Autecology of crenarchaeotal and bacterial clades in marine sediments and microbial mats

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Summary

The focus of this thesis was the autecology of the Miscellaneous Crenarchaeotal Group (MCG), a phylum-level clade of *Archaea* occurring mostly in marine sediments. Sequences of MCG 16S rRNA genes have been retrieved from a wide range of marine and terrestrial habitats, such as deep subsurface sediments, hydrothermal sediments, mud volcanoes, estuaries, hot springs and freshwater lake sediments. MCG members seem to have no general preferences for a particular temperature or salinity. So far, not a single member of the elusive MCG has been cultured. They show a high intragroup diversity with percent identity values of 16S rRNA as low as 77%. Since MCG sequences are frequent in sulfate-methane transition zones (SMTZ) of deep sea subsurface sediments, MCG were assumed to be the dominant archaeal population which might greatly contribute to biogeochemical cycles in the deep biosphere. However, quantitative data on the abundance and activity of MCG are still largely lacking. Therefore, in this doctoral thesis, a polyphasic approach was applied for the quantification and visualization of MCG in marine habitats using different molecular methods such as slot-blot hybridization, quantitative PCR and fluorescence in situ hybridization. MCG-specific oligonucleotide probes and primers were designed and used for the quantification. It was shown that – in general – the relative abundance of MCG strongly increased with depth. In methane-rich surface sediments MCG abundances were below 3% of total *Archaea*. In contrast, MCG constituted a major part of the archaeal community with 15-100% in subsurface SMTZ. My results provide the first quantitative data on the high abundance of MCG in deep subsurface sediments and are inline with a major role of MCG in biogeochemical cycling in these habitats.

In this thesis, cells of MCG were visualized for the first time by CARD-FISH. The cell shape was coccoid and the cell diameter was 0.4-0.5 μm. Signals were weak, but still clearly detectable with CARD-FISH suggesting that MCG are not only abundant, but also active in subsurface sediments. The single cell identification protocol developed in this doctoral thesis will in the future facilitate further quantitative investigations of the autecology of MCG. Further experiments performed in the course of this doctoral thesis addressed the quantification and visualization of particular bacterial populations such as *Chloroflexi* and *Aquificae* in microbial mats and marine sediments.

Zusammenfassung

Schwerpunkt dieser Arbeit waren Untersuchungen zur Autökologie der „Miscellaneous Crenarchaeota Gruppe“ (MCG), einer phylogenetischen Gruppe auf Phylum-Ebene innerhalb der *Archaea*, die hauptsächlich in marinen Sedimenten vorkommt. 16S rRNA Gensequenzen von Vertretern der MCG wurden bereits aus vielen verschiedenen marinen und terrestrischen Habitaten isoliert, wie z.B. aus Sedimenten der tiefen Biosphäre, Hydrothermalquellen, Schlammvulkanen, Flussmündungen, heißen Quellen und Süßwasserseen. Vertreter der MCG scheinen keine generellen Vorlieben für eine bestimmte Temperatur oder Salinität zu haben. Bis heute ist es nicht gelungen auch nur einen Vertreter der MCG zu kultivieren. Die Diversität innerhalb der MCG ist hoch mit einer 16S rRNA Identität von nur 77%. MCG Sequenzen werden oft in Sulfat-Methan-Übergangszonen (SMTZ) in tiefen marinen Sedimenten gefunden. Daher wurde angenommen, dass Vertreter dieser Gruppe die dominierende archaeelle Population darstellen und vermutlich wesentlich zu den biogeochemischen Kreisläufen der tiefen Biosphäre beitragen. Quantitative Daten zur Abundanz und Aktivität von MCG sind bislang kaum vorhanden. In dieser Arbeit wurde deshalb versucht, mithilfe eines polyphasischen Ansatzes (slot-blot Hybridisierung, quantitative PCR und Fluoreszenz in situ Hybridisierung) MCG in marinen Habitaten zu quantifizieren und zu visualisieren. MCG-spezifische Oligonucleotidsonden und Primer wurden entwickelt und für die Quantifizierung eingesetzt. Es konnte gezeigt werden, dass die relative Abundanz der MCG im Allgemeinen deutlich mit steigender Tiefe zunahm. In methanreichen Oberflächensedimenten war die Abundanz von MCG geringer als 3% der gesamten *Archaea* wohingegen MCG den Großteil der archaeellen Population in tiefen SMTZs mit 15-100% stellte. Meine Ergebnisse sind die ersten quantitativen Daten zur Abundanz der MCG in tiefen Sedimenten und zeigen die bedeutende Rolle der MCG für biogeochemische Stoffkreisläufe in diesen Habitaten.

