed study on the gelatinous mats and associated bacterial community has previously been published by Grünke *et al.* (2010). Further, Grünke *et al.* (2010) had identified *Beggiatoa* spp.-type filaments with diameters of 3 to 4 μm (Figure 2F) as well as several filamentous organisms of unknown affiliation. In this study, we identified one 16S rRNA gene sequence that was closely associated with a cluster harboring most marine *Beggiatoa* spp. as well as all known vacuolated attached filaments (VAF) and marine *Thioploca* and *Thiomargarita* spp. (Storegga mat clone NM-S116, Figure 3). A second 16S rRNA gene sequence (Storegga mat clone NM-S134) near the group of giant mat-forming sulfide oxidizers was found to be most closely related to uncultured bacteria from a sulfidic cave mat (EF467566; 86.9% similarity) and iron-containing flocculent mat (FJ535296; 87.1% similarity; Figure 3).

Upon retrieval of another core from a different mat patch (Figure 2C) we observed sulfur-storing, rather stiff filaments that were attached to the sediment and tubeworms (Figure 2G), and that were composed of elongated cylindrical cells with a diameter of approximately 35 μm (Figure 2H, I). These filaments showed semicircular tips and were

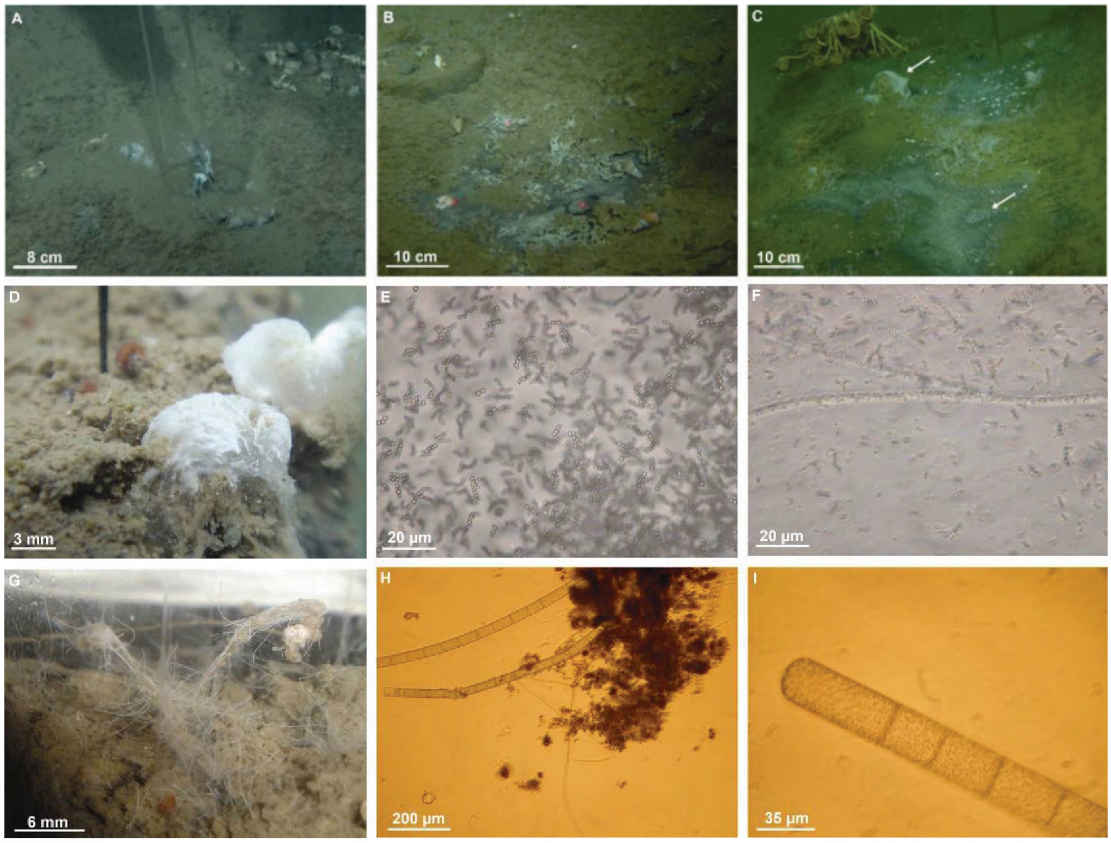

Figure 2: Storegga mats. (A) Small bacterial mat patch harboring gelatinous mats formed by *Thiobacterium* spp. (as shown in panel D). The mat patch was surrounded by dense communities of tubeworms and mollusc shells. Image Source: Ifremer, Vicking 2006. (B) Another example for a gray mat from the Storegga area, which appears to be integrated into a dense community of tubeworms. Image Source: Ifremer, Vicking 2006. (C) This bacterial mat was found in a small depression surrounded by tubeworms and other megafauna. The center of the mat was surrounded by white cotton-ball-like precipitates (right arrow) resembling products of marine *Arcobacter* spp. (Sievert *et al.*, 2007). In addition, patches of supposedly filamentous sulfur bacteria were also present in this area (left arrow). Image Source: Ifremer, Vicking 2006. (D-F) Spherical, gelatinous mats of approximately 1.5 cm diameter (panel D). These mats were formed by *Thiobacterium* spp. (panel E) and have previously been studied by Grünke *et al.* (2010). Other mats were composed of filamentous, sulfur-storing bacteria that resembled *Beggiatoa* spp. (panel F). (G-I) White filaments attached to sediment and tubeworms (panel G). Microscopy revealed the presence of sulfur-storing filaments characterized by elongated cylindrical cells and almost semicircular tips (panel H, I), potentially resembling so-called 'vacuolated attached filaments' (VAF).

morphologically reminiscent of potential VAF observed in the Eastern Mediterranean Sea (Grünke *et al.*, in preparation). Accordingly, we identified a representative of a group of Storegga mat clones (NM-E1935) to be associated with the group of known VAF (98.6 to 99.1% similarity; Figure 3). In addition, we also observed smaller (<5 µm in

diameter), but yet unidentified filamentous bacteria to occur next to the VAF-resembling filaments (Figure 2H). Clone library screening with partial 16S rRNA gene sequences revealed the presence of few putative *Leucothrix* spp.-associated sequences.

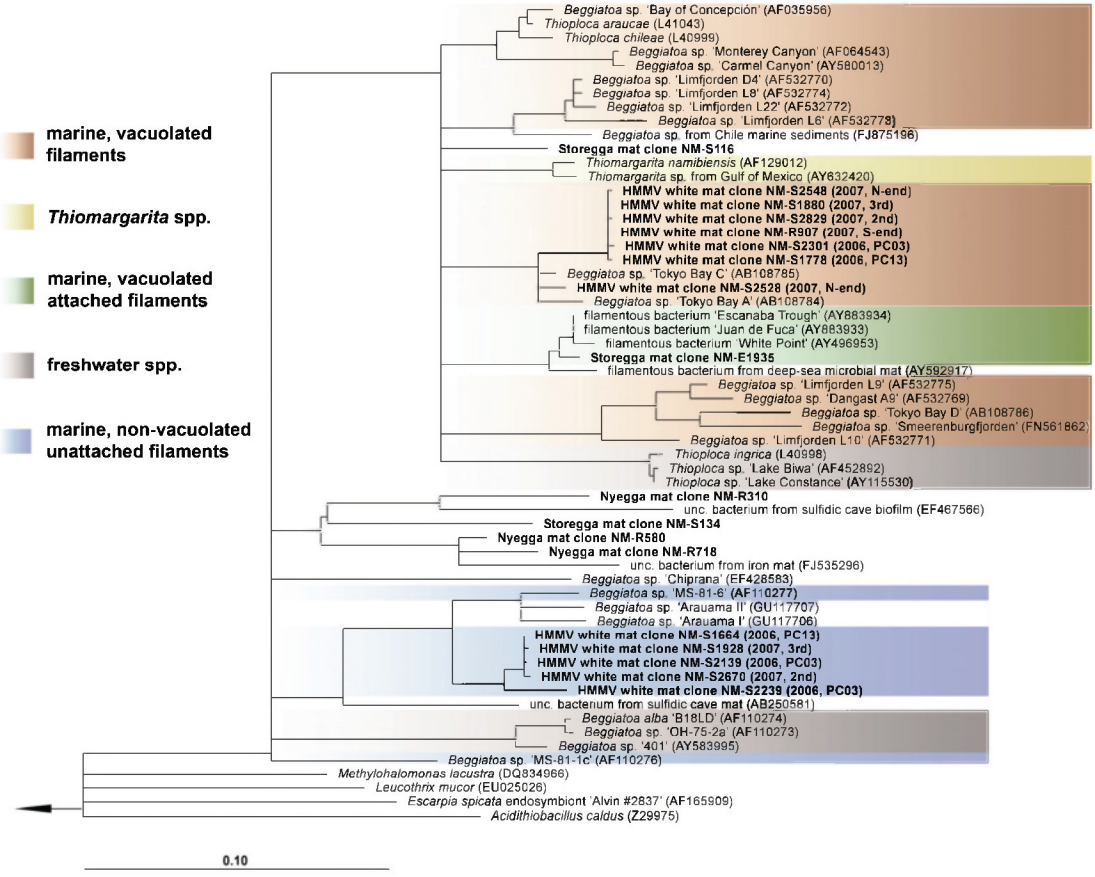

Figure 3: Phylogenetic 16S rRNA gene based tree showing representative sequences obtained from the mats studied here (Storegga mat clones, Nyegga mat clones, HMMV white mat clones; indicated by bold type) to reference sequences within the Gammaproteobacteria. Tree reconstruction was conducted with nearly full length sequences (*E. coli* positions 127 to 1307) by applying neighbour joining, maximum likelihood (RAxML) and maximum parsimony methods as well as different conservatory filters. Short sequences L41043, L40999, AF129012, AY632420, AB108785, AB108784 and AY592917 were added subsequently by applying parsimony criteria. Only selected sequences are shown. Unstable branching orders were visualized by multifurcation. The bar indicates 10% estimated phylogenetic divergence. The color coding refers to previously described species.

Rates of hydrocarbon-fueled SR at the Storegga mats varied between 0.3 to 70.7 mmol m^{-2} d^{-1} (n = 4) integrated over the top 10 cm of seafloor (Table 2). CARD-FISH revealed a dominance of anaerobic methanotrophs belonging to the ANME1-group (>36% of total archaeal cells). Sulfate reducers of the *Desulfosarcina/Desulfococcus* cluster comprised <1% of the bacterial cells (in the upper 5 cm of sediment). Total cell numbers were highest in the upper 2 cm of sediment reaching values of 4.49 to 11.17 × 10^9 cells cm^{-3} (n = 3), and were substantially elevated compared to cell numbers counted in reference sediment (0.66 to 2.09 × 10^9 cells cm^{-3}; n = 2). *Ex situ* determined fluxes of oxygen (4.2 to 35.2 mmol m^{-2} d^{-1}; n = 3) and sulfide (5.0 to 10.1 mmol m^{-2} d^{-1}; n = 2) were clearly elevated compared to fluxes determined at a reference site (Table 2). *Ex situ* microprofiles recorded in a retrieved push core revealed that oxygen and sulfide overlapped and were both consumed within the mat (Figure 6D). The observed decrease in pH indicated oxidation of sulfide.

Table 2: Summary of biogeochemical parameters characterizing thiotrophic mats associated with seeps in the Storegga and Nyegga area and at the Håkon Mosby mud volcano (HMMV).

Sampling site	Measurement/Analysis[a]				Reference
	Microprofiles[b]		Nitrate uptake[c] (mmol m^{-2} d^{-1})	SR[d] (mmol m^{-2} d^{-1})	
	DOU (mmol m^{-2} d^{-1})	Sulfide flux (mmol m^{-2} d^{-1})			
Storegga mats	4.2-35.2 (average: 15.6)	5.0-10.1 (average: 7.6)	ND	0.3-70.7 (average: 23.2)	this study
Nyegga mats	13.8-17.8 (average: 15.8)	4.2-12.3 (average: 8.2)	4.4-7.3	25.3-201.9 (average: 113.6)	this study
Reference Storegga/Nyegga	2	0	0.2	ND	this study
HMMV gray mats	45.2(±8.4)	17-131 (average: 74)	4.5	9.5-108.3 (average: 48.4)	Felden *et al.* (in review), Lichtschlag *et al.* (2010)
Next to HMMV gray mats	35.2(±13)	4.8	ND	ND	Lichtschlag *et al.* (2010)
HMMV white mats	33.7(±8.8)	11.6	1.5-102	2.8-28.7 (average: 12.7)	Felden *et al.* (in review), Lichtschlag *et al.* (2010)
Next to HMMV white mats	9.2(±0.9)	7	ND	ND	Lichtschlag *et al.* (2010)

[a]Abbreviations are as follows: DOU (diffusive oxygen uptake), SR (sulfate reduction), ND (no data).
[b]*In situ* DOU and sulfide fluxes for the HMMV mats were taken from Lichtschlag *et al.* (2010). For the Nyegga mats also *in situ* values are given and represent averages based on 1 (reference sulfide flux) or 2 replicates (mat DOU and sulfide flux, reference DOU) measured in the same mat patch. Values given for the Storegga mats were determined *ex situ* and are based on replicate profiles (n) recorded in 2 different push cores: n = 3 (DOU) and n = 2 (sulfide flux).
[c]Nitrate uptake by the mat-forming community, respective nitrate loss from the water column, was determined *ex situ* in recovered push cores by adding defined amounts of nitrate to the overlying water. Values given for the HMMV sites were taken from nitrate uptake experiments conducted by Lichtschlag *et al.* (2010). Values for the Nyegga mats were determined for 2 separate push cores. Further, a single reference experiment was conducted.
[d]Integrated SR rates were determined *ex situ* for the upper 10 cm of sediment. Average values were calculated from replicate measurements (n): n =4 for Storegga mats, n = 2 for Nyegga mats, n = 6 for HMMV gray mats (2006, 2007), and n = 18 for HMMV white mats (2006, 2007). HMMV SR rates: Lichtschlag *et al.* (2010) and Felden *et al.* (in review).

3.2 Nyegga mats

The complex pockmarks G11 and G12 at Nyegga investigated here showed a deeper bathymetric structure and were between 100 to 300 m in diameter. They appeared more active than the seeps associated with the Storegga gas chimneys, as dense accumulations of siboglinid tubeworms were observed. The Nyegga pockmarks also hosted carbonate cements inhabited by dense filter-feeding communities. The Nyegga mats within the pockmarks were of a similar size as the Storegga mats and were also found to be diverse in their visual appearance (Figure 4A-G). Mat patches were surrounded by small snails and dense populations of thin siboglinid tubeworms. Some mats were associated with *Arcobacter* spp.-resembling sulfur precipitates (e.g. Figure 4B, D, F) or white filamentous patches (e.g. Figure 4A, D, E). Samples for molecular analyses were obtained from a mat patch which was white on the edge and gray in the middle. The white mat parts resembled a *Beggiatoa* mat (Figure 4G). Microscopy of filaments picked from both parts of the mat (white and gray) revealed the presence of sulfur-storing *Beggiatoa* spp.-like filaments of approximately 3 μm in diameter (Figure 4H, I) and unknown transparent filaments (Figure 4I). Molecular analyses targeting the 16S rRNA gene sequences of white-mat-associated *Beggiatoa* spp.-type filaments resulted in few clones, i.e. Nyegga mat clone NM-R718, NM-R580 and NM-R310 (representatives), that were associated with uncultured bacteria from a sulfidic cave mat (EF467566; 87.0 to 90.7% similarity) and iron mat (FJ535296; 87.3 to 93.9% similarity; Figure 3) similar to Storegga mat clone NM-S134. From the gray mat parts no 16S rRNA gene sequences near or directly related to giant filamentous sulfur bacteria could be retrieved. These clone libraries were dominated by Epsilonproteobacteria, mostly assigned to *Sulfurovum* spp. that were also found in samples from the white mat part. In addition, *Arcobacter* spp.-related 16S rRNA gene sequences were found to be associated with both white and gray mat parts.

In situ microprofiling of one Nyegga mat patch (Figure 5) as well as *ex situ* microprofiling in three push cores, each recovered from a different mat patch (Figure 6A-C), showed that oxygen and sulfide were consumed within the mats. However, there was a high variability in the recorded pH profiles, ranging from (i) a pH increase beneath the mat followed by a pH decrease (Figure 5A) to (ii) a decrease in pH (Figure 6A, C) or (iii) a decrease in pH directly beneath the mat followed by an increase in pH in deeper sediment layers (Figure 6B). *In situ* recorded fluxes of sulfide (4.2 to 12.3 mmol m^{-2} d^{-1};

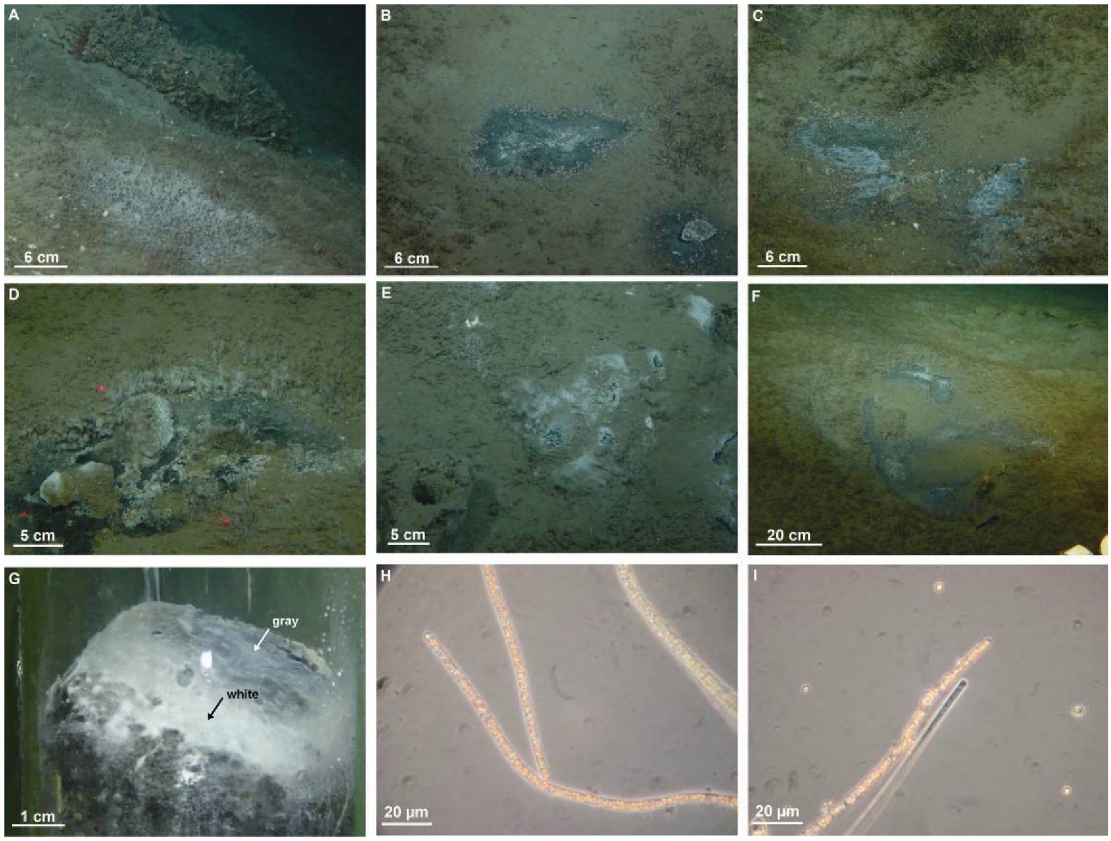

Figure 4: Nyegga mats. (A-F) Different mat types observed at the Nyegga complex pockmarks G11 and G12. All mats were either closely associated with or surrounded by tubeworms. White precipitates resembling sulfur filaments produced by *Arcobacter* (Sievert *et al.*, 2007) were associated with some mats (panel B, D and F). Further, filamentous patches were occasionally visible (panel A, D and E). Image Source: Ifremer, Vicking 2006. (G) Gray and white colored mat sample in a push core retrieved for *ex situ* microprofiling and subsequent sampling. (H) Sulfur-storing filaments associated with the white mat part resembled *Beggiatoa* spp. (I) The same type of sulfur-storing filaments was also associated with the gray mat part. In addition, transparent filaments were visible.

n = 2) and oxygen (13.8 to 17.8 mmol m^{-2} d^{-1}; n = 2) were clearly elevated compared to reference sediment that contained no sulfide (0 mmol m^{-2} d^{-1}; n = 1) and showed a much smaller oxygen flux (2 mmol m^{-2} d^{-1}; n = 2; Table 2). Temperature profiles remained constant with depth at approximately 0.2°C (Figure 5). Nitrate loss from the overlying water column was used as proxy for nitrate uptake by the thiotrophic mat community and was determined in two recovered cores to be in the range of 4.4 to 7.3 mmol m^{-2} d^{-1} (experiment with reference sediment: 0.2 mmol m^{-2} d^{-1}; Table 2).

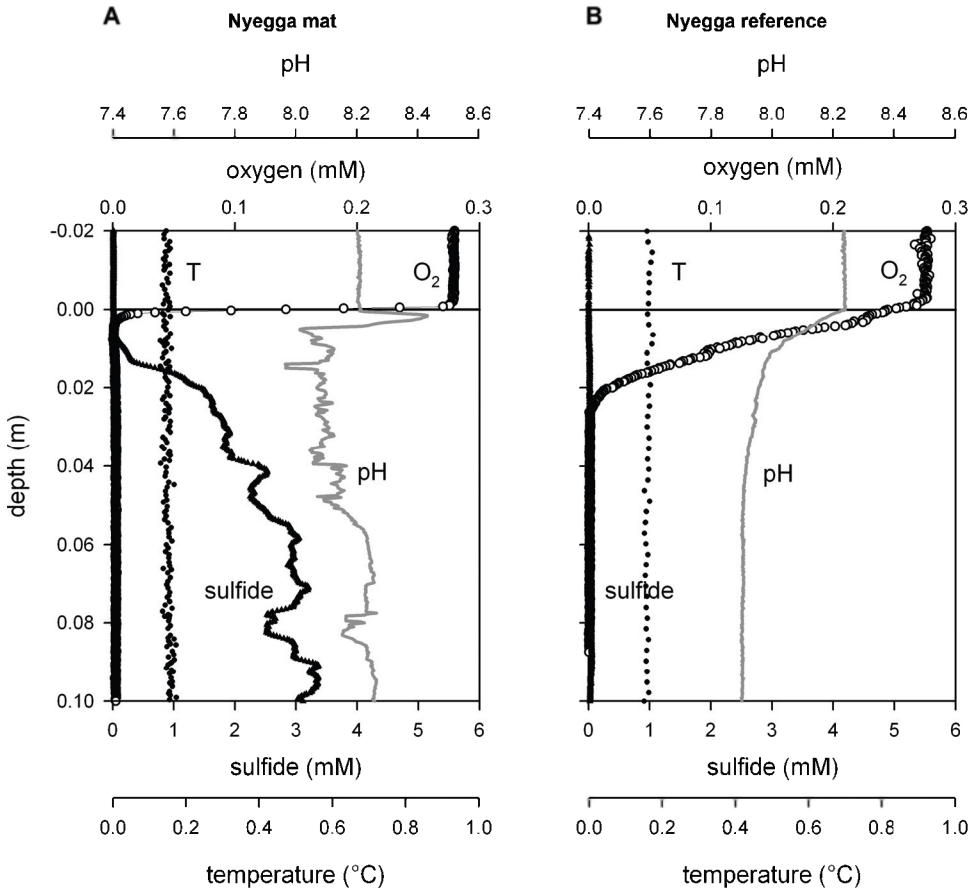

Figure 5: High resolution *in situ* microprofiles of oxygen (O_2), sulfide, pH and temperature (T) recorded (A) on a Nyegga mat in the area of pockmark G12, and (B) on reference sediment between pockmark G11 and G12.