In dieser Arbeit wurden MCG-Zellen mithilfe von CARD-FISH zum ersten Mal visualisiert. MCG sind kokkoide Archaeen mit einem Durchmesser von 0,4 - 0,5 µm. CARD-FISH Signale waren schwach, jedoch klar detektierbar. Dies lässt darauf schließen, dass die MCG-Zellen in tiefen Sedimenten nicht nur abundant, sondern auch physiologisch aktiv sind.

Das in dieser Arbeit entwickelte Protokoll zur Identifizierung von MCG-Zellen wird in Zukunft weitere quantitative Untersuchungen zur Autökologie von MCG erleichtern.

Weitere in dieser Arbeit durchgeführten Experimente umfassen die Quantifizierung and Visualisierung spezieller Bakteriengruppen wie z.B. *Chloroflexi* und *Aquificae* in mikrobiellen Matten und marinen Sedimenten.

1 Introduction

Microbial autecology is the study of the interaction of a distinct population of microorganisms with its biotic and abiotic environment. A population-centric approach to ecology has many advantages, not the least a simplification of an otherwise highly complex network of interactions (Pernthaler and Amann, 2005). Autecologists focus on the identification of a distinct population and the quantification of its abundance. In the last two decades, researchers were focusing mainly on the diversity of microbes in certain habitats and general interactions of microbial communities with their environment. Rather than doing autecology they did synecology which is defined as the study of groups of organisms. The development of molecular biology had allowed for reconstructing the phylogeny of microbes (Woese and Fox, 1977). By comparative analyses of 16S rRNA sequences an unexpectedly high diversity of yet uncultured microorganisms was revealed (Hugenholtz et al., 1998). After a very fruitful period of diversity discovery in natural microbial communities, the major question has now shifted from "Who is out there?" to "How many of what kind?" Also, this doctoral thesis was started with the goal to connect the identity (the "who?") of microbes with their quantity (the "how many?") in exactly what niche (the "where?"). By focusing on distinct groups of microbes, answers can be reached faster, proceeding step by step. The final goal of the microbial autecology would be to understand the role of a distinct microbial population in a particular environment, also including the specific biochemical pathways catalyzed (the "What are they doing?"). Ultimately, deep insights into the entire ecosystem will be achieved by combining autecological and synecological investigations.

In the following, a short overview will be provided on the habitats examined in the course of this thesis.

1.1 Marine sediments

Marine sediments are the largest repositories of nutrients and microorganisms in the oceans. According to the tectonical activities, sea floor forms various geological structures, such as hydrothermal vents, cold seeps and mud volcanoes (Jørgensen and Boetius, 2007; Fig. 1-1).
Coastal sediments including tidal flats, contain high amounts of organic compounds from terrestrial areas and reach up to 50% of total organic carbon accumulation in the ocean (Wollast, 1991). Because of this high nutritional input, microorganisms are abundant and

metabolically highly active. On the other hand, in open ocean below 200 m water depth, light intensity is too low for photosynthesis. Due to the low input of organic material, the sea floor is, in general, oligotrophic although some geologically active sites like hydrothermal vents or cold seeps are hot spots in the oceanic desert which harbor many chemolithoautotrophic and heterotrophic microorganisms.