SR rates for the Nyegga mats varied between 25.3 and 201.9 mmol m^{-2} d^{-1} (n = 2; Table 2). CARD-FISH revealed a dominance of ANME1 (>9% of total archaeal cells) and ANME2 (>1% of total archaeal cells). Sulfate reducers of the *Desulfosarcina/Desulfococcus* cluster comprised <1% of the bacterial cells (in the upper 5 cm of sediment). Total cell counts were generally highest in the upper 2 cm of sediment, reaching values of 1.51 to 16.60 × 10^9 cells cm^{-3} (n = 6), and were elevated compared to cell numbers counted in reference sediment (0.66 to 2.09 × 10^9 cells cm^{-3}; n = 2). Geochemical profiles of pore-water constituents were found to vary between individual mat patches (Figure 7). Nevertheless, chloride concentration usually remained constant with increasing depth, whereas sulfate and sulfide profiles were indicative of their

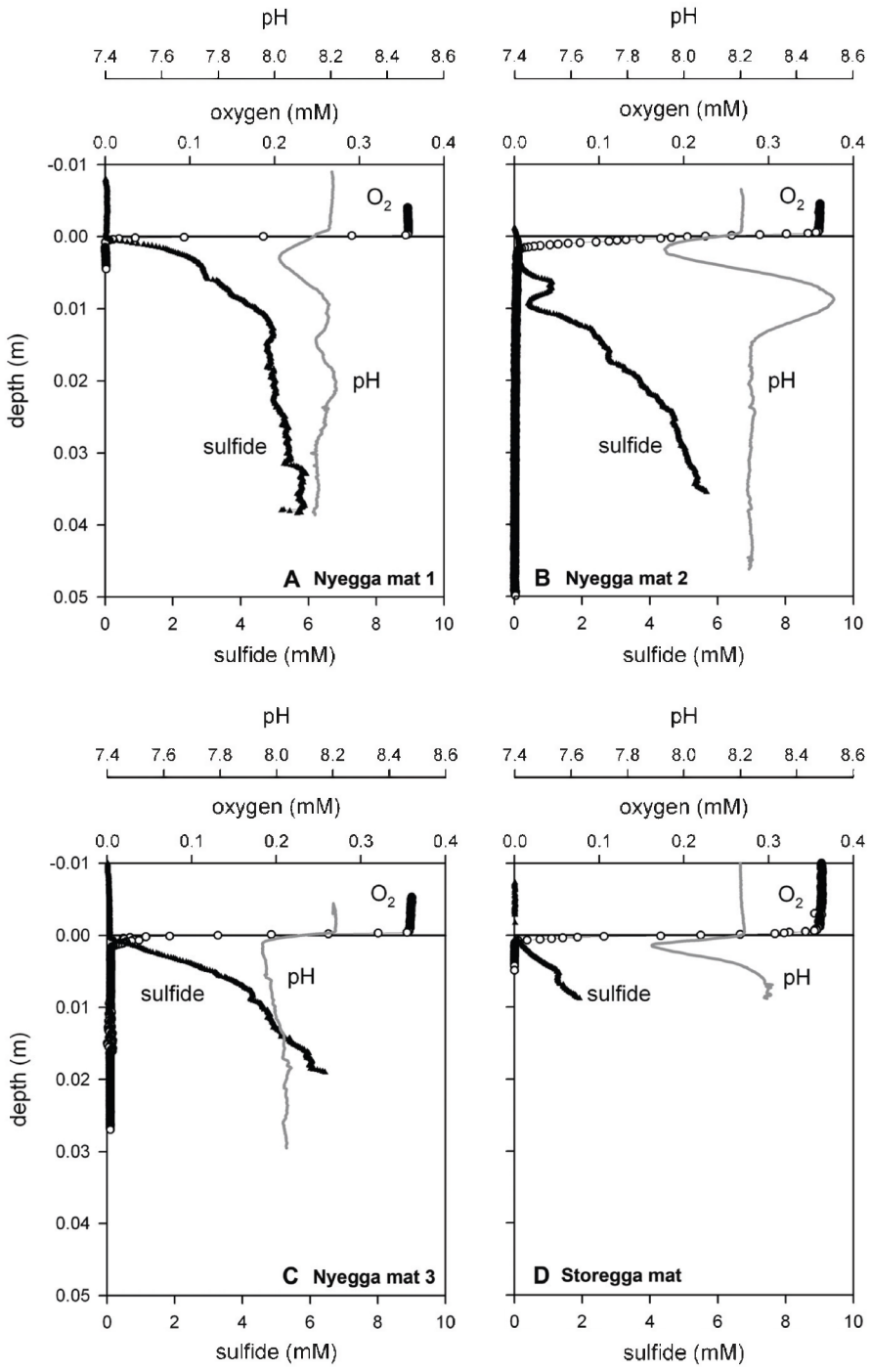

Figure 6: High resolution *ex situ* microprofiles of oxygen (O_2), sulfide and pH recorded in three different cores recovered from the Nyegga area (A-C) and one core recovered from the Storegga area (D).

consumption or production, respectively, by hydrocarbon-fueled SR in the upper 5 cm of sediment underlying the mats (Figure 7A-C). At the reference site no sulfide could be detected (Figure 7C). Elemental sulfur concentrations were highest at the sediment surface (1.2 to 2.9 μmol cm^{-3}; n = 3), except for one core where the maximum concentration was reached at 5 cm sediment depth (1.0 μmol cm^{-3}; n = 1). Elemental sulfur concentration at the surface of non-seep reference sediment was below the detection limit.

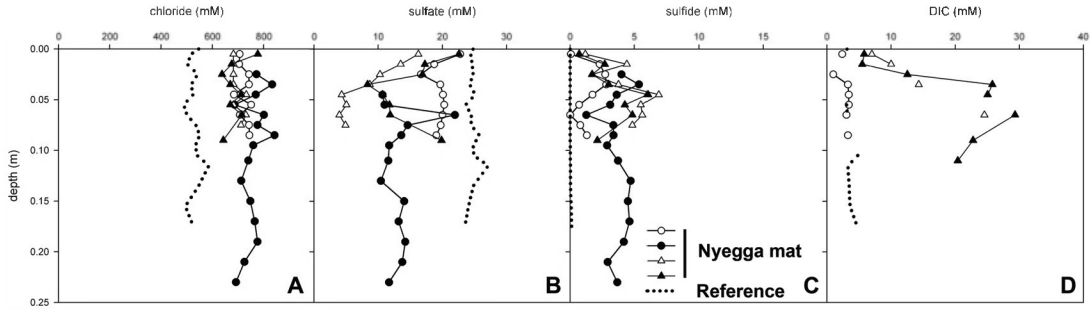

Figure 7: Vertical distribution of chloride, sulfate, total sulfide and dissolved inorganic carbon (DIC) concentrations measured in different Nyegga mats as well as at one reference site. Single profiles are shown.

3.3 HMMV gray mats

Various gray mat patches of 1 to 5 m in size (Figure 8A-C) were observed in the transition zone northwest of the central area of the HMMV, or within the hummocky outer rim. Here, the occurrence of gray mats was confined to areas devoid of siboglinid tubeworms, which cover most of the outer zones of the HMMV. Some of the gray mat patches were partially overgrown by whitish mats (Figure 8A, C). Microscopic analyses showed that the gray mats harbor very different bacteria, amongst them various *Beggiatoa* spp.-resembling filaments (diameters between < 1 μm and up to 10 μm; Figure 8D-F), various transparent filaments (Figure 8E), unidentified giant pigmented filaments (diameter approximately 7 μm; Figure 8G), *Thiomargarita* spp.-resembling cells (diameter approximately 50 μm; Figure 8H) and unidentified single cells (Figure 8I). In addition, on some gray mat samples *Arcobacter* spp.-sulfur precipitates were observed. Despite the

microscopical observations, phylogenetic analyses from the same subsamples from gray mats targeting the 16S rRNA gene sequences of giant sulfide-oxidizing bacteria did not result in any sequence closely related to the group of *Beggiatoa*, VAF, *Thioploca* or *Thiomargarita* spp. (samples obtained in 2006). Clone libraries were mainly dominated by delta- and epsilonproteobacterial phylotypes, the latter including *Arcobacter* spp.-related 16S rRNA gene sequences.

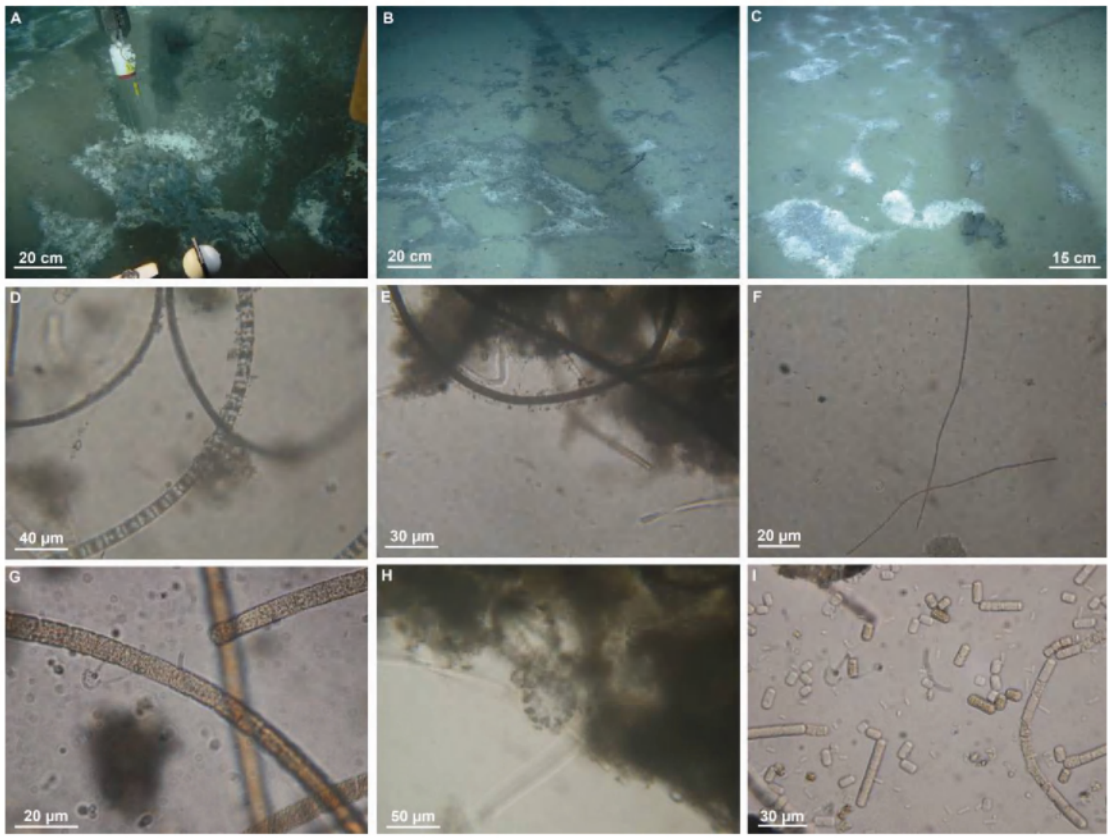

Figure 8: HMMV gray mats. (A-C) Examples of different gray mat patches observed at the HMMV during the Vicking cruise in 2006 (A; Image Source: Ifremer) and ARK-XXII/1b cruise in 2007 (B and C; Image Source: Marum, University Bremen). In some occasions the gray mats were surrounded by a ring of white filamentous (*Beggiatoa*) mats. (D) Two types of filamentous bacteria resembling *Beggiatoa* spp. (ARK-XXIV/2b in 2009). (E) Some of the *Beggiatoa* spp.-resembling filaments were covered by a transparent layer or sheath with attached sediment particles and supposedly other (small) bacteria. In addition, few transparent filaments and one potentially pigmented filament are visible (ARK-XXIV/2b in 2009). (F) Small *Beggiatoa* spp.-resembling filaments with diameters of < 1 µm. These filaments appeared as thin strings of sulfur granules (ARK-XXIV/2b in 2009). (G) Unidentified (and supposedly pigmented) filaments (Vicking cruise, 2006). (H) Single *Thiomargarita* cell of 50 µm diameter (ARK-XXIV/2b in 2009). (I) Unidentified single cells (Vicking cruise in 2006).

In situ microprofiling of oxygen and sulfide was conducted previously and revealed overlapping oxygen-sulfide gradients beneath the mat (de Beer *et al.*, 2006; Lichtschlag *et al.*, 2010). SR rates varied between 9.5 and 108.3 mmol m^{-2} d^{-1} (Table 2; Felden *et al.*, in review; Lichtschlag *et al.*, 2010). CARD-FISH analyses conducted with sediments underlying a gray HMMV mat revealed a dominance of ANME3 (>6% of total archaeal cells), a lower abundance of ANME1 (>3% of total archaeal cells), and no ANME2 or sulfate reducers of the *Desulfosarcina/Desulfococcus* branch. Total cell numbers were generally highest in the upper 2 cm of the sediment underlying the mats, expressing values of 1.35 to 2.46 × 10^9 cells cm^{-3} (n = 3). Total cell numbers determined for non-sulfidic reference sediment were comparable with 0.66 to 2.08 × 10^9 cells cm^{-3} (n = 2).

3.4 HMMV white mats (*Beggiatoa* mats)

Surrounding the flat active center in a circular zone of 10 to 50 m width, dense white mats cover large parts of the flat seafloor characterized by high fluid flow, gassy sediments and high sulfide flux. These giant mats make up almost 15% of the entire HMMV structure. A large white *Beggiatoa* mat sampled in the southeastern part of the HMMV in 2007 covered an area of approximately 850 m^2 (Figure 9). Samples were taken at four different positions, including (i) the northern end (N-end) of the mat that was characterized by the occurrence of only partially connected spots, (ii) an area 40 m away where the mat started to become more dense (2nd station), (iii) an area 100 m away where we observed a dense, thick coverage of the seafloor with *Beggiatoa* filaments (3rd station), and (iv) the southern end (S-end) 158 m away from the N-end where the mat was less thick and increasingly patchy. In the past, HMMV white mats (Figure 9 and 10A) were always referred to as *Beggiatoa* mats, but identification of these bacteria was solely based on morphological resemblance. Here, the molecular analyses targeting the 16S rRNA genes of *Beggiatoa* spp.-resembling filaments (Figure 10B-D) recovered in 2006 and 2007 now confirmed the previous morphological identification of the mat-forming (dominating) filaments as *Beggiatoa* spp. (Figure 3). We detected two types of sulfur-storing filaments, having diameters of approximately 6 to 10 μm and showing different loads of internally stored elemental sulfur. Accordingly, two different genetic 'phylotypes' were retrieved from the mat sample (Figure 3). One was closely associated

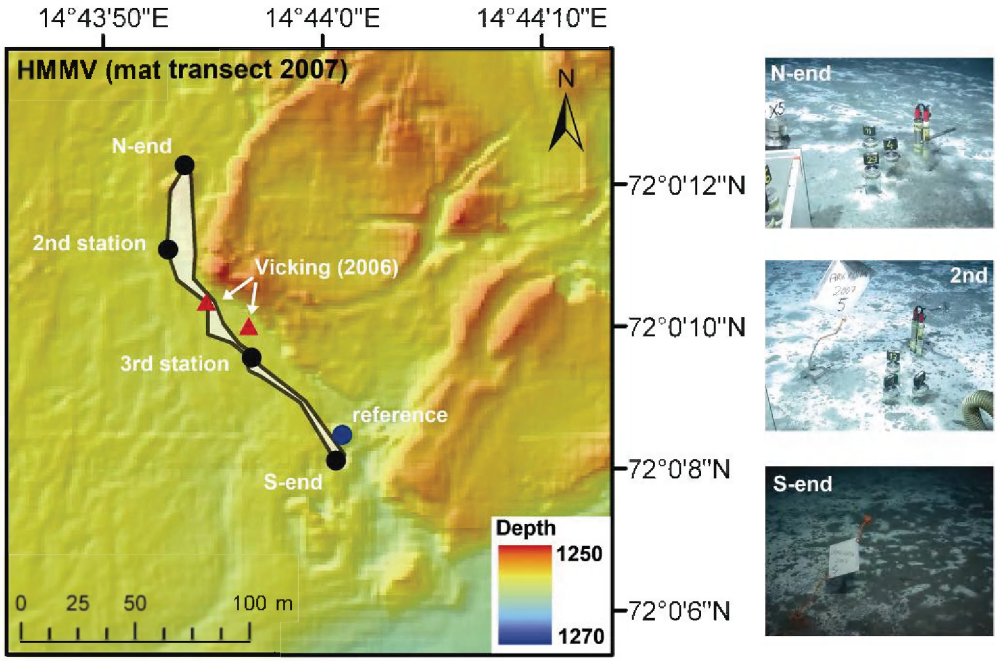

Figure 9: Detail of the HMMV bathymetric map recorded in 2003 (Edy *et al.*, 2004) showing a close up on the southeastern area of the mud volcano in which an extensive white mat was sampled during the ARK-XXII/1b cruise (2007) at four different locations (black circles), including the northern end ('N-end'; loose mat patches), a 2nd station 40 m away ('2nd'; beginning of dense coverage), a 3rd station 100 m away ('3rd'; dense coverage), and the southern end ('S-end'; transition to grayish mat) 158 m away from the N-end, as well as one location next to the mat (blue circle). In addition, sample locations of push cores used for phylogenetic analyses from the Vicking cruise (2006) are indicated (red triangles).

to partial sequences of marine *Beggiatoa* spp. from Tokyo Bay (hereafter named 'phylotype I'; represented by clones NM-S2548, NM-S1880, NM-S2829, NM-S2301, NM-S1778, NM-S2528 and NM-R907). The other (hereafter named 'phylotype II'; represented by clones NM-S1664, NM-S1928, NM-S2139, NM-S2670 and NM-S2239) clustered with a known non-vacuolated marine (salt marsh) *Beggiatoa* sp. (MS-81-6, AF110277; 94.3 to 96.6% similarity) as well as two novel *Beggiatoa* spp. from a hypersaline lagoon (Arauama I and Arauama II, GU117706 and GU117707; 93.4 to 95.0% and 94.2 to 95.8% similarity, respectively) and an uncultured bacterium from a sulfidic cave mat (AB250581; 89.6 to 90.8% similarity). The two phylotypes were related to each other with 87.6 to 90.2% similarity. Both phylotype clusters contain an 'additional' sequence (Figure 3), resulting from similarity values <99.7% (97.5 to 97.7%

similarity for NM-S2239 and 97.8 to 97.9% similarity for NM-S2528, compared to clones obtained in this study for the respective clusters). Further studies will be needed to target the question which phylotype refers to which filamentous morphotype. In addition, we observed numerous unidentified transparent filaments associated with the HMMV white mats (e.g. Figure10C), and clone libraries revealed the presence of *Arcobacter* spp.-associated sequences

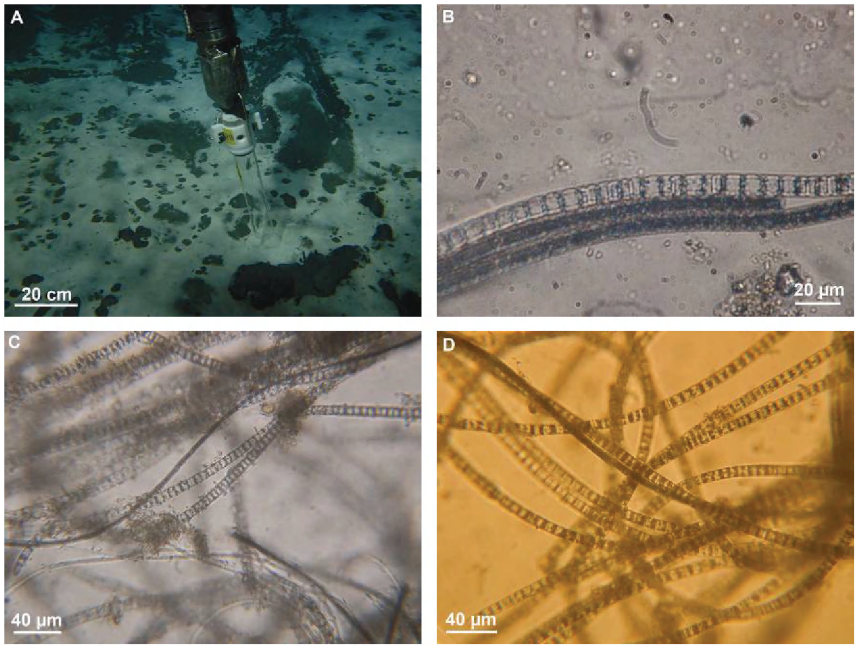

Figure 10: HMMV white (*Beggiatoa*) mats. (A) White *Beggiatoa* mats at the HMMV can extend for several hundreds meters. Inbetween, small patches of uncovered sediment are visible. Image Source: Ifremer, Vicking 2006. (B) Microscopic image of the two observed types of *Beggiatoa* spp.-resembling filaments that dominate the white HMMV mats (Vicking 2006). (C, D) Microscopic images of mat samples recovered during the ARK-XXII/1b cruise (2007) showing the same two types of *Beggiatoa* filaments as well as unidentified transparent filaments.

Total cell numbers, SR rates and temperature gradients beneath the mat did not show significant variations along the transect (Table 3). Cell numbers in the upper 2 cm of sediment were slightly elevated at the S-end, the part of the mat with the least dense coverage of *Beggiatoa* filaments, reaching values of 2.58×10^9 cells cm^{-3} (Table 3). SR rates and temperature gradients were highest in sediments underneath the most densely

covered area (3rd station), reaching values of 20.9 ± 10.2 mmol m^{-2} d^{-1} and 2.73 °C m^{-1}, respectively (Table 3).

Table 3: Comparison of total cell numbers (AODC), sulfate reduction rates (SR) and temperature gradients (T) determined along a transect of one large white mat at the Håkon Mosby mud volcano (HMMV) as well as adjacent sediment.

	Relative transect position[a]				
	HMMV white mat N-end	HMMV white mat 2nd station	HMMV white mat 3rd station	HMMV white mat S-end	next to mat
mat description	loose mat patches	beginning of dense coverage	dense coverage	patchy appearance	no mat visible
AODC[b] (10^9 single cells cm^{-3})	2.24; 1.71	1.54; 0.83	2.04; 2.27	2.58; 2.43	2.87; 2.53
SR[c] (mmol m^{-2} d^{-1})	3.29	14.0 ± 6.8	20.9 ± 10.2	12.2 ± 6.5	ND
T[d] (°C m^{-1})	1.52	2.20	2.73	1.11	0.94

[a]During cruise ARK-XXII/1b in 2007, an extensive white mat discovered in the southeastern area of the HMMV was sampled along a North-South gradient (transect).
[b]Total cell numbers are given for the upper 2 cm (horizons 0-1 cm and 1-2 cm) of sediment underlying the bacterial mat and were retrieved from one push core each.
[c]Integrated SR rates were determined *ex situ* and were calculated for the upper 10 cm of sediment from samples obtained during the ARK-XXII/1b cruise in 2007. Average values were calculated from replicate measurements (n), retrieved from single push cores recovered for each site: n = 1 for N-end, n = 3 for 2nd station, 3rd station and S-end. Data are a subset of SR rates in Felden *et al.* (in review).
[d]Temperature gradients were inferred from measurements conducted with a ROV-operated temperature probe (Felden *et al.*, in review).