In sediments, there is a general order of depletion of electron acceptors used by microorganisms. The order starting from the sediment surface is oxygen, nitrate, manganese and iron minerals, sulfate and bicarbonate (Froelich et al., 1979; Fig. 1-2). Sediment surfaces are subjected to mixing by higher *Eukarya* (bio-irrigation) and sedimentation of organic matter, but in subsurface sediments the availability of the electron acceptors is limited by diffusion from surface sediments and fluid flux from basalts. There are a couple of different depth definitions for separating surface from subsurface sediments which ranged between 10 cmbsf (Whitman et al., 1998) to 1 mbsf (Jørgensen and Boetius, 2007). In this thesis I define depths < 10 cmbsf as surface sediments, deeper layers (>10 cmbsf) as subsurface.

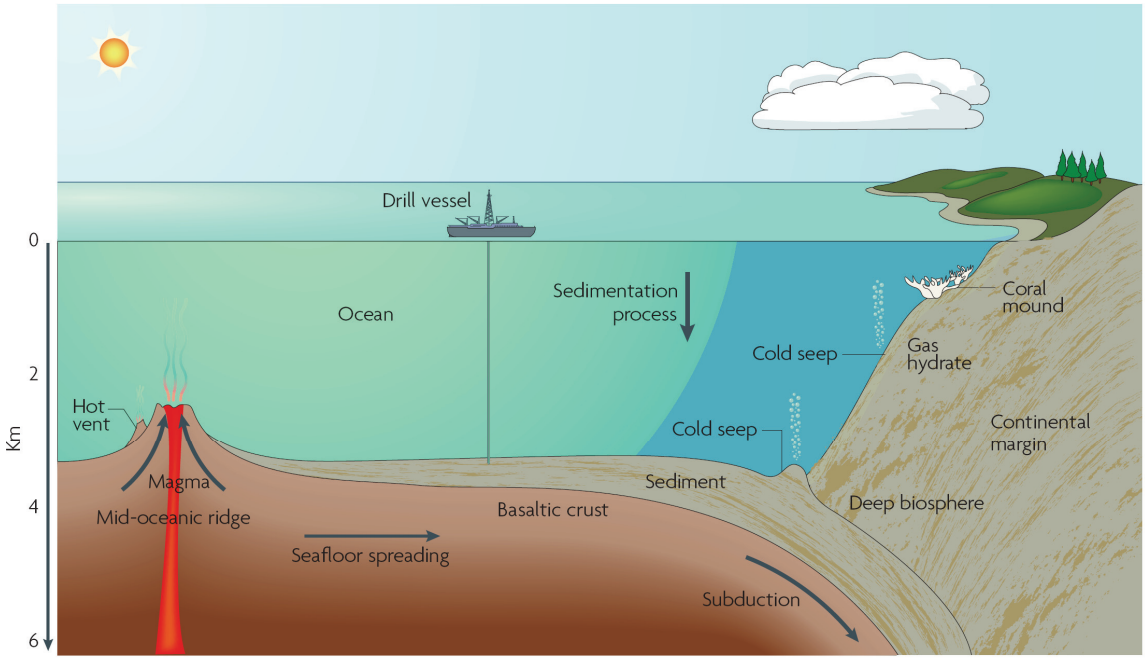

Figure 1-1 Vertical section of the seabed and seafloor structures. (from Jørgensen and Boetius, 2007)

1.2 Key biogeochemical processes in the sea floor

1.2.1 Sulfate reduction

Sulfate is the most abundant electron acceptor in the marine environment, and especially important in anoxic sediments (Jørgensen, 1982). By the activity of distinct *Bacteria* and *Archaea* it is converted into reduced sulfur compounds, most notably sulfide. Since the diffusion of sulfate into deeper sediment layers is limited it can be totally consumed by the activity of microorganisms. Sulfate reduction is the key process in marine sediments, in particular in shallow sediments where it contributes to 50% of organic carbon mineralization (Jørgensen, 1982; Canfield et al., 1993). Most sulfate-reducing bacteria belong to the subclass *Deltaproteobacteria*. Other groups of bacteria, such as *Firmicutes* (gram-positives), *Nitrospirae*, *Thermodesulfovibrio* spp. or *Thermodesulfobacterium* spp. are known to reduce sulfate as well. At high temperature environments such as hydrothermal vents, certain groups of *Archaea* (e.g. class *Archaeoglobi* in *Euryarchaeota*, class *Thermoprotei* in *Crenarchaeota*) also do sulfate reduction (e.g. Muyzer and Stams, 2008). Hot spots of sulfate reduction are cold seeps, where extremely high sulfate-reduction rate (SRR) observed, and is coupling with methane oxidation and sulfate reduction in a 1:1 stochiometric ratio.

1.2.2 Anaerobic oxidation of methane

Most of global methane is stored in marine sediments and permafrost soils (Post et al., 1982), from where only a minor portion (2% of the global flux of methane) is emitted to the atmosphere (Cicerone and Oremland, 1988). In contrast, methane emission to the atmosphere is significantly high in terrestrial habitats such as rice paddy fields. In the marine environment most methane is consumed by the anaerobic methane oxidation (AOM) in the sediment before reaching the water column. Thus, AOM is one of the key processes of suppressing the emission of the green-house gas methane into the atmosphere.

In 2000, it was shown that AOM is catalyzed by dense consortia of *Euryarchaeota* and sulfate-reducing bacteria (Boetius et al., 2000). There are three clades of methanotrophic Euryarchaeota referred to as Anaerobic MEthanotrophs (ANME-1 to ANME-3). They form structured aggregations of either a shell-type or a mat-type (Knittel and Boetius, 2009), or they form mixed-type aggregates. AOM is assumed to function as a reversal of methanogenesis coupled to the reduction of sulfate. The key enzyme of methanogenesis is the methyl-coenzyme M-reductase (MCR) which catalyzes the final step of methanogenesis, the reduction of a methyl moiety attached to the coenzyme M to methane (Ankel-Fuchs et al., 1986; Wolfe, 1991). A homologous protein and gene of the MCR was purified from AOM-catalyzing microbial mats in the Black Sea which are dominated by ANME-1 (Krüger et al.,

2003). The ANME clades were observed to be associated with different sulfate-reducing partners (Knittel et al., 2005), i.e. SEEP-SRB1a, a subcluster of the *Desulfosarcina/Desulfococcus* branch of *Deltaproteobacteria,* or *Desulfobulbus* relatives (Lösekann et al., 2007; Pernthaler et al., 2008; Schreiber et al., 2010). Very recently, the so-called HotSeep1 group, a clade most closely related to sulfur-reducing bacteria of the genera *Desulfurella* and *Hippea*, has been identified as ANME-1 partners in thermophilic AOM enrichment cultures (Holler et al., 2011). Although ANMEs are abundant at methane seeps, only few sequences were found in deep sea Sulfate-Methane Transition Zones (SMTZ) (Nunoura et al., 2008; Roussel et al., 2008). SMTZ were recently recognized as microbiologically highly active layers in subsurface sediments (Jørgensen, 2006). From these sites, in particular crenarchaeotal sequences of the Marine Benthic Group B (MBGB) and Miscellaneous Crenarchaeotal Group (MCG) have been retrieved. Therefore, a contribution of other microbes to AOM in deep sea subsurface has been proposed (Biddle et al., 2006). Other electron acceptors than sulfate for AOM have also been shown; for example nitrate or nitrite (Raghoebarsing et al., 2006; Ettwig et al., 2008) and manganese and iron in marine sediments (Beal et al., 2009). However, the main methane turnover is shown to be sulfate-dependent because of the high concentration of sulfate in the sea water.

The end product of sulfate-depending AOM is sulfide, thus the existence of the sulfide-oxidizing bacteria on the surface sediment is indicative for the active AOM sites, or chemosynthetic communities utilizing the sulfide.

1.2.3 Methanogenesis

In sulfate-free deep layers methanogenic archaea are using carbon dioxide as terminal electron acceptor resulting in methane production. Methane can also be abiotically generated by thermal cracking of hydrocarbons or water-rock interaction under high temperature and pressure in hydrothermal vents (Foustoukos and Seyfried, 2004). However, estimates based on the isotopic composition of atmospheric methane suggest that about 80% is derived from microbial activity (Ehhalt and Schmidt, 1978). Biotic methanogenesis can be based on hydrogen, methylated substances or small organic acids such as acetate. The reaction is oxygen sensitive because of the key enzyme, methyl coenzyme M reductase (MCR) and is conducted solely by methanogenic archaea. Known methanogens are all classified into the phylum *Euryarchaeota*, and most belong to the classes *Methanobacteria*,

Methanococci/Methanothermea, "*Methanomicrobia*" and *Methanopyri*. Methanogenesis is often the terminal step in the biodegradation of organic compounds in anoxic environments.