In situ microprofiling of oxygen and sulfide was conducted previously and revealed spatially separated oxygen-sulfide gradients (by 4 to 6 mm; de Beer *et al.*, 2006; Lichtschlag *et al.*, 2010). SR rates varied between 2.8 and 28.7 mmol m^{-2} d^{-1} (Table 2; Felden *et al.*, in review; Lichtschlag *et al.*, 2010).CARD-FISH analyses conducted with sediments sampled in 2006 from an area similar to the third station of the mat transect 2007 (dense mat coverage) revealed a dominance of ANME3 (>45% of total archaeal cells) and ANME1 (>23% of total archaeal cells) and presence of bacteria belonging to the *Desulfobulbus* branch. No ANME2 or sulfate-reducers of the *Desulfosarcina/Desulfobulbus* group were detected. Total cell numbers were generally highest in the upper 2 cm of sediment and reached 0.21 to 1.69 × 10^9 cells cm^{-3} (n = 3),

i.e. in the same range as determined for a reference site (0.66 to 2.08 × 10^9 cells cm^{-3}, n = 2).

4. Discussion

4.1 Diversity of mat-forming sulfide oxidizers at cold water seeps

Microscopic and 16S rRNA gene based phylogenetic analyses of different thiotrophic mats occurring at cold seep systems of the Norwegian margin revealed a high diversity of sulfur-storing and non-sulfur storing microorganisms. At temperatures close to the freezing point, members of the same types of giant sulfide oxidizers were detected as previously found in warm water systems, e.g. of the Eastern Mediterranean (Grünke *et al.*, in preparation; Omoregie *et al.*, 2008) or in the Guaymas Basin (Nelson *et al.*, 1989). Mat-forming bacteria of the Nordic cold seeps included different types of the giant filamentous sulfur oxidizer *Beggiatoa*, single cells of the giant sulfur bacterium *Thiomargarita*, the gelatinous mat-forming *Thiobacterium* spp., giant sulfur-storing filaments attached to sediment and tubeworms, filamentous sulfur structures reminiscent of *Arcobacter* mats as well as numerous unknown filamentous bacteria. The variation between the observed mat types and their dominant thiotrophs was explained by biogeochemical gradients and most likely also variations in the stability of habitats as discussed below.

4.2 Isolated gray mat patches harbor diverse thiotrophic communities

HMMV gray mats have previously been postulated to be associated with dynamic conditions in gas flux and temperature gradients (Felden *et al.*, in review; Lichtschlag *et al.*, 2010) and are thought to be characterized by closely interacting sulfide oxidation processes (Lichtschlag *et al.*, 2010). Accordingly, in this investigation of the distribution of mat-forming thiotrophs, we observed a considerable heterogeneity between samples of gray mats. Cores from gray mats within one mud volcano structure differed in the dominance of various *Beggiatoa* spp.-like organisms, VAFs and unknown single cell thiotrophs. A similar heterogeneity in the diversity of thiotrophic bacteria and

biogeochemical fluxes was observed between small gray mat patches from the Storegga and Nyegga pockmarks, especially with regard to local pH, profiles of pore-water constituents, as well as in hydrocarbon-fueled SR. Common to the gray mat patches were low to absent temperature gradients, and very high SR rates, higher than those determined for HMMV white *Beggiatoa* mats (Table 2; Felden *et al.*, in review; Lichtschlag *et al.*, 2010).

Nitrate uptake in the Nyegga mats was elevated compared to non-sulfidic reference sediment, was similar compared to HMMV gray mats and was lower than in HMMV white mats (Table 2; Lichtschlag *et al.*, 2010). However, *in situ* and *ex situ* profiles revealed that sulfide and oxygen overlapped and were consumed within the mats (Figures 5 and 6). *In situ* recorded oxygen and sulfide fluxes for a Nyegga mat (Table 2) were in a ratio of 2:1, indicating that enough oxygen was present for a complete oxidation of sulfide to elemental sulfur at the time of the analysis ($2HS^- + O_2 + 2H^+ \rightarrow 2S^0 + 2H_2O$) or sulfate ($HS^- + 2O_2 \rightarrow SO_4^{2-} + H^+$). However, anaerobic sulfide oxidation with nitrate may be a temporarily important process in the Nyegga gray mats.

4.3 Extensive white HMMV mats are dominated by two *Beggiatoa* spp.

In contrast to the substantial heterogeneity observed for the gray mats within and between cold seep locations, the giant white mats of the HMMV showed a rather low spatial variation. Two morphologically distinct filament types resembling *Beggiatoa* spp. were observed within the HMMV white mats (Figure 10B-D). Microprofiling within the white mats had previously given indications for either (i) completely anaerobic sulfide oxidation with nitrate (de Beer *et al.*, 2006), or (ii) a combination of anaerobic sulfide oxidation with nitrate to elemental sulfur in deeper sediment layers and aerobic oxidation of internally stored sulfur to sulfate near the sediment surface (Lichtschlag *et al.*, 2010), as oxygen and sulfide gradients were spatially separated. Chemical sulfide oxidation or precipitation was found to be of minor importance (Lichtschlag *et al.*, 2010). Further, Lichtschlag *et al.* (2010) determined an average internal nitrate content of 110 mmol L^{-1} within *Beggiatoa* filaments. Phylogenetic analyses within this study revealed the presence of several 16S rRNA gene sequences ('phylotype I') closely associated with marine, vacuolated and nitrate-storing *Beggiatoa* spp. of similar diameter (10 µm; Kojima and Fukui, 2003; Sayama, 2001), whereas a second group of sequences ('phylotype II') was

closely associated with a smaller (4 to 5 μm in diameter), non-vacuolated salt marsh *Beggiatoa* sp. (Nelson *et al.*, 1982). It is possible, that at the HMMV two subpopulations of *Beggiatoa* spp. co-exist within the same mat, thereby occupying different ecological niches with respect to their electron acceptors (oxygen and nitrate). This has previously been observed for *Beggiatoa* spp. within phototrophic mats at a hypersaline pond (Garcia-Pichel *et al.*, 1994). However, narrow, vacuolated *Beggiatoa* spp. obtained from hypersaline lake sediments ('Chiprana', Figure 3; Hinck *et al.*, 2007) have been found to be closely related to the narrow, marine, non-vacuolated *Beggiatoa* spp. ('MS-81-1c' and 'MS-81-6'; Figure 3) of which one was the closest relative for the second group of sequences obtained within this study.

Here, we observed the same two *Beggiatoa* spp. types in spatially and temporally separate samples of a white mat at HMMV, recovered in 2006 and 2007 (Figure 10B-D and Figure 3). This finding was supported by the sequences retrieved, with the only exception that no phylotype II sequence was obtained for the N-end and S-end of the mat transect, which is most likely a problem of undersampling the clone library. Accordingly, SR rates, temperature gradients and total cell numbers did not vary significantly along the mat transect (Table 3), indicating rather stable conditions at the point of sampling.

4.4 Mat size, color and spatial separation of mat patches as indicators for habitat dynamics and microbial diversity

In this study, thiotrophic mats associated with gas chimneys in the Storegga area, complex pockmarks in the Nyegga area and the Håkon Mosby mud volcano were compared regarding biogeochemical gradients and the mat-associated bacterial community. Generally, there were two types of mats – isolated, small gray mats in the Storegga and Nyegga area (20 to 50 cm in diameter) as well as in a transition zone of the HMMV (1 to 5 m in diameter), and large white mats (up to 850 m^2) characteristic for a habitat surrounding the active center of the HMMV. The smaller gray mats were visually diverse and appeared to represent almost individual islands of always differing (in appearance or abundance) communities of thiotrophic bacteria, including *Beggiatoa* spp., VAF, *Thiomargarita* cells, *Thiobacterium* spp., *Arcobacter* spp., unknown pigmented filaments or single cells. Contrary, the extensive white HMMV mats were dominated by only two

different types of *Beggiatoa* filaments. An important factor explaining the difference in diversity between the two mat types seems to be sulfate turnover, which produces energy-rich sulfide. SR rates measured for gray mats were much more variable between the individual mat patches. This might indicate, as previously postulated for the HMMV (de Beer *et al.*, 2006; Lichtschlag *et al.*, 2010), that smaller (gray) mats are found in rather dynamic habitats. Under dynamic conditions, either small and highly motile (e.g. *Arcobacter* spp.) or specially adapted (e.g. *Thiomargarita* spp.) sulfide oxidizers might be pioneer colonizers and thrive efficiently when chemical gradients change (Bernard and Fenchel, 1995; Girnth *et al.*, in review; Schulz, 2006; Sievert *et al.*, 2007). These pioneer colonizers might later be replaced by other organisms once conditions become more stable (Bernard and Fenchel, 1995; Sievert *et al.*, 2007). Factors supporting this hypothesis for the Norwegian thiotrophic mats might be (i) the appearance of *Arcobacter* spp. beneath all mat types, (ii) remnants of *Arcobacter* spp.-precipitates in association with gray mats only, and (iii) the observation that gray mat patches of the HMMV were sometimes overgrown by white *Beggiatoa* spp.-type mats. Hence it is suggested that the spatial and temporal variations in biogeochemical gradients at cold seep ecosystems are the main selective force for the diversity of mat-forming thiotrophs. Physical factors, such as temperature and spatial (biogeographical) distance, seemed less important as all main types of mat-forming thiotrophs associated with cold seeps were observed in the polar systems investigated here.

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Supplementary Information

Supplementary Table 1: Sampling and measurement sites in this study.

Mat type	Measurement/Analysis[a]	PANGAEA event label[b]
Storegga mats	*Ex situ* microprofiling (O_2, pH, sulfide, DOU, sulfide flux)	VKGD275/PC-1 (Figure 6D)
	SR	VKGD275/PC-1, VKGD275/PC-3, VKGD275/PC-10, VKGD275/PC-11
	AODC	VKGD275/PC-2, VKGD275/PC-3, VKGD275/PC-4
	CARD-FISH	VKGD275/PC-2, VKGD275/PC-11
	16S rRNA clone libraries	VKGD275/PC-2
	Microscopic analyses[c]	VKGD275/PC-2 (Figure 2D-F), VKGD275/PC-11 (Figure 2G-I)
Nyegga mats	*In situ* microprofiler (O_2, pH, sulfide, T, DOU, sulfide flux)	VKGD272/MIC-1 (Figure 5A)
	Ex situ microprofiling (O_2, pH, sulfide, DOU, sulfide flux)	VKGD272/PC-9, VKGD272/PC-11, VKGD272/PC-32 (Figure 6A-C)
	SR	VKGD272/PC-4, VKGD272/PC-34
	Pore-water analyses (SO_4^{2-}, Cl^-, total sulfide, DIC), elemental sulfur (S^0)	VKGD272/PC-11, VKGD272/PC-24, VKGD272/PC-28, VKGD272/PC-32
	Nitrate uptake experiment	VKGD272/PC-11, VKGD272/PC-32
	AODC	VKGD272/PC-3, VKGD272/PC-4, VKGD272/PC-29, VKGD272/PC-31, VKGD272/PC-32, VKGD272/PC-34
	CARD-FISH	VKGD272/PC-32
	16S rRNA clone libraries	VKGD272/PC-32
	Microscopic analyses[c]	VKGD272/PC-32 (Figure 4G-I)
Reference Storegga/Nyegga	*In situ* microprofiler (O_2, pH, sulfide, T, DOU, sulfide flux)	VKGD272/MIC-2 (Figure 5B)
	Ex situ microprofiling (O_2, pH, sulfide, DOU, sulfide flux)	VKGMTB2
	Pore-water analyses (SO_4^{2-}, Cl^-, total sulfide, DIC), elemental sulfur (S^0)	VKGMTB2
	Nitrate uptake experiment	VKGMTB2
	AODC	VKGMTB1, VKGMTB2
HMMV gray mats	AODC	VKGD277/PC-1, VKGD277/PC-3, VKGD277/PC-10
	CARD-FISH	VKGD277/PC-10
	16S rRNA clone libraries	VKGD277/PC-8, VKGD277/PC-10
	Microscopic analyses[c]	VKGD277/PC-3 (Figure 8I), VKGD277/PC-10 (Figure 8G); PS74/176-1_PUC-118 (Figure 8D, E), PS74/176-1_PUC-121 (Figure 8H), PS74/176-1_PUC-127 (Figure 8F)
Reference HMMV gray mats	AODC	VKGMTB1, VKGMTB2
HMMV white mats	AODC	VKGD276/PC-1, VKGD276/PC-2, VKGD276/PC-3 (mat); PS70/112-1_PUC-4 (N-end), PS70/112-1_PUC-1 (2nd), PS70/112-1_PUC-27 (3rd), PS70/112-1_PUC-17 (S-end)
	CARD-FISH	VKGD276/PC-2, VKGD276/PC-13
	16S rRNA clone libraries	VKGD276/PC-3, VKGD276/PC-13; PS70/112-1_PUC-29 (N-end), PS70/112-1_PUC-7 (2nd), PS70/112-1_PUC-28 (3rd), PS70/112-1_PUC-36 (S-end)
	Microscopic analyses[c]	VKGD276/PC-2 (Figure 10B); PS70/112-1_PUC-29 (Figure 10C), PS70/112-1_PUC-28 (Figure 10D)
Reference HMMV white mats	AODC	VKGMTB1, VKGMTB2; PS70/132-1_PUC-16 (next to mat)

[a]Abbreviations are as follows: T (temperature), DOU (diffusive oxygen uptake), SR (sulfate reduction), DIC (dissolved inorganic carbon), AODC (acridine orange direct cell counts), CARD-FISH (catalyzed reporter deposition fluorescence *in situ* hybridization), rRNA (ribosomal RNA).
[b]Labeling is according to the PANGAEA database (www.pangaea.de). Abbreviations are as follows: VKG (Vicking cruise 2006), PS70 (ARK-XXII/1b cruise 2007), MIC (microprofiler), PC or PUC (push core), MTB (multicorer).
[c]Listed cores refer to data presented as figures in the Results section.

Supplementary Table 2: Oligonucleotide probes and hybridization conditions used for CARD-FISH analyses in this study.

Probe	Probe specificity	Probe sequence (5'-3')	Target Site within 16S rRNA gene[a]	FA[b] (%)	T_h[c]/T_w[d] (°C)	Reference
EUB338(I-III)	Most Bacteria	Equimolar mixture of the following three probes	338-355	30	46/46	Daims *et al.* (1999)
EUB338	Most Bacteria	GCT GCC TCC CGT AGG AGT	338-355	30	46/46	Amann *et al.* (1990)
EUB338-II	Planctomycetales	GCA GCC ACC CGT AGG TGT	338-355	30	46/46	Daims *et al.* (1999)
EUB338-III	Verrucomicrobiales	GCT GCC ACC CGT AGG TGT	338-355	30	46/46	Daims *et al.* (1999)
DSS658	*Desulfosarcina / Desulfococcus*	TCC ACT TCC CTC TCC CAT	658-685	60	46/46	Manz *et al.* (1998)
660[e]	*Desulfobulbus*	GAA TTC CAC TTT CCC CTC TG	660-679	60	46/46	Devereux *et al.* (1992)
NON338	Negative control	ACT CCT ACG GGA GGC AGC	338-355	20	46/46	Wallner *et al.* (1993)
ARCH915	Most Archaea	GTG CTC CCC CGC CAA TTC CT	915-935	30	46/46	Raskin *et al.* (1994)
ANME1-350	ANME-1 archaea	AGT TTT CGC GCC TGA TGC	350-367	40	46/46	Boetius *et al.* (2000)
ANME2a-647	ANME-2 archaea	TCT TCC GGT CCC AAG CCT	647-664	55	46/46	Knittel *et al.* (2005)
ANME3-1249	ANME-3 archaea	TCG GAG TAG GGA CCC ATT	1250-1267	40	46/46	Niemann *et al.* (2006)
ANME3-1249H3	helper probe for ANME3-1249	GTC CCA ATC ATT GTA GCC GGC	1229-1249	40	46/46	Lösekann *et al.* (2007)
ANME3-1249H5	helper probe for ANME3-1249	TTA TGA GAT TAC CAT CTC CTT	1268-1288	40	46/46	Lösekann *et al.* (2007)

[a] *E.coli* positions. rRNA (ribosomal RNA).

[b] Formamide (FA) concentration in the hybridization buffer.

[c] Hybridization temperature.

[d] Washing temperature.

[e] This probe was only applied to samples of the core VKGD276/PC-13.

Chapter 6

Filamentous Sulfur Bacteria, *Beggiatoa* spp., in Arctic Marine Sediments (Svalbard, 79°N)

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Abstract

Eleven fjord sediments on the west coast of the arctic archipelago Svalbard were surveyed, to understand whether large filamentous sulfur bacteria of the genus *Beggiatoa* can thrive at seawater temperatures permanently near the freezing point. Two of the studied sediments had abundant populations of *Beggiatoa* while at six sites only sporadic occurrences were observed. We conclude that *Beggiatoa*, although previously unnoticed, occur widespread in these arctic fjord sediments. The *Beggiatoa* ranged in diameter from 2 to 52 μm and, by those tested, stored nitrate in vacuoles at up to 260 mM concentration. Further, the 16S rRNA gene sequence of a 20 μm wide filament is closely associated with other large, marine, nitrate-storing *Beggiatoa*. The *Beggiatoa* mostly occurred in the upper 2-5 cm of the oxidized surface sediment between the oxygen and the deeper sulfidic zone. In spite of very low or undetectable sulfide concentration, sulfate reduction provided abundant H_2S in this zone. However, *Beggiatoa* only accounted for a small fraction of the sulfide removal. The total living biomass of *Beggiatoa* filaments at one study site was high and varied over three years between 1.13 and 3.36 g m^{-2}. Due to their large size, *Beggiatoa* accounted for up to 15% of the total prokaryotic biomass, even though filament counts at this site were rather low comprising <1/10.000 of the bacterial numbers on a cell basis.

1. Introduction

Filamentous, sulfide-oxidizing bacteria of the genus *Beggiatoa* occur widespread in marine surface sediments that have a sufficiently high production of sulfide from bacterial sulfate reduction. Such sediments occur in the eutrophic coastal zone and in highly productive upwelling systems along continental margins, often associated with low oxygen concentration in the bottom water. *Beggiatoa* spp. also occur as benthic mats in many other marine environments where sulfide is introduced by advective flow, e.g. at cold seeps or at hydrothermal vents (Jørgensen & Boetius, 2007).

Beggiatoa spp. are most often noticed when growing as white mats on the seafloor, yet the most widespread occurrence is probably as scattered filaments hidden within the uppermost few cm of the sediment. This more diffuse distribution requires a designated approach to find and quantify the organisms, although they are conspicuous as they have the size of micro- or meiofauna. It is a property unique for these large, multicellular bacteria that they can be identified and counted by simple light microscopy. By that approach it has been demonstrated that *Beggiatoa* generally occur in the 'suboxic zone' between the few mm thick oxic zone at the sediment surface and the diffusion front of sulfide starting several cm below (Jørgensen, 1977; Mußmann *et al.*, 2003; Jørgensen & Nelson, 2004; Preisler *et al.*, 2007).

Beggiatoa display tactic responses that enable them to avoid both increasing oxygen and increasing sulfide concentration (Møller *et al.*, 1985; Preisler *et al.*, 2007). The microaerophilic organisms only accumulate at the sediment surface when the population density is sufficient high to create a steep oxygen gradient in the diffusive boundary layer, thereby ensuring low oxygen concentration at the exposed mat surface (Jørgensen & Revsbech, 1983). It was proposed that a prerequisite for the sub-surface occurrence of *Beggiatoa* in sediments is the presence of a distinct sulfide zone which prevents filaments from getting lost at depth without a chemotactic clue (Preisler *et al.*, 2007).

The predominant *Beggiatoa* populations in marine sediments are filaments with diameters of several μm to several tens of μm. Filaments of these size groups were found to accumulate nitrate in internal vacuoles, often up to concentrations of several hundred mM (McHatton *et al.*, 1996; Ahmad *et al.*, 1999; Mußmann *et al.*, 2003; Preisler *et al.*, 2007). *Beggiatoa* use nitrate as an alternative electron acceptor when living in the suboxic

zone. Where studied, nitrate is not denitrified but is rather reduced to ammonium (McHatton, 1998). Internal nitrate accumulation and reduction to ammonium is a property shared with other large, marine sulfur bacteria of the genera *Thioploca* with whom *Beggiatoa* form a monophyletic group (Otte *et al.*, 1999; Jørgensen & Nelson, 2004; Jørgensen *et al.*, 2005b).

In coastal environments *Beggiatoa* have so far mostly been observed under temperate conditions where the organic sedimentation is high and where experimental measurements of sulfate reduction demonstrate high sulfide production rates. In these moderately warm sediments *Beggiatoa* have gliding motility and express highly developed phobic responses to oxygen and sulfide. At temperatures above 5-10°C the filaments glide at a velocity of 2-4 μm s^{-1} and effectively migrate within their preferred environmental zone (Dunker *et al.*, submitted). Coastal sediments of the Arctic and Antarctic have temperatures permanently near 0°C which may require a psychrophilic adaptation of the indigenous *Beggiatoa* communities, not only of their metabolic rate but also of their gliding motility, in order to metabolize and orient effectively. Such a psychrophilic adaptation has been demonstrated for arctic communities of sulfate reducing bacteria (Knoblauch *et al.*, 1999; Knoblauch & Jørgensen, 1999) but was not yet described for the large, sulfide oxidizing bacteria such as *Beggiatoa*. The present study therefore aimed to explore the occurrence of *Beggiatoa* spp. in arctic sediments with respect to their distribution, size spectrum and biomass and to understand whether these large bacteria are equally well adapted to permanently cold environments as to temperate environments. Concurrent studies of the temperature regulation of their metabolic rate, expressed through their gliding velocity, will be published elsewhere (Dunker *et al.*, submitted).

2. Materials and Methods

2.1 Field sampling and sediment properties

Sediment was collected from different fjords on the west coast of Spitsbergen, the main island of the archipelago Svalbard bounding the North Atlantic and the Arctic Ocean. The location and relevant characteristics of these sites are given in Fig. 1 and Table 1. All

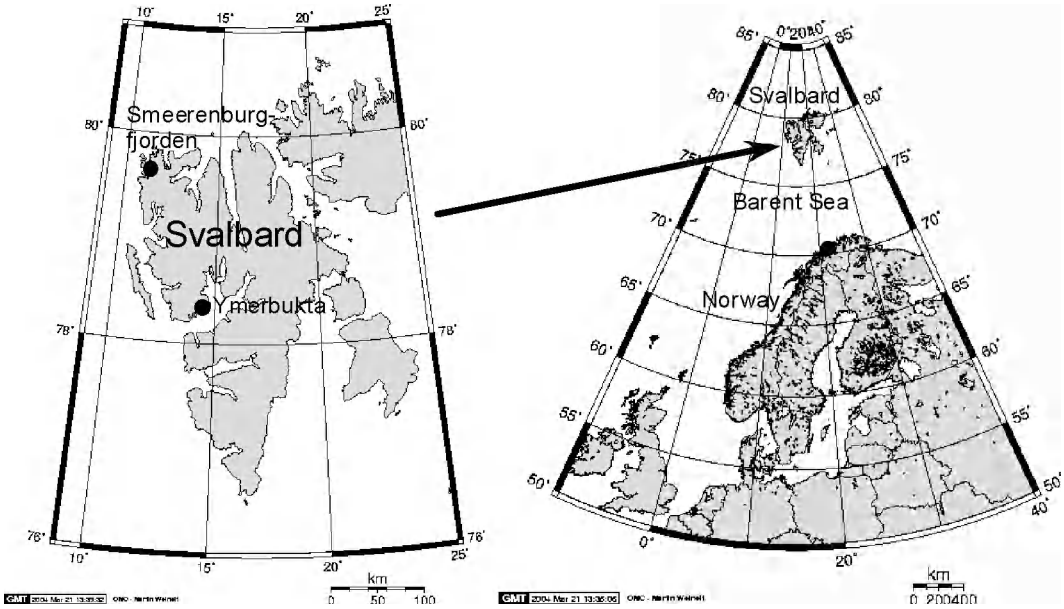

Fig. 1: Map of Svalbard with sampling sites.

sites were located between 78° and 80° northern latitude and, apart from a small lagoon, had temperatures permanently near 0°C. In several of the 70-200 m deep Spitsbergen fjords, cores of 15 cm diameter and 20-30 cm depth were collected with a HAPS corer (Kanneworff & Nicolaisen, 1973) during cruises with MS FARM (Longyearbyen). Sub-cores were taken immediately on board using 26 mm or 36 mm inner diameter acrylic tubes. The cores were kept submerged in seawater at ca 0°C during the following 1-3 days on the ship. Back in the laboratory, cores were transferred to an incubator at 0.5 ±0.5°C until further sub-sampling within 1-2 days.