1.2.4 Oxidation of organic matter using other electron acceptors

Soluble manganese and iron mostly trapped into oxic surface sediments of a few millimeters (muddy sediments) to centimeters depth (sandy sediments), and few released to bottom sediments (Thamdrup et al., 1994). Manganese and iron-reducing microbes are thus mostly active in oxic-anoxic interface in the sediment. In this thesis I rather focus on sulfur and methane cycles in sediments.

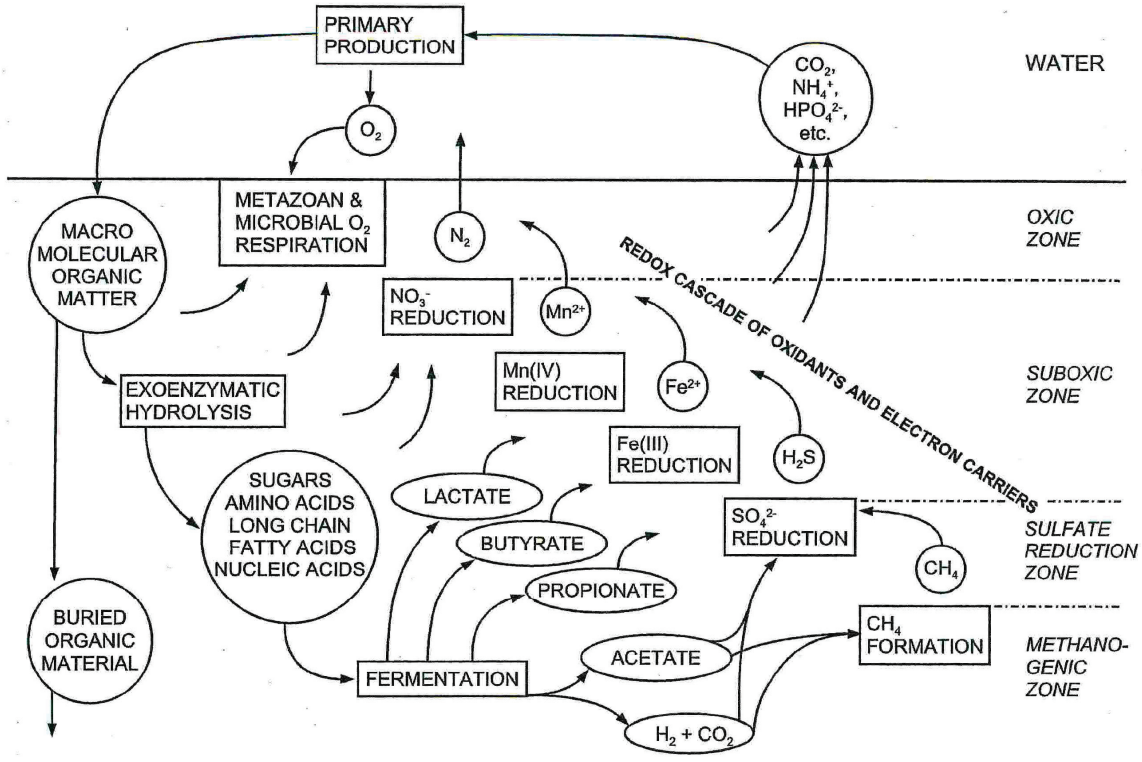

Figure 1-2 Pathways of organic carbon degradation in marine sediments and their relation to the geochemical zonations and the consumption of oxidants. (from Jørgensen 2006, after Fenchel and Jørgensen, 1977)

1.2.5 Organic carbon degradation in subsurface sediments

In deep sea subsurface sediments, the organic carbon content is low and causes a low number of microorganisms. While 25-50% of the organic matter as primal productivity from planktons goes to coastal seas, only about 1% reaches the deep sea floor (Suess, 1980). Due to the great water depth most organic matters has been degraded in water column before it

reaches the seafloor. Remaining organic matter might also be difficult to degrade for most microorganisms. Organic matters sedimented on the seafloor is used for aerobic respiration in the surface, and in the suboxic layer of the sediment, organic matter is hydrolyzed into small molecules such as sugars, amino acids, fatty acids and nucleic acids. Those small organic molecules are used for fermentation or as electron donor for anaerobic respiration such sulfate reduction or methanogenesis (Jørgensen, 2006).

1.3 Microbial key players in surface and subsurface sediments

It has been estimated that the prokaryotes of subseafloor sediments constitutes 50 to 85% of total prokaryotic biomass on earth (Whitman et al., 1998). However, more than 99% of these microbes are not isolated or cultivated yet (Amann et al., 1995).

More than 97% of prokaryotes in marine sediments are habiting the zone shallower than 600 m (Whitman et al., 1998). It was a surprise that microorganisms could live in such deep sediment layers with high pressure, low nutrition and metabolic activity. In general, the total cell numbers decrease from $>10^9$ cells cm^{-3} at the surface to $<10^6$ cells cm^{-3} at deep subsurface sediments (Parkes et al., 1994; D'Hondt et al., 2004), and intact microbial cells have even been detected from depths >600 mbsf.

The knowledge on the deep subsurface ecosystem is still limited. There is, e.g., an ongoing vivid discussion whether *Bacteria* or *Archaea* are more relevant in the deep biosphere. Most reports showed *Archaea* to be more abundant than *Bacteria* (Inagaki et al., 2003; Mauclaire et al., 2004; Biddle et al., 2006; Lipp et al., 2008), but there were also a few reports other way around (Schippers et al., 2005). Due to the low cell numbers in the deep subsurface, the quantification itself is still challenging for those samples. Most of the 16S rRNA gene libraries showed a typical community structure for deep subsurface sediments (Inagaki et al., 2003; Webster et al., 2003; Biddle et al., 2006; Inagaki et al., 2006a; Sørensen and Teske, 2006). Among those sequences, Miscellaneous Crenarchaeotal Group (MCG) (Inagaki et al., 2003) and Marine Benthic Group B (MBGB) (Vetriani et al., 1998) in *Archaea* and *Chloroflexi* (Blazejak and Schippers, 2010) in Bacteria are predominant.

1.4 The phylum *Crenarchaeota*

The domain *Archaea* has been classified into four major phyla, *Euryarchaeota*, *Crenarchaeota*, *Korarchaeota*, and *Nanoarchaeota*. Recently, a separation of *Thaumarchaeota* equivalent to the former Marine Group I (MG-I) from the *Crenarchaeota*

was suggested (Brochier-Armanet et al., 2008). MG-I has been intensively investigated, since it has the first mesophilic crenarchaeotal isolate, *Nitrosopumilus maritimus* (Könneke et al., 2005). Other strains from this clade were detected in terrestrial hot springs (Hatzenpichler et al., 2008) or as sponge symbionts (DeLong et al., 2004). Sometimes they were detected in marine sediments, but did not seem to be abundant.

Crenarchaeota is one of the biggest phylum in the domain *Archaea*. Most cultured representatives of *Crenarchaeota* are so-called extremophiles (hyperthermophiles, acidophiles). However, by comparative sequence analysis of 16S rRNA gene libraries it was revealed that many more unseen clades of *Crenarchaeota* must exist (Hershberger et al., 1996; Cavicchioli, 2006). In the following, major uncultured groups of *Crenarchaeota* are listed.