Porosity, density, and ignition loss of the sediments were determined at 2 cm depth intervals. Core segments of defined volume were transferred to pre-weighed crucibles and the sediment weight was determined before and after drying at 105°C overnight. Porosity was determined from the water loss per volume of sediment upon drying. Porosity data were used for the calculation of sulfate reduction rates.

Table 1: Sampling sites visited during field campaigns in 2003, 2005 and 2008. A general description of the sediment and an indication for the occurrence of *Beggiatoa* are provided. Stations are all located in fjords on the west and north coast of the island Spitsbergen. Water depths and sediment temperature at the time of sampling are indicated. Areal rates of sulfate reduction (SRR) were integrated over the top 0-15 cm of the sediment and represent the mean of three cores. Two numbers are given when measurements were conducted during different years.

Station	Location	Coordinates		Depth (m)	Temp. (°C)	SRR (mmol m^{-2} d^{-1})	Description
J	Smeerenburgfjorden	79°42'815N	011°05'189E	214	0.4	2.71	Light gray until 2-4 cm, dark gray below, silt and very fine sand, rich in macrofauna Many *Beggiatoa* of different diameters
DΛ	Ymerbukta	78°16'61N	014°02'69E	0.3	6.5	2.04*	Light brown until 2-4 cm, silt with fine sand of 30-80 µm, gray to black fine sand below, patches of *Beggiatoa* or cyanobacteria overlying black sediment Many *Beggiatoa* of mostly 5 and 12 µm
A	Adventfjorden	78°15'44N	015°30'90E	69	0.4	4.48	Gray-brown until 5-6 cm, mottled gray below; silt and fine sand of 30-100 µm diameter, not very sulfidic No *Beggiatoa*
O	Borebukta	78°19'557N	014°27'760E	94	1.4	2.50/1.59	Gray-brown until 3 cm, gray below, silt and very fine sand of <30 µm One *Beggiatoa* filament of 6 µm on top
BN	Ymerbukta S	78°15'352N	013°58'417E	104	0.3	1.99	Gray-brown until 4 cm, gradually into gray below, silt-clay, no sand, pelletized Three *Beggiatoa* of 15-20 µm in top 2 cm
E	St. Jonsfjorden W	78°32'599N	012°17'909E	168	1.6	1.87	0-3 cm gray-brown, gray below, silt and fine sand One *Beggiatoa* filament of 4 µm at 2 cm
F	Kongsfjorden	78°55'234N	012°13'912E	114	1.8	1.33/1.11	Silty sediment colored red from hematite Few scattered *Beggiatoa* of 7 and 20 µm
S	Blomstrandhalvoya N	78°59'929N	011°59'213E	77	0.0	2.40	Red-gray, with depth dark gray mottled in red, silt and fine sand of 50-150 µm Few *Beggiatoa* of 4 and 7 µm in top 2 cm
R	Blomstrandhalvoya S	78°57'424N	012°09'873E	90	0.0	1.32	Red, with depth gray mottled in red, silt and very fine sand of <30 µm No *Beggiatoa*
K	Raudfjorden	79°46'144N	012°04'433E	154	-0.5	2.09	Gray-brown to 6 cm, mottled gray below, silt No *Beggiatoa*
I	Magdalenefjorden	79°34'052N	011°03'597E	124	-1.0	1.87	Gray-brown to 5 cm, mottled gray below, silt Three *Beggiatoa* of 18-20 µm

*Data from Sawicka *et al.*, in press, measured in 2007.

2.2 Biomass determination

Sediment sub-cores were sectioned into 0.5 cm depth increments. Subsamples of 20-30 mg were taken with a clean mini-spatula, transferred to a tarred microscope slide and weighed to ±0.2 mg accuracy. The sediment was immediately wetted with drops of seawater, mechanically suspended and smeared over the surface of the slide and finally covered with a large cover slip. *Beggiatoa* filaments were quantified by direct light microscopy according to Jørgensen (1977) by scanning the entire slide systematically using the 10× objective. The length (10 ×) and diameter (40 ×) of all detected filaments were measured by using a calibrated ocular micrometer. Only motile, colorless, multicellular filaments with distinct sulfur inclusions were counted as *Beggiatoa*. Some *Beggiatoa*, in particular those of smaller diameters, may thereby have been missed and the counts thus represent minimum values.

Beggiatoa filaments were also quantified by a modified approach whereby a weighed sediment sample of ca 0.5 g was added to 10 ml seawater and suspended. A 0.35 g subsample was then smeared on a glass slide and the filaments counted. This procedure improved the statistical representation of the bacterial density in case these had a patchy distribution. Determination of length and diameter was also done by digital photography using imaging software (ImageTool, The University of Texas Health Science Center, San Antonio, USA),

For quantification, diameters were grouped in 2-3 μm increments. Based on the mean dimensions of each diameter group, *Beggiatoa* biomass was calculated using the cylinder volumes of the filaments and assuming a density of 1 g cm^{-3}. All population data were recalculated from gram wet weight to volume (cm^3) of sediment based on determined sediment densities. Adding up the data over the entire depth interval revealed the population size as fresh biomass per sediment surface area (g m^{-2}).

2.3 Phylogenetic analyses

Single filaments intended for phylogenetic analyses were stored separately at -20°C, preserved in 1 × TE buffer (Promega Corporation, Madison, WI). Prior to PCR amplification, each filament was separated from the TE buffer by centrifugation for 3 min at 5000 × *g* and dissolving in 5 μL of PCR-grade water (Sigma-Aldrich Biochemie GmbH, Hamburg, Germany). The complete volume of 5 μL was then used as template in the following amplification reaction. Universal bacterial primers GM3F (5'-AGAGTTTGATCMTGGC-3'; Muyzer *et al.*, 1995) and GM4R (5'-TACCTTGTTACGACTT-3'; Muyzer *et al.*, 1995) were used for amplification of nearly full length 16S rRNA gene sequences. Amplification reactions were set up as follows: 1 × MasterTaq Buffer with 1.5 mM Mg^{2+}, 0.3 mg mL^{-1} BSA (Sigma-Aldrich Biochemie GmbH), 250 μM of each dNTP (Roche, Mannheim, Germany), 0.5 μM of each primer (Biomers, Ulm, Germany) and 0.025 U μL^{-1} MasterTaq (5Prime, Hamburg, Germany) in a total volume of 50 μL. After an initial denaturation for 15 min at 95°C, 30 cycles of 95°C/1 min, 42°C/1 min and 72°C/3 min were performed before a final elongation step at 72°C for 10 min.

PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), ligated into pGEM-T Easy vector (Promega Corporation) and

transformed into One Shot TOP10 Chemically Competent *E. coli* (Invitrogen Corporation, Karlsruhe, Germany) according to the manufacturer's instructions. Three representative clones were selected for plasmid preparation (Montage Plasmid Miniprep$_{HTS}$ Kit; Millipore GmbH, Schwalbach, Germany). Purified plasmids were subjected to *Taq* cycle sequencing with an ABI Prism 3130x Genetic Analyzer (Applied Biosystems, Foster City, CA).

Partial sequences were assembled with Sequencher 4.6 software (Gene Codes Corporation, Ann Arbor, MI) and manually checked. Examination for chimeric signals was done by using the Pintail program (Ashelford *et al.,* 2005) with nearest neighbors obtained with the SILVA-based Webaligner (http://www.arb-silva.de/aligner/; Pruesse *et al.*, 2007). No genuine chimeric signals were detected. All three sequences were nearly identical (similarity values of 99.5-99.7%). Finally, clone S3678 was chosen for further phylogenetic analyses.

A phylogenetic tree was constructed with the neighbor joining and maximum likelihood (RAxML) algorithm, as included in the ARB software package (Ludwig *et al.*, 2004). Initial calculations were conducted with nearly full length sequences (≥ 1200 bp) and by applying different filters. Partial sequences L41043, L40999 and AF129012 were subsequently inserted by applying parsimony criteria and without allowing changes in the overall tree topology. Deltaproteobacterial sequences were used as outgroup. A consensus tree based on the different reconstruction approaches was built, wherein unstable branching orders were visualized by multifurcation.

The sequence of the uncultured *Beggiatoa* sp. clone S3678 from Smeerenburgfjorden (Station J) has been submitted to the EMBL database under accession no. FN561862.

2.4 Chemical measurements

Sediment sub-cores were sectioned in 1 cm depth increments and pore water was obtained by squeezing through 0.45 μm pore size membrane filters under N_2 according to Reeburgh (1967). Sulfate was measured by non-suppressed ion chromatography (Waters IC with conductivity detector). A subsample was diluted 30-50-fold in double distilled water and membrane-filtered just before analysis.

Profiles of O_2, H_2S and pH were measured with microsensors in cores mounted in a mini-flume which provided a constant temperature of 0°C and a water flow of 1-2 cm s^{-1}

at 1 cm above the sediment surface (Jørgensen *et al.*, 2005). O_2 and H_2S were measured with Clark-type microelectrodes. The O_2 electrode had an internal reference and a guard cathode (Revsbech, 1989). The tip size of the O_2 sensors was 10-20 μm, the stirring sensitivity <1% and the 90% response time ~1 s. A two-point calibration was made by positioning the sensor in air-saturated seawater and in anoxic sediment. The H_2S electrode (Jeroschewski *et al.*, 1996) had a tip diameter of 20-50 μm, stirring sensitivity <2 %, and a 90 % response time of ~3 s. The H_2S electrode was calibrated in anoxic, stirred 0.2 M phosphate buffer at pH 7.5 to which appropriate volumes of 100 mM sodium sulfide solution were added. The electrode current was read after each addition and a subsample of the calibration solution was fixed in 2% Zn-acetate for later photometric analysis (Cline, 1969). Total sulfide was calculated from the H_2S concentrations using the sediment pH and a pK_1 of 6.87 as calculated from the seawater salinity and temperature of the incubated cores (Millero et al., 1988). The pH was measured with freshly filled liquid ion exchange microsensors (de Beer *et al.*, 1997), calibrated with standard pH buffers.

With the help of a dissection microscope, all sensors were positioned vertically above the sediment surface. Data acquisition started above the sediment and the electrodes were moved downwards in 100 μm steps by using a computer-controlled motor (Faulhaber, Germany). The O_2 and H_2S electrode currents were read by a pA-meter and the pH potential was determined with a mV-meter. Data were transferred to an A-D converter (National Instruments, USA) and stored on a laptop computer.

For the determination of nitrate concentrations in *Beggiatoa* vacuoles, 50-100 filaments were picked for each analysis with a clean glass needle into 1 mL of a NaCl-solution isotonic to seawater. After gentle centrifugation, 0.9 mL of the supernatant water was collected separately. Water and *Beggiatoa* samples were acidified at pH 1 with 6 M HCl and stored frozen. Three freeze-thaw cycles between liquid N_2 and 90°C warm water ensured breakage of the vacuoles and release of the nitrate. Nitrate was analyzed with a Chemoluminescence NO/NOx Analyser (Eco Physics, Germany) with the isotonic NaCl solution as control. The supernatant samples were also analyzed to check for nitrate leakage before freezing. The mean biovolume of *Beggiatoa* filaments was determined by photographing at random individual filaments (n=73) from a bulk sample of the mat. The length and width of each photographed filament was determined with

calibrated image analysis software (ImageTool). The mean biovolume was used to calculate the internal nitrate concentration.

2.5 Sulfate reduction rates

Sulfate reduction rates (SRR) were measured by whole core injection using ^{35}S-labelled sulfate (Jørgensen, 1978). At each 1 cm depth interval, 2 µl carrier-free $^{35}SO_4^{2-}$ tracer (~100 kBq) was injected and the core was incubated for 8-12 h at *in situ* temperature. Sulfate reduction was stopped by mixing 1 or 2 cm depth sections with 10 ml cold Zn-acetate (20% w/v) and freezing. The samples were later treated with cold chromium distillation according to Kallmeyer *et al.* (2004). Sulfate reduction rates were calculated according to Jørgensen (1978)(1978):

$$SRR = [sulfate] \times (^{35}S\text{-}CRS/^{35}S\text{-}sulfate) \times (1.06/t)\ nmol\ SO_4^{2-}\ cm^{-3}\ d^{-1} \quad (Eq.\ 1)$$

where [sulfate] is the sulfate concentration in nmol cm^{-3} of wet sediment, ^{35}S-CRS is the radioactivity of total reduced sulfur at the end of the incubation, ^{35}S-sulfate is the initial radioactivity of sulfate added to the experiment, 1.06 is a correction factor for the expected isotope discrimination against ^{35}S-sulfate versus the bulk ^{32}S-sulfate by the sulfate-reducing bacteria, and t is the incubation time measured in days.

3. Results

Filamentous bacteria observed and quantified microscopically in sediments from Svalbard fjords were recorded as belonging to the genus *Beggiatoa* based on the following criteria (Strohl, 2005): A) The filaments were freely motile and were not surrounded by a visible sheath common to several filaments. B) The filaments ranged in diameter from 2 to 50 µm, were multicellular, and had rounded, never tapered, terminal cells. C) The cells were always rich in light-refracting, spherical sulfur globules. In the wider filaments, with diameters >5 µm, sulfur globules were distributed in the periphery of the individual cells,

typical of the morphology of *Beggiatoa* spp. containing nitrate vacuoles. Filaments of similar appearance, but devoid of sulfur globules, were not counted.

3.1 Occurrence of *Beggiatoa* around the arctic archipelago Svalbard

We made a survey of *Beggiatoa* in fjords on the west and north coast of the main Svalbard island, Spitsbergen. Sediment cores were sampled, generally in the deeper and central part of the fjords, and the cores were screened for *Beggiatoa* using the described approach. For each site, a total of 5-10 samples of ca 30 mg wet sediment each were screened from the oxidized surface zone. Thus, a total of 150-300 mg sediment was completely screened per station. The results are summarized in Table 1.

The main conclusion from this survey was that only two out of eleven investigated sites, i.e. Smeerenburgfjorden and Ymerbukta, had abundant *Beggiatoa* during all three sampling years. At six sites, only one or a few *Beggiatoa* were found with diameters mostly ranging from 4 to 20 μm. At the remaining three sites no *Beggiatoa* were found. Given the volume of sediment screened, those sites had statistically <3-6 filaments cm^{-3}, if any.

3.2 Filament frequency and dimensions

During the entire study, close to one thousand *Beggiatoa* filaments were counted and measured. In Fig. 2 the frequency distribution and mean dimensions are shown after an arbitrary classification of all filaments into different diameter groups. When compiling data from all locations and years, there was an almost continuous variation in diameters but a strong variation in their frequency (Fig. 2A). The two narrowest size classes, 2-3 μm and 4-5 μm, were the most abundant and comprised 77% of all *Beggiatoa* filaments counted. Filaments narrower that 2 μm were not found, although they were particularly searched for in several of the sediment samples. There were notably many *Beggiatoa* with diameters of 11-22 μm. A small number of very wide filaments of >23 μm diameter were also found. The widest filament encountered was 52 μm in diameter. The mean filament length of each diameter class increased with the filament width (Fig. 2B). The narrowest filaments of 2-5 μm had a mean length of 0.25 mm while the widest were about 1.5 mm long. The longest individual filaments were >3 mm in length.

The biomass (living wet weight) of individual filaments was calculated based on their measured volume, assuming a density of 1 g cm^{-3}. It should be noted that a large part of

this biomass is not active cytoplasm, but is comprised of vacuoles inside the cells. As shown in Fig. 2C, the mean biomass of whole filaments increased >1000-fold from 1.3 ng of the 2-3 μm class to 1500 ng of the 30-50 μm class. Due to the large difference in biomass per filament between narrow and wide diameters (Fig. 2C), the widest filaments often dominated the biomass in spite of their low numbers. Typically, the 5% widest filaments comprised 50% of the total *Beggiatoa* biomass. It is striking that, in spite of the small mean individual filament lengths ranging from 0.25 to 1.5 mm, the accumulated length of all filaments reached 50 cm per cm^3 of sediment and was typically a few tens of cm per cm^3.

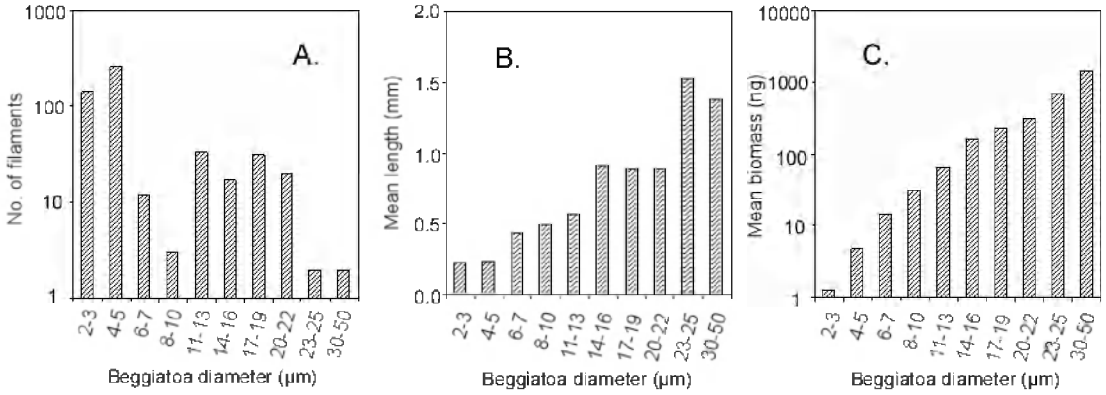

Fig. 2: Size frequency and dimensions of all *Beggiatoa* measured. A) Frequency distribution of filament diameters. B) Mean filament lengths of different size groups. C) Mean fresh biomass of different size groups. Notice log scales in frames A) and C).

There was a rather constant ratio between filament (i.e. cell) diameter and cell length (i.e., the height of the cylinder-shaped individual cells) for the different size classes. The narrow filaments had longer cylindrical cells than the wide filaments which had flatter disk-shaped cells. Thus, among the 2-5 μm wide filaments the mean cell diameter was 3.7±1.1 μm and the mean cell length was 5.4±0.4 μm. Among the 17-22 μm wide filaments the mean cell diameter was 19.2±0.5 μm and the mean cell length was 9.0±1.2 μm. The mean volume of the two size classes of cells was 18 μm^3 and 830 μm^3, respectively, i.e. 100 and 5000-fold larger than the mean size of other sediment bacteria with a mean volume of 0.2 μm^3. The mean number of cells in each filament was 50 cells

for the 2-5 μm size class and 160 cells for the 17-22 μm size class. The latter, multicellular filaments were thereby close to a million-fold larger than the mean size of other sediment bacteria.

3.3 Smeerenburgfjorden *Beggiatoa* community

Station J was situated in central Smeerenburgfjorden at 214 m water depth (Table 1) and was particularly rich in *Beggiatoa*. The station was sampled during three different summers in 2003, 2005 and 2008. The Smeerenburgfjorden is a channel on the north-west coast of Spitsbergen and connects to the ocean both towards the west and the north. The sediment was a silty mud mixed with fine sand and rich in burrowing macrofauna, particularly in tube-building polychaetes. The upper 2-4 cm of sediment was oxidized and light gray to brown in color while the sediment below was light to dark gray and black due to iron sulfides with a mottled appearance due to heterogeneity caused by ventilation and mixing by the fauna.

During sampling in 2003 and 2005 *Beggiatoa* occurred until 2.5 cm depth in the oxidized surface sediments (Fig. 3). *Beggiatoa* filaments with a narrow size range of 2-2.5 μm width dominated in numbers in 2005 and made up 70% of all *Beggiatoa* counted there. In the more sulfidic (based on coloration) of two sediment cores analyzed in 2005, nearly all of these narrow filaments were found in the top 0-5 mm while the wider filaments of >5 μm mostly occurred sub-surface at 0.5-2.5 cm depth (Fig. 3A). Filament numbers reached 1600 cm^{-3} for the 2-2.5 μm group and 500 cm^{-3} for the >5 μm wide group. Due to the large size difference, however, the >5 μm Beggiatoa completely dominated the biomass (Fig. 3B). Comparison with 2003 data shows a high reproducibility in biomass distribution between these two years (Fig. 3C).

In order to understand the parameters controlling *Beggiatoa* distribution, high-resolution microprofiles of oxygen, sulfide and pH were recorded in 2008 in sediment cores retrieved from Station J. The oxidized zone was deeper that year, 5 cm judging from sediment color. The primary oxygen front penetrated only 1-2 mm into the sediment (Fig. 4A). Free sulfide (total H_2S) was detected from 2 mm depth and downwards at very low concentration, reaching 3 μM at 2-3 cm depth. The pH was 7.9 in the overlying seawater and showed a sharp minimum of 7.4 at the oxic-anoxic interface at 1.5 mm. A broad pH maximum of 8.0 occurred in the middle of the oxidized

zone. Although free H_2S was hardly detectable, sulfate reduction took place throughout the sediment reaching maximum values of up to 17 nmol cm^{-3} d^{-1} in 2-3 cm depth (Fig. 4B). *Beggiatoa* were observed throughout the oxidized zone with high abundances and biomass down to 4.5 cm (Fig. 4C).

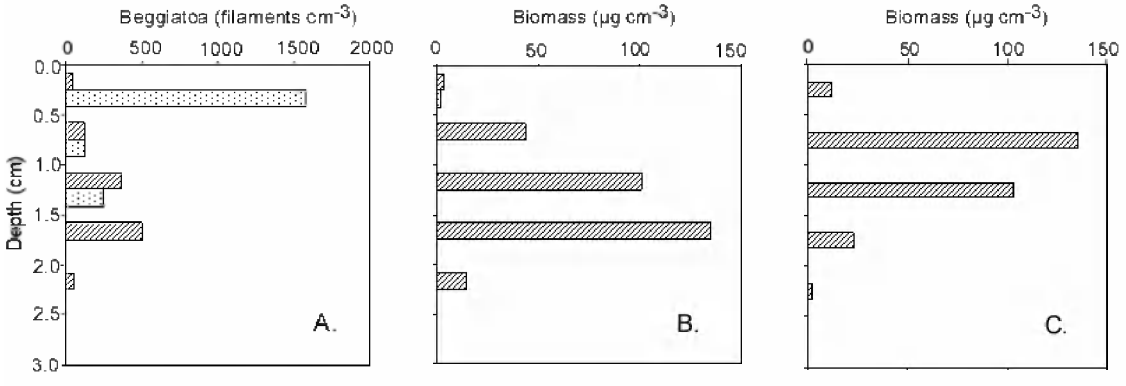

Fig. 3: Depth distribution of *Beggiatoa* in sediment of Station J, Smeerenburgfjorden, during two sampling seasons. A) Numbers of filaments in 2005. B) Biomass of filaments in 2005. In A) and B) dotted bars show 2-2.5 µm wide filaments while hatched bars show >5 µm wide filaments. C) Biomass of filaments in 2003.

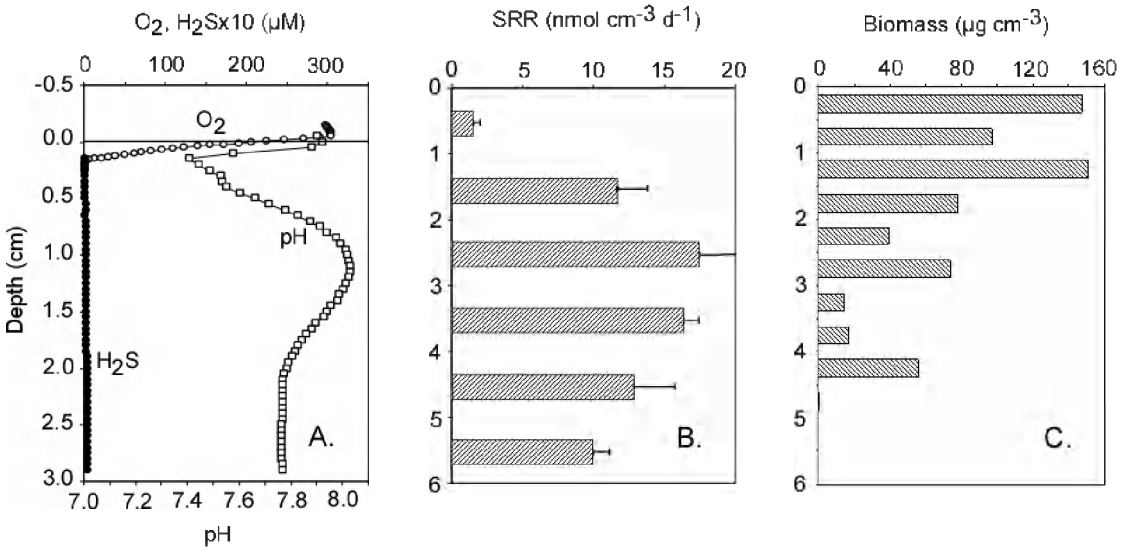

Fig. 4: Station J, Smeerenburgfjorden, in 2008. A) Microsensor measurements of O_2, total H_2S and pH. B) Sulfate reduction rates (SRR) measured by ^{35}S-tracer technique (depth resolution 1 cm). C) Depth distribution of *Beggiatoa* wet biomass. Note difference in depth scales.

The maximum biomass of *Beggiatoa* varied between the three years from 70 to 150 μg cm^{-3} (Fig. 3 and 4). The total biomass of *Beggiatoa* (in g of wet biomass per m^2) was 1.38 g m^{-2} in 2003, 1.13 g m^{-2} in 2005 (mean of two cores with 0.76 and 1.49 g m^{-2}, respectively) and 3.36 g m^{-2} in 2008.

3.4 Ymerbukta *Beggiatoa* community

The other site visited during all three years was a protected lagoon in Ymerbukta on the north coast of Isfjorden (Station DA, Table 1). Due to the shallow water depth, 20-30 cm, and the 24 h of daylight during summer, the sediment surface was relatively warm, 6-7°C at the time of sampling. The sediment was mixed silt and fine sand of mostly 20-80 μm grain size and was light brown and oxidized to 4 cm depth. The sediment below was grey to black fine sand.

Beggiatoa occurred throughout the 4 cm deep oxidized zone (Fig. 5) and two distinct size classes prevailed. As in Smeerenburgfjorden, there was a distinct difference in the depth distributions of narrow and wide *Beggiatoa*. There was a predominance of *Beggiatoa* filaments with a narrow size range of 5±0.5 μm width which comprised 90% of all filaments counted at this site. Most of the wider *Beggiatoa* were 12 μm in diameter. The narrow 5 μm *Beggiatoa* had highest density at 1-2 cm depth while the wider 12 μm *Beggiatoa* had maximum at 2-3 cm depth (Fig. 5A). Due to their much larger individual filament size, the biomass of the 12 μm filaments was overall highest (Fig. 5B). The mean areal biomass was 0.167 g m^{-2} of which the 5 μm wide filaments accounted for 40%.

On the sediment surface were also scattered patches of white mats of *Beggiatoa*, typically a few hundred cm^2 in size and dominated by 2 μm and 8-10 μm wide filaments. Some patches were green to blue-green and were dominated by *Oscillatoria*-like cyanobacteria with filaments of mostly 18 or 25 μm in diameter. Interestingly, these filamentous cyanobacteria often occurred down to >4 cm depth in the sediment. The cyanobacterial patches also contained many *Spirulina*-like cyanobacteria, some *Beggiatoa*, and different pennate diatoms. The sediment beneath the white and green patches was gray to black and highly sulfidic.

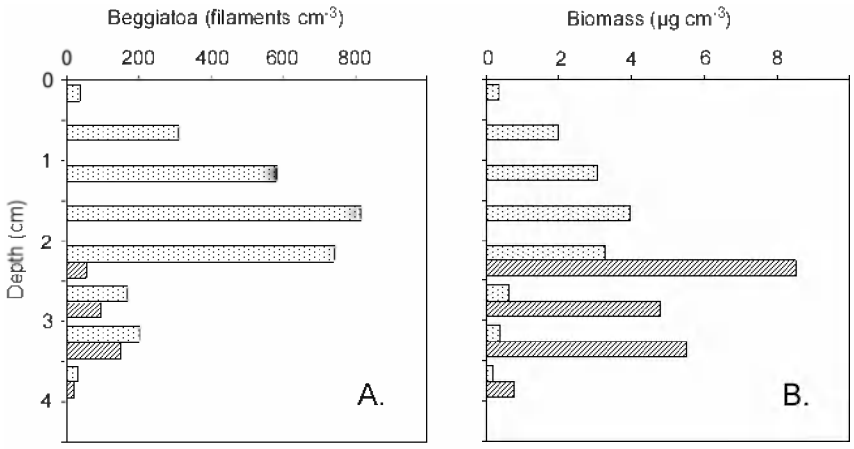

Fig. 5: Depth distributions of *Beggiatoa* in sediment at Station DA, Ymerbukta lagoon, 2005. A) Numbers of filaments per cm³ sediment. B) Biomass of filaments per cm³ sediment. Dotted bars: 5 µm filaments. Hatched bars: >5 µm filaments. Mean of two cores.

In a studied patch with *Beggiatoa* mat on the surface oxygen penetrated only to 0.4 mm depth (Fig. 6A), while the penetration depth just outside the visible *Beggiatoa* mat was 0.8 mm (Fig. 6B). In both cases, sulfide overlapped a few hundred µm with oxygen but did not reach the sediment surface. The *Beggiatoa* mat was only about 1 mm thick and covered the O_2-H_2S interface and the uppermost front of the sulfide zone. Maximum sulfide concentration was found at 1 cm depth and was twice as high below the *Beggiatoa* mat (1100 µM, Fig. 6A) as just outside the mat (600 µM, Fig. 6B). These near-surface peaks of sulfide were extremely high and indicated intensive sulfate reduction driven by a high pool of organic matter in the sediment. Accordingly, we found abundant remains of decomposing macroalgae buried just under the sediment surface, probably brought into the lagoon during storms and covered by fine-grained sediment during calm weather. The steep decrease in sulfide below the peak indicates that a large pool of reactive Fe(III) was also mixed into the sediment and was precipitating different iron-sulfide minerals and, thus, causing the black color of the sediment.

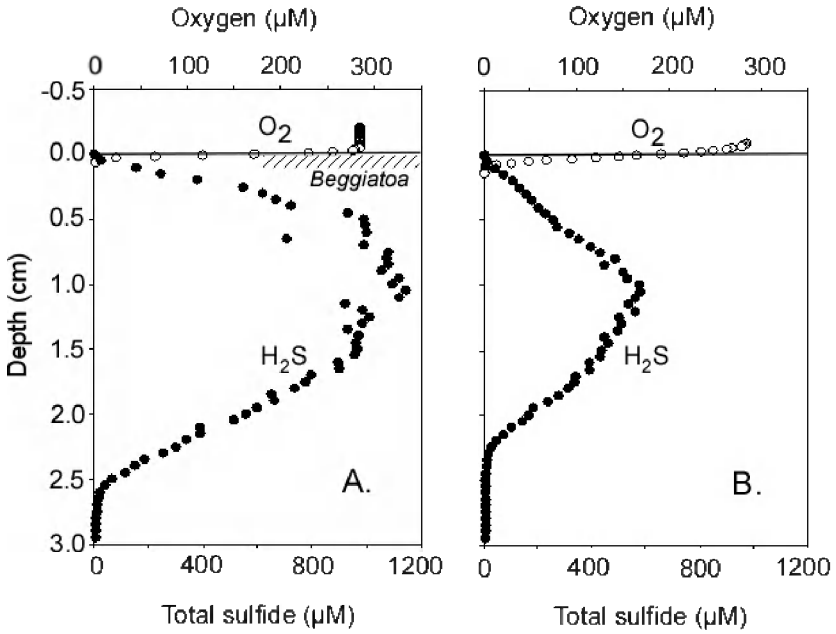

Fig. 6: Oxygen and sulfide (total H_2S) profiles in sediment from a shallow lagoon in Ymerbukta (Station DA). A) Patch of white *Beggiatoa* mat. B) Sediment just outside of the visible *Beggiatoa* mat.

3.5 Nitrate-accumulating arctic *Beggiatoa*

Beggiatoa filaments of different diameters were used to either measure the internal nitrate concentration or to determine their phylogenetic affiliation. Two parallel batches of 16-20 µm wide filaments from Station J in Smeerenburgfjorden contained 86 and 134 mM nitrate, respectively, while a batch of 13-15 µm wide filaments contained 260 mM nitrate. The mean nitrate concentration for these large *Beggiatoa* was thus ca 130 µM. In contrast, *Beggiatoa* of 8-10 µm diameter from a mat in Ymerbukta contained only 2.7 ±0.2 mM nitrate.

Phylogenetic analysis of 20 µm wide *Beggiatoa* from Smeerenburgfjorden (clone S3678; Fig. 7) revealed that the closest relatives include nitrate-storing *Beggiatoa* spp. from the brackish Limfjorden, Denmark (AF532775) and from an intertidal mud flat at Dangast, German Wadden Sea (AF532769). All these *Beggiatoa* are relatively large, with diameters between 9-17 µm, and accumulate nitrate in intracellular vacuoles (Mußmann *et al.*, 2003). Information on possible nitrate-storage in the closest relative, a marine,

uncultured *Beggiatoa* from Tokyo Bay (AB108786), was not given by the original investigators, but seems likely due to the presence of an internal vacuole (Kojima & Fukui, 2003).

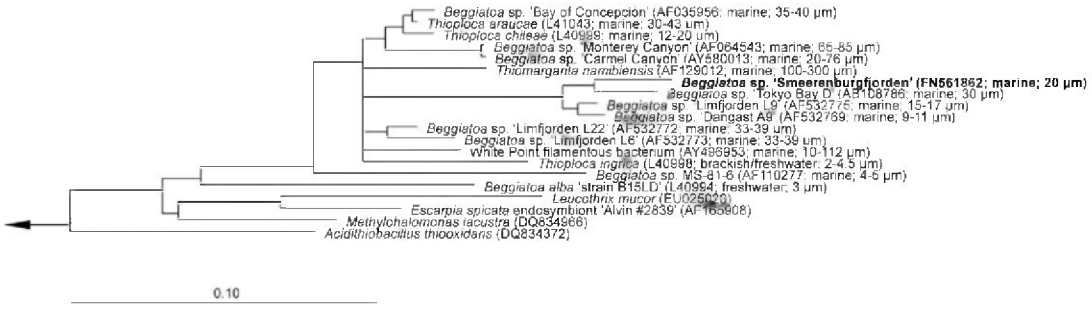

Fig. 7: Phylogenetic 16S rRNA gene based tree showing the affiliation of the uncultured *Beggiatoa* sp. clone S3678 from Smeerenburgfjorden in Svalbard (FN561862, indicated by bold type) to selected reference sequences within the Gammaproteobacteria. Following each accession number, information on the general habitat (marine/freshwater) and cell diameter is given in parentheses. The bar indicates 10% estimated phylogenetic divergence.

4. Discussion

The fjords of Svalbard provide some of the most extreme arctic, coastal sediments, given the latitudes ranging between 78° and 80° N, continuous daylight in summer for 3-4 months and corresponding dark night in winter, and seawater temperatures always near the freezing point. Our study demonstrates for the first time the widespread, yet scattered occurrence of large filamentous sulfur bacteria, *Beggiatoa* spp., in such permanently cold sediments. An earlier study of a conspicuous white bacterial mat of unidentified Gammaproteobacteria in Young Sound on the NE coast of Greenland also reported the presence of *Beggiatoa*, however in very low numbers (Glud *et al.*, 2003).

The occurrence of *Beggiatoa* is best known from sites where sulfide reaches the sediment surface and where thin, visible mats are formed at the narrow oxygen-sulfide interface (Jørgensen & Revsbech, 1983). Previously, white mats of sulfur bacteria have been observed in the cold deep-sea and also in the Arctic. These mats are, however,

often associated with seep systems where sulfidic pore fluid emerges at the sediment surface. A remarkable example is the Håkon Mosby mud volcano in the deep North Atlantic west of Spitsbergen, where dense *Beggiatoa* mats have been observed at 1250 m water depth thriving at a temperature of -0.5°C (de Beer *et al.,* 2006; Niemann *et al*, 2006). It is therefore not a new finding that marine *Beggiatoa* can live at near-zero temperatures provided that sufficient sulfide is available. In this study, *Beggiatoa* mats on the sediment surface were only observed in the shallow lagoon of Ymerbukta where microbial sulfate reduction coupled to decomposing macroalgae just beneath the sediment surface provided a high sulfide flux from below. At most other stations *Beggiatoa* were found to thrive within the sediment at depths down to 2-5 cm.

4.1 Controls on *Beggiatoa* distribution

There is no indication that low temperature is directly inhibitory to the growth and distribution of *Beggiatoa.* The arctic communities of *Beggiatoa* are apparently cold adapted and at some sites large communities are able to thrive at temperatures permanently near the freezing point. Recent measurements of the gliding speed of 8-10 μm wide Svalbard *Beggiatoa* showed that these were moderately psychrophilic with optimum at 17°C. They continued their gliding motility even down to -5°C and immediately recovered, without motility loss, from a transient freezing at that temperature (Dunker *et al.,* submitted). Since the upper part of the lagoonal sediment in Ymerbukta freezes solid in winter, it is an important observation that both *Beggiatoa* and sulfate reducing bacteria from this environment survive freezing without or with only little detrimental effect on their metabolic rates (Dunker *et al.*, submitted; Sawicka *et al.,* in press; Mountford *et al.* 2003). By comparison, *Beggiatoa* from a sediment of the temperate North Sea were immobilized, and probably killed, by transient freezing, but survived freezing provided that the population had been cold adapted over a few months (Dunker *et al.*, submitted).

The availability of H_2S is a critical for *Beggiatoa* to establish a community in marine sediments. The sulfate reduction rates measured in the permanently cold Svalbard sediments, 1.3-4.5 mmol m^{-2} d^{-1} (Table 1), tend to be of similar magnitude as rates measured in temperate sediments (Canfield *et al.,* 2005; Jørgensen & Kasten, 2006). The key control on these rates is provided by the phytoplankton productivity in the water column and thus the deposition and burial of degradable organic matter in the sediment.

The fjords of Svalbard are relatively deep, typically 70-200 m, with steep rocky coasts and hardly any terrestrial organic material coming from the barren and extensively ice and snow-covered land. Yet, the primary productivity during the ice-free season is relatively high and the rates of microbial metabolism (oxygen uptake, metal reduction and sulfate reduction) in the sediments is correspondingly high (Glud *et al.,* 1998; Vandieken *et al.,* 2006; Arnosti & Jørgensen, 2006).

The most common, yet mostly unnoticed occurrence of marine *Beggiatoa* is within the top several cm of slightly oxidized sediment (Jørgensen, 1977). This zone is characterized by the abundance of oxidized iron minerals which provide the sediment with a light gray to brown color. The zone generally has no detectable sulfide, yet often a high rate of sulfate reduction. The produced sulfide is thus turned over immediately, mostly by reaction with oxidized iron minerals, but also by *Beggiatoa.* It is a prerequisite for the occurrence of *Beggiatoa* in this zone that the filaments are able to glide through the sediment. It has been noticed in temperate sediments that a mixture of silt and very fine sand may physically exclude *Beggiatoa* because gliding motility is not possible (Jørgensen, 1977).

The occurrence of visible *Beggiatoa* mats on the sediment surface was associated with buried macroalgae which were the source of intense sulfide production. The oxygen flux into the *Beggiatoa* mat was more than twice the oxygen flux just outside the *Beggiatoa* patch (61 and 24 mmol m^{-2} d^{-1}, respectively, Fig. 6). The corresponding sulfide fluxes were 34 and 13 mmol m^{-2} d^{-1}, respectively, yielding flux ratios of 2.1:1 (O_2:H_2S) in the mat and 1.8:1 outside the mat. This corresponds in both cases to a complete oxidation of sulfide to sulfate with oxygen which would imply a 2:1 stoichiometry:

$$2\ O_2 + H_2S \rightarrow SO_4^{2-} + 2\ H^+ \qquad \text{(Eq. 2)}$$

A flux ratio of 2:1 or slightly higher was also found by microsensor studies of a temperate *Beggiatoa* mat (Jørgensen & Revsbech, 1983). This stoichiometry is characteristic of a community in steady state where the *Beggiatoa* biomass or their elemental sulfur content is not increasing (Nelson *et al.,* 1986). The complete sulfide oxidation to sulfate outside of the *Beggiatoa* mat could be due to other, non-conspicuous sulfide oxidizing bacteria, for example of the *Thiobacillus* or *Thiomicrospira* group. A purely

chemical sulfide oxidation would expectedly not lead to a quantitative conversion to sulfate but rather to sulfur compounds of intermediate oxidation state, such as elemental sulfur or thiosulfate.

In the sediments where the oxygen and sulfide were separated by an intermediate oxidized zone inhabited by *Beggiatoa* (Fig. 4) the pH profile showed a sharp minimum near the oxygen front. Since there was no significant gradient of free sulfide, the pH minimum may be due to the oxidation of elemental sulfur to sulfate in *Beggiatoa* as proposed by Sayama *et al.* (2005). It may also be due to the re-oxidation of free Fe^{2+} produced by iron reduction in the intermediate zone. Preisler *et al.* (2007) concluded from a similar pH minimum in a *Beggiatoa* inhabited Baltic Sea sediment that the oxidant for Fe^{2+} was MnO_2 rather than O_2, both of which would produce excess H^+ and thus a pH minimum. On the contrary, other oxidation processes with MnO_2 involving pyrite, iron sulfide or organic matter consume H^+ and may generate the broad pH maximum in the oxidized zone (Preisler *et al.*, 2007)

In Table 1 we compare the occurrence of *Beggiatoa* to the sulfate reduction rates, i.e. to the rate of sulfide formation and, thus, presumably to the availability of sulfide for these sulfide oxidizing bacteria. Station A in Adventfjorden had the highest areal rates of sulfate reduction, yet *Beggiatoa* were not found. Perhaps this sediment was physically not accessible to *Beggiatoa* due to the lack of pore space for gliding motility. Smeerenburgfjorden sediment with abundant *Beggiatoa* was also in the higher end of the SRR range, but overall there was no clear correlation between rates of sulfide production and the occurrence of *Beggiatoa* (Table 1).

4.2 Ecology of arctic *Beggiatoa*

The ability to store nitrate enable the large marine *Beggiatoa* of Svalbard to thrive in the oxidized but anoxic zone within the upper 2-4 cm of the sediment. Near the sediment surface they may take up nitrate which in the bottom seawater in fjords on the west coast of Svalbard is mostly present at 1-10 μM concentration (Eilertsen *et al.*, 1989; Wang *et al.*, 2009). In the sediment below, they may effectively utilize the sulfide produced from bacterial sulfate reduction which proceeds throughout this zone although sulfide is near or below detection. Thus, in Smeerenburgfjorden sulfate reduction rates reached 17 nmol cm^{-3} d^{-1} at 2-3 cm depth where *Beggiatoa* were abundant, but where free

sulfide concentrations did not exceed 3 μM (Fig. 4). With a measured porosity of 0.72 at that depth, this rate corresponds to 24 μM SO_4^{2-} reduced per day or 2 μM SO_4^{2-} reduced per hour. The turnover time of total sulfide in the *Beggiatoa* zone is therefore in the order of one hour. The experimentally determined SRR data show that *Beggiatoa* do indeed have sulfide available which they can oxidize with intracellularly stored nitrate. The low concentration and relatively fast turnover of free sulfide shows that the sulfide was oxidized at the depth where it was produced and did not diffuse away.

Even at the highest density of *Beggiatoa* found in Smeerenburgfjorden, they probably did not play an important role for the overall sulfide oxidation, as the following calculations show. The cell-specific rate of nitrate reduction in marine *Beggiatoa* of 24-30 μm diameter from a temperate sediment at 15°C was found to be 13 mM NO_3^- d^{-1} (Preisler *et al.*, 2007). Arctic *Beggiatoa* of this size class living at 0°C may have ca 5-fold lower metabolic rate, estimated from the difference in gliding speed (Dunker *et al.*, submitted), i.e. about 3 mM NO_3^- d^{-1}. Since the dominant biomass of *Beggiatoa* in Smeerenburgfjorden belonged to the wider size range (Fig. 3B) which stored on the order of 130 mM NO_3^-, this rate of nitrate reduction could keep the filaments supplied with electron acceptor for (130/3 =) ca 40 days without a refill. If the filaments carry out dissimilatory nitrate reduction to ammonium and oxidize sulfide completely to sulfate, then the stoichiometry of nitrate reduction to sulfide oxidation is 1:1 and the cell specific rate of sulfide oxidation would also be about 3 mM sulfide d^{-1}:

$$H_2S + NO_3^- + H_2O \rightarrow SO_4^{2-} + NH_4^+ \qquad \text{(Eq. 3)}$$

This rate can be compared to the rate of sulfide production from the measured sulfate reduction. At 0-1 cm depth in the Smeerenburgfjorden sediment the biomass of *Beggiatoa* was 150 μg cm^{-3}. This is equal to 0.15 mm^3 of *Beggiatoa* biovolume per cm^3 of sediment and these *Beggiatoa* could oxidize ($0.15 \times 3 \times 10^{-6}$ =) 0.5 nmol sulfide cm^{-3} d^{-1}. The measured SRR at 0-1 cm was equal to 2 nmol H_2S cm^{-3} d^{-1} produced, i.e. 4-fold higher. The same calculation for the 2-3 cm depth interval provide: 60 μg cm^{-3} of *Beggiatoa* biomass which could oxidize 0.2 nmol sulfide cm^{-3} d^{-1}. The measured SRR at 2-3 cm was equal to 17 nmol H_2S cm^{-3} d^{-1} or nearly 100-fold higher. The conclusion is that, even at their highest biomass density in Smeerenburgfjorden, the *Beggiatoa* did not contribute

significantly to the overall rate of sulfide oxidation. This is the same conclusion as reached for a rather similar *Beggiatoa* population in a Baltic Sea sediment (Preisler *et al.*, 2007).

Mat-forming *Beggiatoa* of 8-10 μm diameter in Ymerbukta stored only 2.7 mM nitrate. It is possible that their chemoautotrophic life at the narrow interface of overlapping O_2 and H_2S (Fig. 6) is not as selective for a large internal nitrate storage as the subsurface life in Smeerenburgfjorden sediment with many hours or days of gliding away from the oxic surface zone.

4.3 *Beggiatoa* – members of the "rare biosphere"?

During the microscopic search for *Beggiatoa* in Svalbard sediments we also looked for other cells containing light refractive globules that could indicate sulfur bacteria with elemental sulfur inclusions. This search for morphologically conspicuous sulfur bacteria was generally without positive results. In one core from Smeerenburgfjorden, however, we found a distinct sheath which harbored five filaments of 23 μm diameter. The sheath was 3.5 mm long and 70 μm wide and the individual filaments were 0.5-1.1 mm in length and rich in light refractive inclusions. The terminal cells were rounded and it was thus not possible to distinguish the filaments morphologically from free-living *Beggiatoa*. The presence of a common sheath for a bundle of filaments, however, is diagnostic of the genus *Thioploca* and the diameter of 23 μm classified the filaments taxonomically to the species *T. chileae* (Jørgensen *et al.*, 2005b). In contrast to the observed filaments, *Thioploca* spp. most often have tapered terminal cells.

The scattered occurrence of *Beggiatoa* of highly variable diameters in many sediments indicates that these bacteria are widely present in the Arctic, but in low numbers. A different approach than used here would be required to scan a larger sediment volume (>>0.1 g) in the search for *Beggiatoa*, e.g. by extracting DNA and searching for *Beggiatoa*-related 16S rRNA genes, provided that appropriate primers for amplification could be established. Such an environmental genomic approach, however, misses the unique advantage provided by the distinct morphology of *Beggiatoa*. *Beggiatoa* is of the size of micro- and meiofauna, rather than of the size of normal bacteria, and the organisms can therefore be quantified with similar sample volumes and techniques. Due to their extraordinary size, *Beggiatoa* are rarely recorded in marine sediments although they are

widely distributed. The main reason is that they are not detected by normal direct bacterial counts using fluorescent stains such as acridine orange or DAPI. This becomes clear from the following example.

The densities of *Beggiatoa* filaments in Svalbard varied from <10 to 1000 filaments cm^{-3} sediment. By direct fluorescence counts of bacteria, about 1 μg of sediment may be scanned under the light microscope at 1000 × magnification. As the cell density of bacteria in normal fjord sediments is $>10^9$ cells cm^{-3} this would suffice to count >1000 bacteria. However, in 1 μg of sediment the chance of finding just one *Beggiatoa* at the above densities is $<10^{-5}$ to 10^{-3}, i.e. highly unlikely. By the counting of *Beggiatoa* we routinely counted all filaments in 30 mg of sediment, i.e. in a 10^5-fold larger sediment volume than is needed to count all other bacteria.

Even at their highest density, *Beggiatoa* filaments comprise only a millionth of all bacteria in the sediment. Yet, due to the very large biomass of each filament, 1-1000 ng, *Beggiatoa* may comprise a significant fraction of the total prokaryotic biomass. In Smeerenburgfjorden sediment the mean total bacterial numbers were 3.4×10^9 cells cm^{-3} sediment in the top 0-2 cm (Ravenschlag *et al.*, 2000). With a typical biovolume of 0.2 μm^3 of sediment bacteria (Kuwae & Hosokawa, 1999) the living biomass per bacterial cell is 0.2 pg. The total prokaryotic biomass (excluding *Beggiatoa*) is thus 700 μg cm^{-3}. The total biomass of *Beggiatoa* at 0.5 cm depth was 50-100 μg cm^{-3}, i.e. 7-15% of the total prokaryotic biomass of other bacteria. This shows that, although a normal direct count of total bacteria in the sediment would not have detected any *Beggiatoa*, they may still account for a significant fraction of the total bacterial biomass. With respect to biomass and metabolic activity they may thus be an important component of the microbial community. With respect to numbers they are less than a millionth (on a per cell basis less than 1/10.000) and belong to the "rare biosphere" (Sogin *et al.*, 2006), even at their highest densities.

Conclusions

Our results show that large, nitrate-accumulating *Beggiatoa* occur widespread in permanently cold sediments of the high Arctic, similar to temperate sediments (Mußmann *et al.*, 2003; Preisler *et al.*, 2007). The bacteria are not inhibited in their metabolic rate or in their motility and chemotactic behavior by temperatures near

freezing, but are well adapted to the cold (Dunker *et al.*, submitted). Only in two out of eleven investigated fjord sediments were *Beggiatoa* abundant and arctic *Beggiatoa* were not found to contribute significantly to sulfide oxidation in the studied sediments. Nevertheless, our findings provide a new and interesting perspective on the biogeography of *Beggiatoa* by showing that these giant, conspicuous sulfur bacteria occur frequently as members of the rare biosphere in marine sediments.

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Chapter 7

Discussion

7.1 Giant Sulfide Oxidizers

Among the mat-forming sulfide-oxidizing bacteria, members of the genera *Beggiatoa*, *Thioploca* and *Thiomargarita* as well as the novel group of vacuolated attached filaments (VAF) are very conspicuous as they can be seen without using a microscope and represent 'giants' within a world that usually remains hidden. For example, *Beggiatoa* are characterized by individual filaments that can have diameters of up to 200 µm and reach lengths of up to 10 cm (Teske and Nelson, 2006). *Thioploca* filaments can be as thick as 80 µm and 2 to 5 cm long (Teske and Nelson, 2006). Individual cells of the genus *Thiomargarita* may even reach diameters of up to 750 µm (Schulz *et al.*, 1999). VAF have been found to reach diameters of up to 112 µm (Kalanetra *et al.*, 2004). A summary of characteristic members of these organisms is presented in Table 1.

Beggiatoa

Occurrence. Historically, *Beggiatoa* spp. play an important role in the field of microbiology, as the concept of chemolitho(auto)trophy developed by Winogradsky in the late 19th century was based on his observations of sulfide oxidation by these very organisms (Winogradsky, 1887; Teske and Nelson, 2006). *Beggiatoa* spp. are known from a variety of shallow-water and deep-sea marine habitats as well as from different freshwater habitats (Teske and Nelson, 2006). In freshwater environments they occur, for example, at sulfur springs (Macalady *et al.*, 2006), in ditches, wetlands and lake sediments (Pringsheim, 1964; Strohl and Larkin, 1978; Sweerts *et al.*, 1990). Within the marine environment, *Beggiatoa* spp. have been found at hydrothermal vents (Nelson *et al.*, 1989; Figure 7.1A), at cold seeps (Larkin and Henk, 1996; Ahmad *et al.*, 1999; Niemann *et al.*, 2006; Jerosch *et al.*, 2007; Figure 7.1B), at whale falls (Deming *et al.*, 1997), in upwelling areas (Teske *et al.*, 1999; Brüchert *et al.*, 2003), in

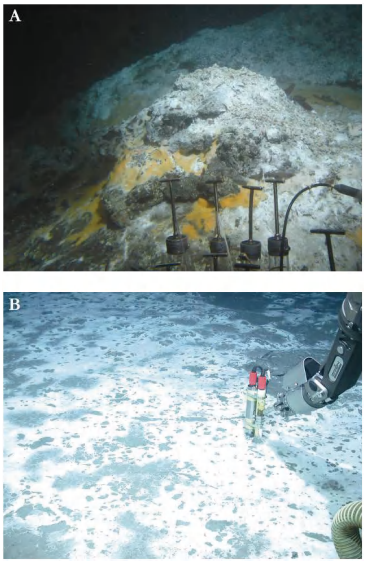


Figure 7.1: *Beggiatoa* mats at hydrothermal vents in the Guaymas Basin (A; Source: WHOI) and at the Håkon Mosby mud volcano (B; Source: Marum).

salt marshes (Nelson *et al.*, 1982) and in coastal sediments (Sayama, 2001; Mußmann *et al.*, 2003; Preisler *et al.*, 2007).

Morphology and Cell Structure. *Beggiatoa* spp. are multicellular, filamentous, gliding bacteria (Figure 7.2). Their cell, respective filament, diameter may vary between 1 and 200 µm, their filament length is estimated between a few micrometers and 10 cm (Teske and Nelson, 2006). Narrow *Beggiatoa* filaments are often composed of rather cylindrical cells, but crosswalls may not always be clearly visible if filaments are filled with elemental sulfur or PHB. Wider *Beggiatoa* flaments usually have more disc-shaped cells and terminal cells at the filament tip can appear slightly tapered when compared with the rounded terminal cells of narrower species (Teske and Nelson, 2006). All *Beggiatoa* spp. observed so far store elemental sulfur internally. Further, inclusions of PHB (poly-β-hydroxybutyrate) and polyphosphate have also been observed in some species (Teske and Nelson, 2006 and references therein). An important feature of several *Beggiatoa* spp. is the occurrence of a central vacuole that has been found to be used for the accumulation of nitrate, which can be up to 4 orders of magnitude higher than ambient seawater concentration (McHatton *et al.*, 1996; Teske *et al.*, 1999; Mußmann *et al.*, 2003; Hinck *et al.*, 2007). Currently under investigation is the mode of 'pigmentation' in *Beggiatoa* spp. forming e.g. yellow and orange mats at hydrocarbon seeps in the Gulf of Mexico (Larkin and Henk, 1996; Nikolaus *et al.*, 2003) or at the Guaymas Basin hydrothermal vent field (Jannasch *et al.*, 1989). Generally, *Beggiatoa* spp. belong to the 'colorless sulfur bacteria' as they lack photosynthetic pigments (Teske and Nelson, 2006), and it is not yet clear what may cause the color of these *Beggiatoa* spp. dominated mats.

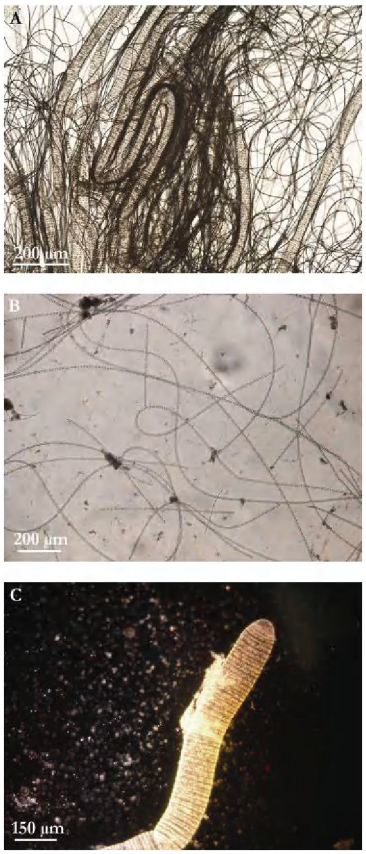


Figure 7.2: *Beggiatoa* filaments obtained from whale bones (A), white mats at the Håkon Mosby mud volcano (B) and white mats at the Amon mud volcano (C).

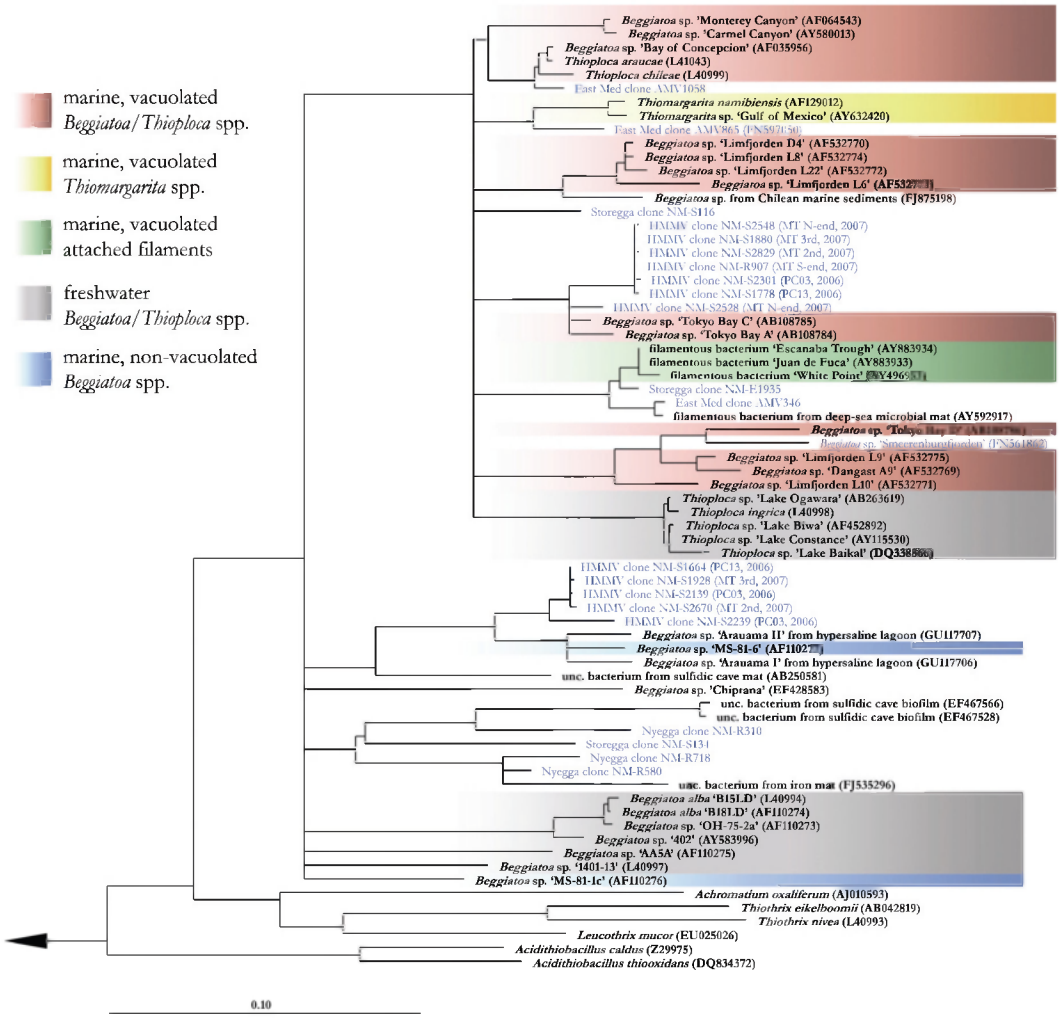


Figure 7.3: Phylogenetic 16S rRNA gene based tree showing characteristic members of the genera *Beggiatoa*, *Thioploca* and *Thiomargarita* as well as vacuolated attached filaments within the Gammaproteobacteria (see also Table 1). Novel 16S rRNA gene sequences obtained within this thesis from different sulfide oxidizer mats are shown in blue. AMV (Amon mud volcano; Chapters 3 & 4). Nyegga, Storegga and HMMV (Håkon Mosby mud volcano; Chapter 5), Smeerenburgfjorden (Chapter 6). Tree reconstruction was conducted with nearly full length sequences by applying neighbor joining and maximum likelihood (RAxML) as well as different conservatory filters within the ARB software package (Ludwig *et al.*, 2004). The final tree is based on 1182 valid columns (*E. coli* positions 126 to 1307) with neighbor joining. Partial sequences were subsequently added by applying parsimony criteria. Only selected sequences are shown. Unstable branching orders are visualized as multifurcation.

Phylogeny. Historically, differentiation between different *Beggiatoa* spp. within a certain habitat has been based on observations of their filament diameter (Larkin and Strohl, 1983; Strohl, 2005), a technique often still used today. Phylogenetically, the genus *Beggiatoa* is assigned to the Gammaproteobacteria, order Thiotrichales, and is closely

related to other giant sulfide oxidizers of the genera *Thioploca* and *Thiomargarita* (Figure 7.3; Strohl, 2005). Characteristic *Beggiatoa* spp. with known 16S rRNA gene based phylogeny are listed in Table 1. Generally, one can distinguish between freshwater and marine (vacuolated and non-vacuolated) strains also based on their 16S rRNA gene sequences (Figure 7.3).

Physiology and Ecology. Beggiatoa spp. cover a wide range of metabolic reactions and are generally divided into three groups (Strohl, 2005; Teske and Nelson, 2006): (i) thin, heterotrophic and non-vacuolated freshwater strains (e.g. B18LD, B15LD, OH-75-2a; Table 1), (ii) thin, autotrophic and non-vacuolated marine strains (MS-81-1c and MS-81-6; Table 1), and (iii) large (> 10 μm filament diameter), supposedly autotrophic, vacuolated and nitrate-accumulating strains that so far remain uncultured (e.g. *Beggiatoa* spp. observed in Monterey Canyon or the Bay of Concepción; Table 1). In addition, recently also thin (6 to 8 μm wide), vacuolated and nitrate-accumulating *Beggiatoa* spp. ('Chiprana'; Table 1) have been found in phototrophic hypersaline mats, their true metabolism though still being unknown (Hinck *et al.*, 2007).

Beggiatoa spp. oxidize sulfide to elemental sulfur, which they can store internally. In autotrophic strains, sulfur may further be oxidized to sulfate in order to gain energy for CO_2 fixation and growth. In heterotrophic freshwater strains, sulfide oxidation may serve as detoxifying mechanism, or sulfur may be used as 'short-term electron acceptor' under anaerobic growth conditions (Strohl, 2005 and references therein; Teske and Nelson, 2006 and references therein). Generally, oxygen is used as terminal electron acceptor in sulfide oxidation (Chapter 1: Eqs. 1.3 to 1.5). However, also nitrate may be used for sulfide oxidation, as it was postulated for thin freshwater strains (Sweerts *et al.*, 1990; Kamp *et al.*, 2006) and for marine, vacuolated *Beggiatoa* spp. that accumulate nitrate internally (Sayama, 2001; Sayama *et al.*, 2005). Whether nitrate is dissimilatory reduced to ammonium (Chapter 1: Eqs. 1.6 to 1.8) or is converted to dinitrogen gas (Chapter 1: Eqs. 1.9 & 1.10), is not totally clear yet. Reduction of nitrate to ammonia has been shown to occur in *Beggiatoa* spp. from coastal marine sediments off Japan (Sayama, 2001; Sayama *et al.*, 2005) and represents the commonly accepted mechanism (Teske and Nelson, 2006). As such, *Beggiatoa* spp. in shallow coastal areas may have a significant impact on eutrophication in these habitats (Sayama, 2001).

Members of the genus *Beggiatoa* are so-called 'gradient organisms' that thrive at oxic-anoxic interfaces and that are bound to the chemical gradients of their electron donor sulfide and their electron acceptors oxygen or nitrate. Tactic responses to light (phobic response, Nelson and Castenholz, 1982), oxygen (microaerophilic growth in the range of 1 to 2.5 µM oxygen, Møller *et al.*, 1985; Nelson *et al.*, 1986b) and sulfide (e.g. repelled by concentrations >1 µM, Preisler *et al.*, 2007) thereby enable *Beggiatoa* spp. to thrive in their preferred micro-niche. *Beggiatoa* spp. may thrive in narrow layers at the oxygen-sulfide interface (Figure 7.4A; Jørgensen and Revsbech, 1983; Nelson *et al.*, 1986a; Fenchel and Bernard, 1995), but may also bridge gaps between sulfide in deeper sediment layers and oxygen or nitrate at the sediment surface by means of their gliding motility and their ability to store large amounts of reductant (sulfur) and oxidant (nitrate in vacuolated species) inside their cells (Figure 7.4B; McHatton *et al.*, 1996; Mußmann *et al.*, 2003; Preisler *et al.*, 2007). In the latter scenario, vacuolated filaments may oxidize sulfide under anoxic conditions with internally stored nitrate to elemental sulfur, and sulfur further to sulfate by using oxygen at the sediment surface (Sayama *et al.*, 2005).

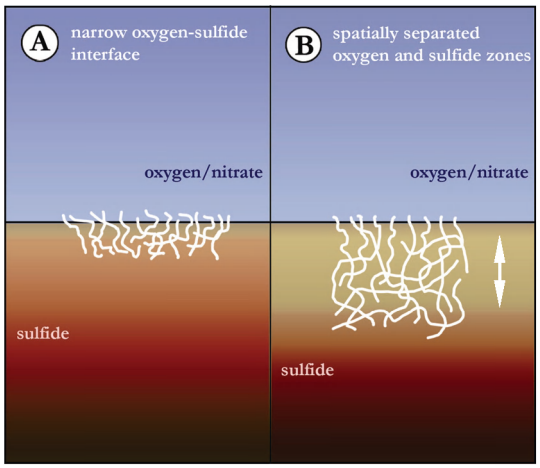
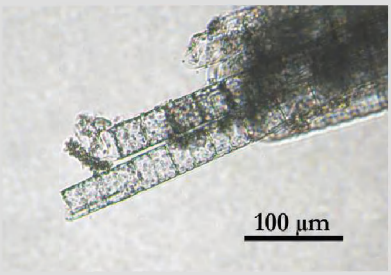


Figure 7.4: Schematic illustration of the main ecological niches occupied by *Beggiatoa* species. (A) Small freshwater and marine *Beggiatoa* spp. may position themselves in a narrow layer at the oxygen-sulfide interface. Sulfide and oxygen are consumed within the mat. (B) Vacuolated *Beggiatoa* spp. may glide to deeper sulfide layers, where they can oxidize sulfide to sulfur under anoxic conditions with accumulated nitrate. At the sediment surface they can oxidize internally stored sulfur further to sulfate with oxygen and replenish their internal nitrate reservoirs. This has been suggested especially for wide, marine *Beggiatoa* species.

Within this thesis, knowledge was gained on the occurrence and ecological niches of different *Beggiatoa* spp. found, e.g., (i) at the Eastern Mediterranean mud volcano Amon (Chapter 4), (ii) in deep-sea thiotrophic mats off Norway (Chapter 5), and (v) in shallow coastal sediments off the arctic archipelago Svalbard (Chapter 6), by combining molecular and biogeochemical data. Currently under investigation are *Beggiatoa* spp. forming mats on whale bones and recovered from hydrothermal vents (Guaymas Basin).

The genus *Thioploca*.



Morphologically and phylogenetically closely related to the genus *Beggiatoa* are members of the genus *Thioploca*. *Thioploca* spp. are also giant, multicellular, filamentous bacteria, but whose filaments occur in bundles surrounded by a common gelatinous sheath (Teske and Nelson, 2006). Outside of their sheath, *Thioploca* spp. are hardly distinguishable from *Beggiatoa* spp. (Teske *et al.*, 1999; Schulz *et al.*, 2000). *Thioploca* filaments are known from freshwater and marine habitats (examples are given in Table 1; Teske and Nelson, 2006 and references therein). Some of the largest mats formed by sulfide-oxidizing bacteria are the *Thioploca* mats observed along the Pacific coast of South America on the Chilean and Peruvian shelf, covering an estimated area of 40,000 km² (Huettel *et al.*, 1996). Here, marine *Thioploca* spp. shuttle within their sheaths between the overlying oxygen-depleted, but nitrate-rich, bottom water and the sulfidic zone within deeper (5 to 15 cm) sediment layers where they oxidize sulfide anaerobically with nitrate that they can accumulate up to 500 mM within internal vacuoles (Fossing *et al.*, 1995; Huettel *et al.*, 1996; Jørgensen and Gallardo, 1999). Within this thesis, no filament bundles reminiscent of *Thioploca* spp. were observed. Picture by Verena Salman (MPI Bremen).

Thiomargarita

Occurrence. The genus *Thiomargarita* was first discovered in 1997 in Namibian shelf sediments (100 m water depth, Schulz *et al.*, 1999). Owing to the conspicuous appearance of the organisms as a string of spherical cells (Figure 7.5), they were named *Thiomargarita namibiensis* – 'sulfur pearl of Namibia' (Schulz, 2006). Thereafter, dense populations of *Thiomargarita* spp. have only been found at hydrocarbon seeps in the Gulf of Mexico (525 to 640 m water depth, Kalanetra *et al.*, 2005). Despite their extraordinary cell size, populations of *Thiomargarita* spp., though, initially appeared rather inconspicuous in these habitats as they did not form dense mats on the seafloor. Further, the occurrence of single *Thiomargarita* spp.-resembling cells was observed in microbial mats at the Häkon Mosby mud volcano off Norway (1,250 m water depth, de Beer *et al.*, 2006).

Figure 7.5: Chain of *T. namibiensis* cells. Image: V. Salman (MPI Bremen).

Morphology and Cell Structure. The genus *Thiomargarita* includes some of the largest single-cell bacteria known today among Earth's prokaryotes (according to biovolume; Schulz *et al.*, 1999). *Thiomargarita* cells are non-motile and appear mostly spherical, but may also

show barrel-like or amorphous shapes (Schulz, 2006). They are either organized in chains held together by a mucus sheath (Figure 7.5), or appear as clusters or individuals (Schulz *et al.*, 1999; Kalanetra *et al.*, 2005; Schulz, 2006). Most cells had diameters between 100 and 400 µm (Schulz *et al.*, 1999; Kalanetra *et al.*, 2005; de Beer *et al.*, 2006), but occasionally *T. namibiensis* could reach diameters of up to 750 µm (Schulz *et al.*, 1999). All *Thiomargarita* spp. observed so far store elemental sulfur internally. In *T. namibiensis* also smaller inclusions resembling polyphosphate and polyglucose were documented (Schulz and Schulz, 2005). The occurrence of a large, central vacuole within the cells was described for *T. namibiensis* and *Thiomargarita* spp. from the Gulf of Mexico (Schulz *et al.*, 1999; Kalanetra *et al.*, 2005).

Phylogeny. So far, two partial 16S rRNA gene sequences derived from *T. namibiensis* and the Gulf of Mexico *Thiomargarita* spp. are published (Table 1). Phylogenetically, both *Thiomargarita* spp. are most closely related to the large, vacuolated, marine species of *Thioploca* and *Beggiatoa* within the order Thiotrichales of the Gammaproteobacteria (Figure 7.3; Kalanetra *et al.*, 2005; Schulz, 2006).

Physiology and Ecology. *Thiomargarita* oxidize sulfide or internally stored elemental sulfur by using either oxygen or internally stored nitrate (Chapter 1: Eqs. 1.3-1.10; Schulz *et al.*, 1999; Schulz and de Beer, 2002; Schulz, 2006). Whether *Thiomargarita* spp. reduce nitrate to ammonia or nitrogen gas is not known yet (Schulz, 2006). The internal accumulation of sulfur and nitrate enables *Thiomargarita* spp. to thrive in habitats where their electron donor and acceptor are temporarily separated, and to survive long periods of starvation (Schulz *et al.*, 1999). In the Namibian shelf, *T. namibiensis* are thought to rely on periodic resuspension of sulfidic sediments (Figure 7.6), presumably caused by fluid and gas eruptions, to contact the oxygenated and nitrate-bearing bottom water (Schulz *et al.*, 1999; Schulz, 2006).

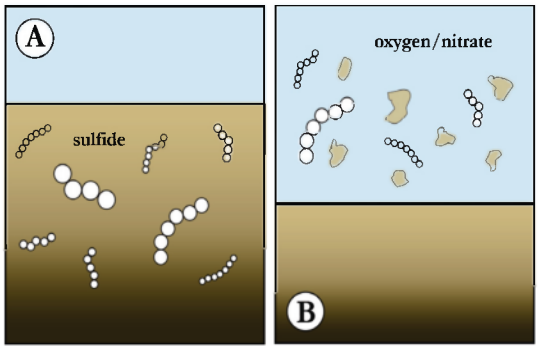


Figure 7.6: Schematic illustration of the ecological niche occupied by *T. namibiensis*. The immobile cells are mostly buried within the sulfidic sediment (A) and contact their electron acceptors only during externally induced resuspension events (B). Scheme modified after Schulz (2006).

Owing to their ability to deal with such disturbances and to endure peaks of oxygen (Schulz *et al.*, 1999; Schulz, 2006) as well as exposure to high sulfide concentrations (100 to 800 µM, Schulz *et al.*, 1999; 200 to 19,000 µM, Kalanetra *et al.*, 2005), *Thiomargarita* spp. seem to be especially well adapted to dynamic habitats. In addition to their ecological importance in the marine sulfur and nitrogen cycles, *T. namibiensis* have also been found to be involved in the cycling of phosphorus, where these organisms may play an important role in the formation of phosphorites through the release of phosphate under anoxic conditions (Schulz and Schulz, 2005; Schulz, 2006).

Within this thesis, a novel *Thiomargarita* population was discovered on top of a sulfidic mud outflow at the flank of the deep-sea mud volcano Amon (AMV) in the Eastern Mediterranean Sea (Figure 7.7; Chapter 3). With diameters ranging between 24 to 65 µm, *Thiomargarita* cells from the Amon mud volcano were substantially smaller than cells of previously described populations off Namibia (Schulz *et al.*, 1999) and in the Gulf of Mexico (Kalanetra *et al.*, 2005). A retrieved 16S rRNA gene fragment is monophyletic with the two previously published *Thiomargarita* sequences (Figure 7.3), and could be assigned to the *Thiomargarita* spp.-resembling cells by fluorescence *in situ* hybridization. In contrast to periodically resuspended *T. namibiensis* populations off Namibia (Schulz *et al.*, 1999; Schulz, 2006), *Thiomargarita* cells at the Amon mud volcano seemed to remain fixed at the sediment surface while environmental conditions changed around them due to periodic brine flow (Figure 7.8). These observations further strengthened previous hypotheses on *Thiomargarita* spp. to be especially well adapted to dynamic

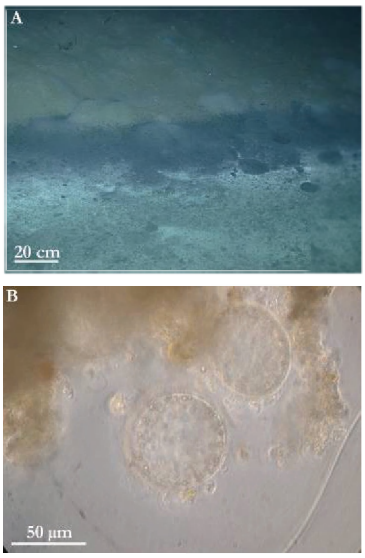


Figure 7.7: Rim of a white mat on top of blackish, sulfidic muds (A), dominated by single spherical cells resembling *Thiomargarita* species (B).

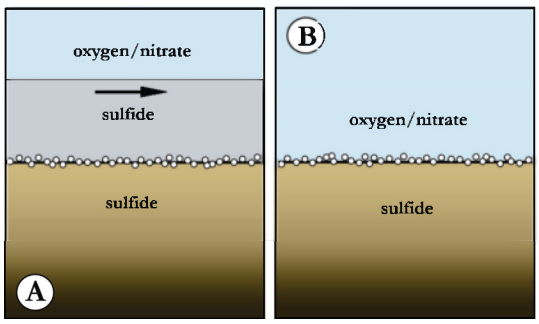


Figure 7.8: Proposed ecological niche for *Thiomargarita* cells at the AMV, where sulfidic brine overflows (A) alternate with phases of contact to oxygenated bottom water (B).

habitats (de Beer *et al.*, 2006; Schulz, 2006) where they might have an advantage over other sulfide oxidizers that are bound to stable chemical gradients (such as *Beggiatoa* spp. and *Thioploca* spp.) or constantly overlapping gradients of electron donor and acceptor (such as supposedly marine *Arcobacter* spp.).

Vacuolated attached filaments (VAF)

Occurrence. A few years ago, Kalanetra *et al.* (2004) defined a new type of filamentous, sulfur-storing bacterium – the so-called 'vacuolated attached filaments' (VAF). These bacteria had been observed near shallow (5 to 7 m water depth) hydrothermal vents off White Point, California, where they formed mats on various biotic and abiotic surfaces (Jacq *et al.*, 1989; Kalanetra *et al.*, 2004). Since then, no other observations attributed to this group of organisms were reported. However, phylogenetic analyses suggest at least one further occurrence of VAF in a deep-sea microbial mat at the Milano mud volcano in the Eastern Mediterranean Sea, even though the original publishers described them as 'unidentified filamentous bacteria' (Heijs *et al.*, 2005).

Figure 7.9: VAF-like bacteria attached to tubeworms that were recovered from the Storegga area off Norway.

Morphology and Cell Structure. Generally, the morphology of the described filamentous bacteria seemed to be between that of the genera *Beggiatoa* and *Thiothrix*. Filament diameter ranged between 4 and 112 µm. All filaments were non-motile, stored sulfur internally and appeared vacuolated. Occasionally, internal sulfur storage showed a gradient from non-existent at the base of the filament to dense at the filament tip (Kalanetra *et al.*, 2004).

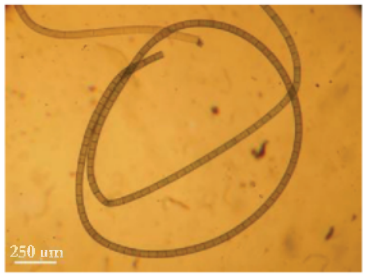


Figure 7.10: Potential VAF from a white mat at the Amon mud volcano in the Eastern Mediterranean Sea.

Phylogeny. VAF from White Point (Table 1), as well as 16S rRNA gene sequences retrieved from filamentous bacteria of the Juan de Fuca Ridge, the Escanaba Trough and unidentified filamentous bacteria from the Eastern Mediterranean Sea form a separate cluster within the group of vacuolated, marine *Beggiatoa*, *Thioploca* and *Thiomargarita* spp. (Figure 7.3).

Physiology and Ecology. So far, all available information on the physiological properties of VAF refer to their occurrence at the shallow hydrothermal vents off White Point (Kalanetra *et al.*, 2004). Despite the presence of a large central vacuole, VAF were not found to accumulate nitrate above ambient seawater concentration, which is in contrast to all other known vacuolated sulfide oxidizers VAF are related to (Figure 7.3). The exact role of vacuoles in VAF remains unknown, but Kalanetra *et al.* (2004) suggested their involvement in either storage of oxygen or filament buoyancy. As such, VAF may oxidize sulfide or internally stored sulfur only aerobically with oxygen (Chapter 1: Eqs. 1.3 to 1.5) and may be adapted to only very brief temporal separation of reductant and oxidant.

Within this thesis, filamentous bacteria potentially resembling VAF were obtained (i) from white microbial mats found near the center of the deep-sea mud volcano Amon in the Eastern Mediterranean Sea (Figures 7.3 & 7.10; Chapter 4), and (ii) from the Storegga area off Norway where they were attached to tubeworms and the surrounding sediment (Figures 7.3 & 7.9; Chapter 5). These observations were very interesting, as here VAF occurred in habitats where sulfide was always consumed within the mat and did not seem to penetrate the sediment surface (as indicated by *in situ* and *ex situ* microsensor measurements). Future studies therefore need to address questions regarding the possible existence of microsulfidic niches within the mats, as well as the extension of VAF into the sediment and their potential in accumulating nitrate internally (even though previously not observed).

Table 1 Characteristic sulfide oxidizers of the genera *Beggiatoa, Thioploca, Thiomargarita, Thiobacterium* and *Arcobacter,* and members of the group of vacuolated attached filaments (VAF)

Name/Site	Isolation source	Water depth (m)	Temp (°C)	Filament/cell size (µm)	Vacuolation	Internal nitrate accumulation	Metabolism	Accession nr. of 16S rRNA gene	Reference
BEGGIATOA									
Freshwater *Beggiatoa* spp.									
AA5A	Davis, California, USA	ND	ND	2-2.5	No	ND	Facultative lithoheterotroph	AF110275	Ahmad *et al*. (2006)
B18LD	Baton Rouge, Louisiana, USA	ND	ND	3.5	No	ND	Heterotroph	AF110274	Strohl and Larkin (1978), Strohl *et al*. (1981), Mezzino *et al*. (1984), Ahmad *et al*. (2006)
B15LD	Baton Rouge, Louisiana, USA	ND	ND	2.8-3.2	No	ND	Heterotroph	L40994	Strohl and Larkin (1978), Mezzino *et al*. (1984)
1401-13	Germany	ND	ND	2.5	No	ND	Heterotroph	L40997	Pringsheim (1964)
OH-75-2a	Warm Spring, Oregon, USA	ND	ND	1.5-2.5	No	ND	Heterotroph	AF110273	Nelson and Castenholz (1981), Nelson *et al*. (1982), Ahmad *et al*. (2006),
402	Freshwater stream	ND	ND	ND	No	ND	Facultative chemolithotroph	AY583996	Grabovich *et al*. (2001) and references therein
Chiprana	Hypersaline inland Lake Chiprana, Spain	0.5	ND	6-8	Yes	4-44 mM	ND	EF428583	Hinck *et al*. (2007)
Marine *Beggiatoa* spp.									
MS-81-1c	Salt marsh, New England, USA	ND	ND	1.6-2.2	No	≤ 0.3 µM	Obligate lithoautotroph	AF110276	Nelson *et al*. (1982), Hagen and Nelson (1996), McHatton *et al*. (1996), Ahmad *et al*. (2006),
MS-81-6	Salt marsh, New England, USA	ND	ND	4.0-5.1	No	ND	Facultative lithoautotroph	AF110277	Nelson *et al*. (1982), Hagen and Nelson (1996), McHatton *et al*. (1996), Ahmad *et al*. (2006),
Tokyo Bay A, C	Marine sediment, Tokyo Bay, Japan	10, 0.5	ND	10	ND	ND	ND	AB108784, AB108785	Kojima and Fukui (2003)
Tokyo Bay	Marine sediment, Tokyo Bay, Japan	10	ND	9	Yes	105 ± 36 mM	ND	ND	Sayama (2001)
Tokyo Bay D	Marine sediment, Tokyo Bay, Japan	16	ND	30	Yes	ND	ND	AB108786	Kojima and Fukui (2003)
Limfjorden	Brackish Limfjorden, Denmark	ND	ND	5-40[a]	Yes	156 ± 71 mM[a]	ND	AF532770-AF532775[a]	Mußmann *et al*. (2003)
Dangast A9	Intertidal mud flat Dangast, Germany	ND	ND	9-11	Yes	288 ± 80 mM	ND	AF532769	Mußmann *et al*. (2003)
Bay of Concepción	OMZ sediments, Bay of Concepción, Chile	25	ND	35-40	Yes	15-116 mM	ND	AF035956	Teske *et al*. (1999)
Monterey Canyon	Clam Field Seep, Monterey, California, USA	900	4	65-85[b]	Yes	160 ± 20 mM	ND	AF064543	McHatton *et al*. (1996), Ahmad *et al*. (1999)
Guaymas	Guaymas Basin hydrothermal vent field	2,000	3-20	88-130[c]	Yes	130 ± 10 mM	ND	ND	McHatton *et al*. (1996)
Carmel Canyon	Cold seep, California, USA	900	ND	20-76	Yes	16.6 ± 4 mM	ND	AY580013	Kalanetra *et al*. (2004)

For all *Beggiatoa* spp. 'Filament/cell size' refers to filament diameter (width). ND, no data.

[a]Filament diameters of 5-40 µm refer to all observed *Beggiatoa* spp. Internal nitrate content was determined for filaments of 9-12 µm width. Sequencing was conducted with filaments of 10-40 µm. Positive hybridization with specific probes designed for the obtained 16S rRNA gene sequences was achieved for filaments of 36 ± 3 µm (AF532770, AF532772-774) and 16 ± 0.5 µm (AF532771, AF532775) diameter.

[b]Most of the observed *Beggiatoa* filaments had a filament width of 65-85 µm. However, few narrower filaments of 20-30 µm were also observed. The available 16S rRNA gene sequence was obtained from the large (and more abundant) *Beggiatoa* spp. (Ahmad *et al*., 1999).

[c]The given range of filament diameters corresponds to the dominating width class of *Beggiatoa* spp., but also narrower filaments of 27 µm diameter were observed (McHatton *et al*., 1996). Previously, Nelson *et al*. (1989) had described three different size classes for *Beggiatoa* spp. in the Guaymas Basin: 24-32 µm, 40-42 µm and 115-122 µm.

Table 1 continued

Name/Site	Isolation source	Water depth (m)	Temp. (°C)	Filament/ cell size (μm)	Vacuolation	Internal nitrate accumulation	Metabolism	Accession nr. of 16S rRNA gene	Reference
THIOPLOCA									
Freshwater *Thioploca* spp.									
Thioploca ingrica	Randersfjord, Denmark Lake Erie, USA	ND	ND	3-4	Yes	ND	ND	L40998	Maier and Murray (1965), Teske *et al*. (1995)
Lake Ogawara	Brackish lake Ogawara, Japan	16	ND	3-4	ND	ND	ND	AB263619	Kojima *et al*. (2006)
Lake Biwa	Freshwater lake Biwa, Japan	90	ND	3-5.6	ND	ND	ND	AF452892	Kojima *et al*. (2003)
Lake Constance	Freshwater lake Constance, Germany	22	ND	3-5.2	ND	ND	ND	AY115530	Kojima *et al*. (2003)
Lake Baikal	Lake Baikal, Frolikha Bay freshwater vents, Russia	23-415	5-6	2-5	Yes	136	ND	DQ338566	Zemskaya *et al*. (2001)
Marine *Thioploca* spp.									
Thioploca chilae	Continental shelf off Peru and Chile (OMZ)	50-300	10	12-20	Yes	150-500 mM[d]	ND[e]	L40999	Maier *et al*. (1990), Fossing *et al*. (1995), Teske *et al*. (1995), McHatton *et al*. (1996), Otte *et al*. (1999)
Thioploca araucae	Continental shelf off Peru and Chile (OMZ)	50-300	10	30-43	Yes	150-500 mM[d]	ND[e]	L41043	Maier *et al*. (1990), Fossing *et al*. (1995), Teske *et al*. (1995), McHatton *et al*. (1996), Otte *et al*. (1999)
THIOMARGARITA									
Marine *Thiomargarita* spp.									
Thiomargarita namibiensis	Fluidic shelf sediments off Namibia (OMZ)	100	ND	100-750	Yes	100-800 mM	ND	AF129012	Schulz *et al*. (1999), Schulz (2006)
Gulf of Mexico	Hydrocarbon seeps in the Gulf of Mexico	525-640	ND	180-375	Yes	460 mM	ND	AY632420	Kalanetra *et al*. (2005)
VAF									
White Point	Shallow hydrothermal vents, White Point, California, USA	5-7	15-30	4-112	Yes	Not detected	ND	AY496953	Kalanetra *et al*. (2004)
THIOBACTERIUM									
Thiobacterium bovistum (*Bacterium bovista*)	Winogradsky column with marine sediment and water, Italy	ND	ND	0.6-1.5 × 2-5	ND	ND	ND	ND	Molisch (1912), Janke (1924)
Thiodendron mucosum	Sulfur spring, polluted waters, tide pools, Florida, USA	ND	12.5-29	1.0-2.5 × 3-9	ND	ND	ND	ND	Lackey and Lackey (1961), Lackey *et al*. (1965)
Thiobacterium sp.	Thermal spring, Austria	ND	43.5-45.5	0.4-0.5 × 4-5	ND	ND	ND	ND	Vouk *et al*. (1967), Scheminzky *et al*. (1972)
Thiobacterium sp.	Hydrothermal vent, North Fiji Basin	2671	ND	0.5 × 1.5 (isolate)	ND	ND	ND	ND	Seki and Naganuma (1989)
ARCOBACTER									
Marine, mat-forming *Arcobacter* spp.									
Candidatus Arcobacter sulfidicus	Coastal seawater	ND	ND	0.5 × 2.0 (isolate)	ND	ND	Chemoautotroph	AY035822	Wirsen *et al*. (2002), Sievert *et al*. (2007)

For all *Thioploca* spp. and VAF 'Filament/cell size' refers to filament diameter (width). For *Thiomargarita* spp. this refers to cell diameter. For *Thiobacterium* spp. and *Arcobacter* spp. cell dimensions (width × length) are given. ND, no data.

[d]Nitrate values were collectively determined for both marine *Thioploca* species.

[e]Otte *et al*. (1999) suggest facultative chemolithoautotrophic growth and capability of mixotrophic growth, but do not differentiate between the two marine *Thioploca* species.

7.2 Other Mat-forming Sulfide Oxidizers

Thiobacterium

Figure 7.11: *Thiobacterium* mat observed off Greece in a shallow-water cave.

Occurrence. The genus *Thiobacterium* was first described by Molisch in 1912 (Molisch, 1912). At the surface of Winogradsky column enrichments prepared with seawater and sediment from the harbor of Trieste he observed the formation of small, but very conspicuous, spherically shaped gelatinous masses. Embedded in the skin of those structures were non-motile rods containing inclusions of elemental sulfur. Due to the morphological analogy of the gelatinous spheres to the fungal genus *Bovista,* Molisch named the newly observed organism *Bacterium bovista*, which was later revised to *Thiobacterium bovistum* (Janke, 1924; Gherna *et al.*, 1989; La Rivière and Kuenen, 1989; Kuenen, 2005). Thereafter, *Thiobacterium* spp.-resembling mats and organisms were found in many other marine and continental locations world-wide, including thermal and sulfur springs (Devidé, 1952; Lackey and Lackey, 1961; Lackey *et al.*, 1965; Vouk *et al.*, 1967; Anagnostidis, 1968; Scheminzky *et al.*, 1972; Fjerdingstad, 1979), and sulfidic marine and brackish waters (Molisch, 1912; Lackey *et al.*, 1965; Seki and Naganuma, 1989).

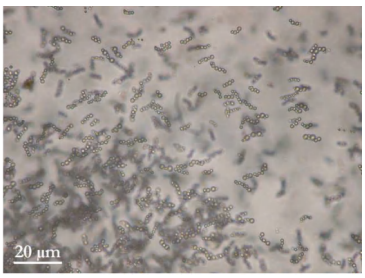


Figure 7.12: *Thiobacterium* spp. from a spherical mat recovered off Norway.

Morphology and Cell Structure. Generally two different morphologies of the gelatinous mats have been described, including spherically shaped (Molisch, 1912; Devidé, 1952; Vouk *et al.*, 1967; Anagnostidis, 1968; Scheminzky *et al.*, 1972; Seki and Naganuma, 1989) and dendroid masses (Lackey and Lackey, 1961; Lackey *et al.*, 1965). Embedded in the gelatinous matrix are non-motile rod-shaped cells of 0.4-2.5 × 2.5-9 µm in size which may contain up to 20 sulfur granules. The cells have been found to be located either in the outer 'skin' of the gelatinous masses (Molisch, 1912), or throughout the whole mat (Devidé, 1952). In addition, Molisch (1912) further described motile bacteria of almost the same appearance, but not being embedded within a gelatinous matrix (*Bacillus thiogenus*). The occurrence of

motile organisms resembling *Thiobacterium* spp. has also been described from other studies (Lackey and Lackey, 1961). Another important observation is, that upon recovery of gelatinous mats, the cells may eventually lose their internal sulfur granules with time (Lackey and Lackey, 1961).

Phylogeny. So far, no 16S rRNA gene sequence has been clearly attributed to the genus *Thiobacterium*, why their true taxonomic position is still uncertain. In Bergey's manual of systematic bacteriology (Kuenen, 2005) and the Encyclopedia of Life (http://www.eol.org/pages/97513), *Thiobacterium* spp. are currently classified as members of the Gammaproteobacteria (family Thiotrichaceae). In The Prokaryotes, *Thiobacterium* spp. are listed as 'morphologically conspicuous sulfur-oxidizing bacteria' (La Rivière and Schmidt, 2006).

Physiology and Ecology. So far, no *Thiobacterium* sp. (respective mat) has been successfully maintained in culture for a longer period of time. Only one study reports about the growth characteristics of a *Thiobacterium* sp. obtained from the plume of hydrothermal vents in the North Fiji Basin (~ 2,700 m water depth, Seki and Naganuma, 1989). Therein, it was suggested that *Thiobacterium* spp. under aerobic conditions may express a eurythermally mesophilic and slightly halophilic behavior. Most importantly, this study could follow cell growth and formation of the gelatinous masses, as well as the final disintegration of the structures, within laboratory experiments. This indicated that *Thiobacterium* cells are themselves forming the gelatinous masses, most likely to retain a favorable spatial position in their occupied sulfidic habitats. A few characteristic *Thiobacterium* spp. are listed in Table 1.

Within this thesis, spherically shaped gelatinous mats formed by *Thiobacterium* spp.-resembling cells were obtained from three different habitats, including (i) a recovered whale bone where the mat had formed within a few days after the bone had been scraped free from mats of *Beggiatoa* spp. and *Arcobacter* spp, (ii) the Storegga area off Norway where the mats were found on the surface of methane-seep sediment (recovered from 745 m water depth), and (iii) a shallow-water cave off Greece (23 m water depth; Figure 7.11) where the mats covered an estimated area of 5 to 7.5 m^2 on the cave walls

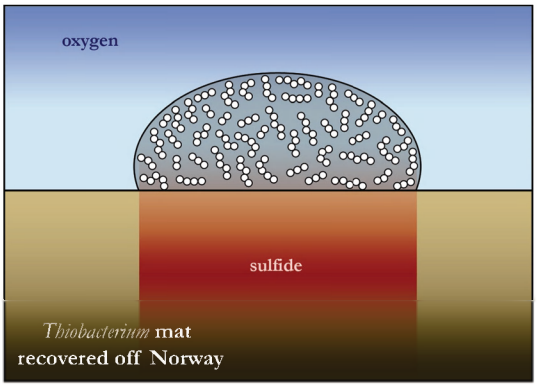


Figure 7.13: *Thiobacterium* mats recovered off Norway had formed where sulfide reached the sediment surface (as indicated by *ex situ* microprofiling). Oxygen penetrated into the mat only a few millimeters (in a 3D-view around the whole sphere) and did not overlap with sulfide within the sphere. (Chapter 2)

(Chapter 2). The size of the *Thiobacterium* mats ranged from a few millimeters (whale bone, cave) to the centimeter scale (seep sediment, cave) and even decimeter scale (cave). The appearance of the rod-shaped cells was consistent with previous findings (Figure 7.12). Positive hybridization of *Thiobacterium* cells with a probe targeting most known Gammaproteobacteria confirmed their phylogenetic affiliation with this class. As shown by microprofiling through the Storegga seep mats, they had formed in an area where sulfide reached the sediment surface, and sulfide was depleted within the mat (Figure 7.13). Strangely, sulfide and oxygen did not overlap within the mat and the mechanisms driving sulfide oxidation in *Thiobacterium* spp. will require special attention in the future.

Arcobacter

Occurrence. The genus *Arcobacter* is considered to be quite unusual (Vandamme *et al.*, 2005), as it combines human- and animal-associated species, bearing a potential as foodborne pathogens (Lehner et al., 2005), with species occupying various environmental niches such as roots and sediments in a salt marsh (McClung *et al.*, 1983), oil field brine (Gevertz *et al.*, 2000), coastal waters (Fera *et al.*, 2004), shelf waters (Lavik *et al.*, 2009), or a hypersaline lagoon (Donachie *et al.*, 2005). As mat-forming organisms, *Arcobacter* spp. have frequently been described in co-occurrence with filamentous sulfur formation. Such mats have been observed, for example, at shallow and deep-sea hydrothermal vents (Sievert *et al.*, 1999; Taylor *et al.*, 1999; Moussard *et al.*, 2006), at marine cold seeps (Robinson *et al.*, 2004), or at a mud volcano-associated brine seep (Omoregie *et al.*, 2008).

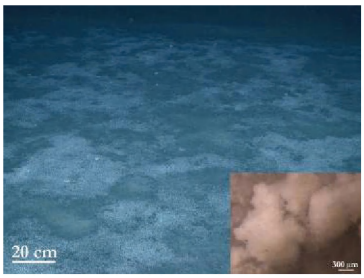


Figure 7.14: *Arcobacter* mat in the Eastern Mediterranean Sea

Within the following paragraphs, the focus will be on mat-forming *Arcobacter* spp., especially on the comprehensively studied coastal strain '*Candidatus* Arcobacter sulfidicus' (Taylor and Wirsen, 1997; Wirsen *et al.*, 2002; Sievert *et al.*, 2007).

Morphology and Cell Structure. '*Candidatus* Arcobacter sulfidicus' (CAS) were described as highly motile, slightly vibrio-shaped organisms (0.5 × 2.0 µm) with four polar flagella. Further, the bacteria were observed to have the ability to attach and detach from solid surfaces (Wirsen *et al.*, 2002).

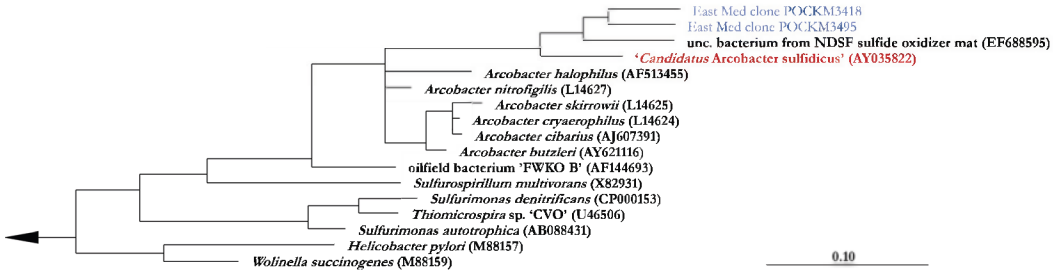


Figure 7.15: Phylogenetic 16S rRNA gene based tree showing characteristic members of the genus *Arcobacter* within the Epsilonproteobacteria (see also Table 1). Novel 16S rRNA gene sequences obtained within this thesis from an *Arcobacter* mat in the Eastern Mediterranean Sea are shown in blue (Chapter 4). Tree reconstruction was conducted with nearly full length sequences by applying neighbor joining and maximum likelihood (RAxML) as well as different conservatory filters within the ARB software package (Ludwig *et al.*, 2004). The final tree is based on 1267 valid columns (*E. coli* positions 83 to 1349) with RAxML. Only selected sequences are shown. Unstable branching orders are visualized as multifurcation.

Phylogeny. Generally, *Arcobacter* spp. belong to the Epsilonproteobacteria (Vandamme *et al.*, 2005). Phylogenetic analyses with a 16S rRNA gene sequence obtained for CAS revealed a close relation of the strain to other known *Arcobacter* spp. (Table 1; Figure 7.15; Wirsen *et al.*, 2002).

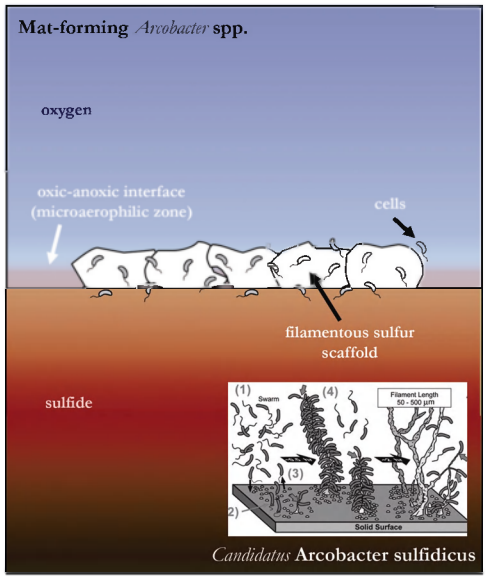


Figure 7.16: Schematic illustration of the postulated ecological niche for marine, mat-forming *Arcobacter* species. The best studied strain among this group is '*Candidatus* Arcobacter sulfidicus' (Sievert *et al.*, 2007), which during laboratory experiments effectively aggregate within the oxic-anoxic interface and oxidize sulfide incompletely to filamentous sulfur (inserted panel shows filamentous sulfur formation; taken from Sievert *et al.* (2007)).

Physiology and Ecology. CAS was described as chemoautotrophic, microaerophilic organism that mainly oxidizes sulfide incompletely to elemental sulfur (Wirsen *et al.*, 2002; Sievert *et al.*, 2007). Filamentous sulfur formation by CAS has been studied in gradient cultures and microslide capillaries (Taylor and Wirsen, 1997; Sievert *et al.*, 2007). Following the attachment to a surface, CAS excrete small droplets or thin filaments of sulfur that with time aggregate into so-called 'protofilaments'. Further sulfur deposition thickens the filaments until they form a scaffold-like structure (Sievert *et al.*, 2007). It was further shown that CAS effectively aggregate within the oxic-anoxic interface of oxygen-sulfide gradients (Sievert *et al.*, 2007). They were found to tolerate high hydrogen sulfide concentrations (1 to 2 mM) and to be able to grow at low oxygen concentrations (1 to 10 µM), which may be an important advantage over other sulfide-oxidizing bacteria in nature (Sievert *et al.*, 2007). The formation of a filamentous sulfur scaffold is thought to retain the organisms in their favorable oxygen-sulfide interface region, even within high-fluid-flow environments (Taylor and Wirsen, 1997; Wirsen *et al.*, 2002; Sievert *et al.*, 2007). As such, CAS may be pioneer colonizers in dynamic habitats (Sievert *et al.*, 2007). Furthermore, sulfur formation may represent a way of detoxification, allowing CAS to thrive at higher sulfide concentrations (Sievert *et al.*, 2007).

Generally, marine mat-forming *Arcobacter* spp. are thought to oxidize sulfide with oxygen (Chapter 1: Eq. 1.3). Nitrate reduction seems to be known among the genus *Arcobacter* (Vandamme *et al.*, 1991), but has not been shown for mat-forming species yet. However, studies conducted with isolates from oil field brine (Gevertz *et al.*, 2000) and observations on a bloom of chemolithotrophic bacteria in African shelf waters

containing *Arcobacter* spp. (Lavik *et al.*, 2009) suggest that *Arcobacter* spp. may be able to use nitrate as alternative electron acceptor for sulfide oxidation.

Within this thesis, filamentous sulfur mats, respective *Arcobacter* mats, were observed in many of the investigated habitats, including cold seeps off Norway and a pockmark-dominated area in the Eastern Mediterranean Sea (Figure 7.14; Chapter 4). In the latter habitat, the largest of all *Arcobacter* mats was observed (extending over 60 × 100 m). *In situ* microprofiling revealed overlapping gradients of oxygen and sulfide within the mat (Figure 7.16), being in accordance with previous observations for CAS in laboratory studies. Two 16S rRNA gene sequences closely related to other *Arcobacter* spp., including CAS, could be obtained (Figure 7.15). In addition, *Arcobacter* spp.-related 16S rRNA gene sequences were often also obtained from other mat habitats, supposedly as a result of sediment contamination, raising the question about their true abundance in seep sediments as well as the possibility of species succession within sulfide oxidizer mats.

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Chapter 8

Concluding Remarks

Concluding Remarks

In the oceans, microbial mats formed by sulfide-oxidizing bacteria are often found at the interface between so-called 'reduced habitats' that are characterized by high concentrations of energy-rich reduced compounds, like sulfide, and oxic zones providing electron acceptors for the oxidation of sulfide. Within this thesis, five different mat-forming sulfide-oxidizing bacteria that were found at cold seeps or in shallow-water areas along the European continental margin were investigated, including the giant filamentous *Beggiatoa* (Teske and Nelson, 2006), the giant spherical 'sulfur pearl' *Thiomargarita* (Schulz, 2006), giant vacuolated attached filaments (VAF, Kalanetra *et al.*, 2004), rod-shaped *Thiobacterium* spp. forming gelatinous mats (La Rivière and Kuenen, 1989) and vibrio-shaped *Arcobacter* forming filamentous sulfur scaffolds (Sievert *et al.*, 2007). Morphological, phylogenetic (16S rRNA gene based) and geochemical analyses were combined within this thesis to describe their diversity and to broaden our knowledge on the geological drivers of these organisms in marine sulfidic habitats. Therein, the focus was on further clarifying niche boundaries and ecological niche differentiation between the different sulfide oxidizers.

Identification of mat-forming sulfide oxidizers

When I started this thesis, I was not only interested in the diversity of the mat-forming sulfide-oxidizing bacteria and their geological drivers in marine habitats, but also wanted to resolve some of the known obstacles in species identification. For example, no 16S rRNA gene sequence existed for the long-known, but poorly investigated, genus *Thiobacterium*. VAF and *Thiomargarita* had been respectively described from only one (Kalanetra *et al.*, 2004), and three (Schulz *et al.*, 1999; Kalanetra *et al.*, 2005; de Beer *et al.*, 2006), habitats. Further, classification of the genera *Beggiatoa* and *Thioploca* seemed by far not completed and was based on a 'working definition on the basis of current insufficient knowledge' (Teske and Nelson, 2006).

As observations on these organisms can be very rare or they occur in such remote locations as the deep-sea, recovering bacterial mat samples in sufficient quality and amount for comprehensive analyses is often the limiting factor. In addition, long-term cultivation so far has only been achieved for two marine *Beggiatoa* strains (and several

freshwater strains, Teske and Nelson, 2006), none of which belong to the group of wide, vacuolated marine species investigated in this thesis (Chapter 7: Figure 7.3 and Table 1). No cultivated strains of *Thiobacterium* and VAF are available. *Thiomargarita* spp. have been maintained for several years in their original sediments, but no pure culture exists up to date (Schulz *et al.*, 1999; Kalanetra *et al.*, 2005). Marine, mat-forming *Arcobacter* spp. are mainly characterized based on studies with the coastal strain '*Candidatus* Arcobacter sulfidicus', which has not been deposited at any cell culture collection, but could be successfully grown in laboratory studies to follow filamentous sulfur formation (Sievert *et al.*, 2007). Hence, cell and mat material can be very limited and identification of these organisms is often restricted to observations on their morphology as well as 16S rRNA gene sequencing, both cost-efficient, well-tested and commonly applied techniques.

Methodological Challenges. Within this thesis, polymerase chain reaction (PCR) amplifications were generally conducted with universal bacterial primers, as no real group-specific primers selecting for *Beggiatoa* spp., VAF, or marine mat-forming *Arcobacter* spp. exist. Primer development did not seem convenient, as for each group it would have been based on a low number of described species and published 16S rRNA gene sequences, possibly making the primers too specific. Further, as previously stated, no 16S rRNA gene sequence clearly attributed to a *Thiobacterium* spp. was available. *Thiomargarita* spp. constituted a special case, as difficulties in amplifying *Thiomargarita* 16S rRNA gene sequences had already previously been encountered and were ascribed to epibiont contamination (Kalanetra *et al.*, 2005; Schulz, 2006). Furthermore, none of the published *Thiomargarita* sequences exceeds *E. coli* position 848, suggesting that certain 3'-specific traits or modifications of their 16S rRNA gene might hinder amplification. Therefore, a set of specific primers targeting a larger group of vacuolated sulfide oxidizers was used in this thesis for amplification of a partial sequence (Chapter 3).

The yield of clones containing a 16S rRNA gene sequence that phylogenetically was attributable to one of the investigated sulfide oxidizers was found to vary approximately between <1% and >90%. Depending on the bacterium under investigation, this may have had the following reasons: For *Beggiatoa* spp. it was found to be best, when directly inserting the filaments into the PCR (without prior DNA extraction). As such, PCR amplicons can be obtained from a single filament or bulk mat samples. Even though

successfully conducted also onboard R/V *Polarstern* (Chapter 5), PCR amplification is generally restricted to the home laboratories making longer storage of the filaments and subsequent freezing and thawing unavoidable. Most difficulties, though, possibly arise from the fact that remnants of sediment and other unicellular bacteria often cannot be completely separated from the filaments. As these bacteria may additionally, or even preferentially, be amplified, this potentially reduces the number of desired *Beggiatoa* spp.-type 16S rRNA gene sequences in a clone library, a problem also known for the giant sulfide oxidizer *Thiomargarita* (see above). Generally, co-amplification of associated bacteria seems to be an issue attributable to all investigated sulfide oxidizers in this thesis. *Thiobacterium* cells are embedded in a gelatinous matrix from which they hardly can be detached. Within the matrix, numerous other bacteria are 'trapped' (Chapter 2; Vouk *et al.*, 1967), which are then also introduced into PCR. In addition, mats and cells of the genus *Thiobacterium* are known to disintegrate after a certain time (Lackey and Lackey, 1961; Seki and Naganuma, 1989), further complicating phylogenetic studies. *Arcobacter* cells are mostly loosely associated with their filamentous sulfur scaffolds and upon recovery from the deep-sea most of them might become detached, why only low numbers may be introduced into a PCR. Amplifying *Arcobacter* spp. 16S rRNA genes from the underlying sediment seemed easier, but may impair identification of the mat-forming population.

In addition, the amplification of potentially chimeric sequences increases the time for retrieving suitable phylogenetic information characterizing the mat-forming sulfide oxidizers. For example within this thesis, analyses conducted with a single picked 135 µm wide *Beggiatoa* spp.-resembling filament recovered from the Amon mud volcano off Egypt (Chapter 4) resulted in three different 16S rRNA gene sequences (data not shown in the manuscript). Two of the sequences were potential chimeras. Due to the limited amount of sample material and the inability to isolate single cells, no FISH analyses could be performed to resolve this problem and to identify the 16S rRNA gene sequence characterizing this type of sulfide oxidizer. However, it could also be possible that this species contained several different types of 16S rRNA genes (possibly reflecting multiple operons) and that chimera detection was a result of limitations in the applied algorithm (Ashelford *et al.*, 2005).

Overall, the search for 16S rRNA gene sequences identifying the mat-forming sulfide oxidizers within this thesis was a time-consuming, and sometimes unpredictable process because of the high diversity of bacteria associated with or attached to mat-forming sulfide oxidizers. In addition, first fluorescence *in situ* hybridization (FISH) studies indicated that linking phylogeny and morphology might be as difficult as finding the phylogenetic marker sequences beforehand. This might, for example, be based on higher-order structures in the 16S rRNA genes or ribosomes hindering probe binding, or increased autofluorescence of the cells raising the need for catalyzed reporter deposition (CARD-) FISH procedures that enhances the FISH signals for clear discrimination between target and non-target (Amann and Fuchs, 2008). As such, these analyses will need to be addressed in future studies and were not incorporated in this thesis.

Open Questions. As the previous paragraph indicated, further resolving the obstacles in species identification of mat-forming sulfide-oxidizing bacteria needed to be postponed to future studies. Some questions remain open, e.g. whether *Beggiatoa* and *Thioploca* may constitute the same genus, wherein sheath formation or loss might be a facultative adaptation to certain environmental conditions (Teske *et al.*, 1999; Ahmad *et al.*, 2006). This raises the need for detailed ecological studies and finding new strategies for laboratory cultures in which sheath formation can be followed and possibly also be stimulated or repressed under certain conditions. Another unresolved question includes, whether filament diameter can still be validly used for distinguishing different *Beggiatoa* and *Thioploca* species (Larkin and Strohl, 1983; Strohl, 2005). Within a certain habitat, this might at first appear to be reasonable. For example, Limfjorden *Beggiatoa* spp. of 9 to 17 μm diameter group separately from 33 to 40 μm wide filaments (Mußmann *et al.*, 2003), the same applying to 10 μm and 30 μm wide *Beggiatoa* spp. from Tokyo Bay (Kojima and Fukui, 2003). However, Mußmann *et al.* (2003) also observed that filaments of same width did not hybridize with the same probe, and within this study two different phylotypes of *Beggiatoa* spp. were obtained from filaments with almost identical diameter (Chapter 5). Furthermore, genomic analyses conducted with two *Beggiatoa* filaments of similar filament diameter indicated a clear phylogenetic difference between them (Mußmann *et al.*, 2007), emphasizing the need for species identification to go beyond morphological traits and potentially also beyond 16S rRNA gene analyses. In addition,

the phylogenetic relatedness of all wide, vacuolated, marine species of *Beggiatoa*, *Thioploca*, *Thiomargarita* and VAF needs to be clarified. Based on 16S rRNA gene analyses they are monophyletic, but form several stable subgroups within this cluster (Chapter 7: Figure 7.3). Finally, single cell techniques may provide a valuable opportunity in the future to facilitate, for example, the taxonomic classification of the genus *Thiobacterium* and to investigate potential microdiversity in the giant mat-forming sulfide-oxidizing bacteria.

Niche characterization for mat-forming sulfide oxidizers

Within this thesis, morphological and phylogenetic analyses were combined with geochemical analyses, mainly regarding oxygen-sulfide gradients, to infer on the ecological niches occupied by the different mat-forming sulfide-oxidizing bacteria. Most of the studies were conducted with microbial mats observed in deep-sea habitats, such as Storegga and Nyegga cold seeps (~700 m water depth), the Håkon Mosby mud volcano (~1,250 m water depth), the Amon mud volcano (~1,100 m water depth) and a pockmark-dominated area on the Nile Deep Sea Fan (~1,700 m water depth). As sample recovery from such remote locations is often limited or might disturb geochemical gradients, and as cultivation of the mat-forming bacteria may be difficult, *in situ* technologies have become of great importance (Boetius and Wenzhöfer, 2009). Within this thesis, high resolution microprofiles of oxygen and sulfide were recorded with an ROV (remotely operated vehicle)-operated microprofiler (Treude *et al.*, 2009). Investigation of the ecological niches occupied by *Beggiatoa* spp. generally supported previously gained knowledge on these organisms (Chapters 4, 5 & 6), i.e. that they can thrive in narrow oxygen-sulfide gradients, but may also shuttle between deeper sulfidic sediment layers and electron acceptor (oxygen or nitrate) at the sediment surface (Teske and Nelson, 2006). In addition, two potential types of VAF were found in habitats where no sulfide emanated into the bottom water (Chapters 4 & 5), raising the question how VAF in these habitats could gain access to their energy source sulfide. Furthermore, this thesis presents the first *in situ* recorded gradients of oxygen and sulfide for deep-sea *Arcobacter* and *Thiomargarita* mats. Marine mat-forming *Arcobacter* spp. in the Eastern Mediterranean Sea dominated when oxygen and sulfide gradients overlapped within the bottom water (Chapter 4), supporting previous observations in laboratory studies

(Sievert *et al.*, 2007). Eastern Mediterranean *Thiomargarita* spp. populated a dynamic habitat with temporarily changing supply of oxygen and sulfide (Chapter 3).

By occupying such defined niches, it seems intriguing how such specialized organisms can sometimes occur as virtual monocultures and in such high numbers, i.e. dense microbial mats, on the seafloor. Following species succession (Bernard and Fenchel, 1995) and chemical gradients within a defined mat during long-term observations would therefore be an important task in the future. This could eventually give more insight into the beginning of mat formation, if and at what point chemical gradients may change and pave the way for the occurrence or dominance of other sulfide oxidizers, as well as potential cell-cell-communication regulating optimum cell density within such a mat.

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(1) Eastern Mediterranean Sea (Nile Deep Sea Fan), BIONIL expedition, leg M70/2a (21.10.-06.11.2006), R/V Meteor, MUMM2, ESF EUROCORES EuroMargin

(2) Håkon Mosby mud volcano, ARK-XXII/1b expedition (23.06.-09.07.2007), R/V Polarstern, MUMM2, HERMES

(3) Guaymas Basin hydrothermal vent field, AT15-40 expedition (05.12.-19.12.2008), R/V Atlantis, NSF Biological Oceanography

Poster and Oral Presentations

(1) S. Grünke, H. Røy, A. Ramette, A. Boetius (2007, poster presentation)
Diversity of giant sulfide-oxidizing bacteria at continental margins
HERMES 2nd Annual Meeting, Faro, Portugal

(2) S. Grünke, H. Røy, A. Ramette, A. Boetius (2007, oral presentation)
Diversity of deep-water ecosystems: Investigating mat-forming giant sulfide-oxidizing bacteria at cold seeps
EGU General Assembly, Vienna, Austria

(3) S. Grünke, H. Røy, A. Ramette, A. Boetius (2007, poster presentation)
Diversity of giant sulfide-oxidizing bacteria at cold seeps
1st EuroDIVERSITY Annual Conference, Marne-la-Vallée (Paris), France

(4) S. Grünke, A. Ramette, A. Boetius (2008, oral presentation)
Diversity of mat-forming sulfide-oxidizing bacteria at European cold seeps
HERMES 3rd Annual Meeting, Faro, Portugal

(5) S. Grünke, A. Ramette, A. Boetius (2008, poster presentation)
Diversity of mat-forming giant sulfide-oxidizing bacteria in chemosynthetic ecosystems along Europe's continental margins
EGU General Assembly, Vienna, Austria

(6) S. Grünke, A. Ramette, A. Boetius (2008, oral presentation)
Thiobacterium – *Unknown sulfur oxidizers producing conspicuous gelatinous structures in sulfidic habitats*
The 12th International Symposium on Microbial Ecology - ISME 12, Cairns, Australia

(7) S. Grünke, A.-C. Girnth, J. Felden, A. Lichtschlag, A. Ramette, A. Boetius (2009, poster presentation)

Diversity of mat-forming sulfide-oxidizing bacteria on deep water continental margins

GRC for Applied and Environmental Microbiology, Mount Holyoke, USA

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