The marine benthic group B (also referred to as Deep Sea Archaeal Group, DSAG) is known to be widely distributed in the marine realm, mostly in sediments. This is in contrast to terrestrial or freshwater environments where this group is not occurring. They were so far found in organic-rich coastal sediments, deep subsurface sediments (Inagaki et al., 2003), Nankai Trough (Reed et al., 2002; Newberry et al., 2004), cold methane seep sediments, in methanotrophic microbial mats in the Black Sea (Knittel et al., 2005), organic poor sediments, hydrothermal vents at Juan de Fuca Ridge (Huber et al., 2002), Izu-Ogasawara Arc (Takai and Horikoshi, 1999), Mid-Atlantic-Ridge (Reysenbach et al., 2000), Guaymas Basin (Ehrhardt et al., 2007), coastal intertidal sediments, cold marine surface (but 3-27 cm) sediments in Northwest Atlantic (Vetriani et al., 1999).

The Miscellaneous Crenarchaetal group (MCG) is another one of the dominant groups of *Crenarchaeota* in marine sediments. As the name indicates, the ecological roles and metabolisms of the diverse representatives are still unclear and no isolate is available. MCG *Crenarchaeota* were often found in deep subsurface sediments from, e.g., the Okhotsk sea (Inagaki et al., 2003), Nankai Trough (Reed et al., 2002; Newberry et al., 2004), Juan de Fuca marine basalt (Huber et al., 2006), terrestrial subsurface(Chandler et al., 1998), and hydrothermal vents such as Guaymas Basin (Ehrhardt et al., 2007). They were found not only in marine but also in terrestrial (e.g. at Yellowstone hot springs (Barns et al., 1996) and limnic habitats (Jiang et al., 2008). This group is phylogenetically as diverse as the phylum *Proteobacteria*. This explains the wide distribution of MCG regardless of environmental factors such as temperature and salinity. They were mostly detected in SMTZ, and suggested to be heterotrophic organisms (Biddle et al., 2006). Currently, incorporation of ^{13}C-acetate by MCG subgroups have been shown by stable-isotope probing experiments on tidal sediment

slurries from the sulfate-reduction zone (Webster et al., 2010). This group of *Crenarchaeota* might be involved in AOM in the SMTZ, but for the functional investigation further studies are required. A single fosmid which contains the 16S rRNA sequence of MCG has been analyzed and shown to contain putative bacteriochlorophyll a synthase (Meng et al., 2008). However, MCG crenarchaeota have been detected mainly in dark, deep subsurface sediments and it is unlikely that they perform photosynthesis using this gene. So far the function of the group remains unknown.

1.5 The phylum *Chloroflexi*

The phylum *Chloroflexi* forms a deep branch within the domain *Bacteria*. It is phylogenetically also as diverse as the phylum *Proteobacteria* (Dojka et al., 2000) and currently divided into six major classes, *Anaerolineae* (Yamada et al., 2006), *Caldilineae* (Yamada et al., 2006), "Chloroflexi", "Dehalococcoidetes", *Ktedonobacteria* (Cavaletti et al., 2006), and *Thermomicrobia* (Hugenholtz and Stackebrandt, 2004). Based on comparative 16S rRNA sequence analysis Rappé and Giovannoni (2003) divided the phylum in eight sub-groups. The phylogenetic diversity of *Chloroflexi* reflects their metabolic diversity. Members of this phylum can perform respiration, fermentation, dehalorespiration as well as anoxygenic photosynthesis. The habitats are diverse as well including hot springs (Nakagawa and Fukui, 2002, 2003; Kubo et al., 2011), wastewater treatment reactors (Yamada et al., 2005), or microbial mats with/without cyanobacteria. Cultivated representatives are mostly limited to thermophiles of filamentous or coccoid morphotypes.

Sequences of marine *Chloroflexi* have been often detected from water column (Morris et al., 2004; Varela et al., 2008), cold marine sediments (Dang et al., 2009), tidal flat sediments (Wilms et al., 2006), and as symbionts in marine sponges (Siegl and Hentschel, 2010). Even in deep sea subsurface sediments, they are often detected in clone libraries and sometimes they seem to dominate the habitat (Inagaki et al., 2006a; Li et al., 2008; Blazejak and Schippers, 2010). A metagenomic approach using pyrosequencing supported the finding of *Chloroflexi* as abundant group in deep subsurface sediment (1 to 50 mbsf) of Peru Margin (Biddle et al., 2008). However, the abundance and functions of marine *Chloroflexi* are poorly understood.

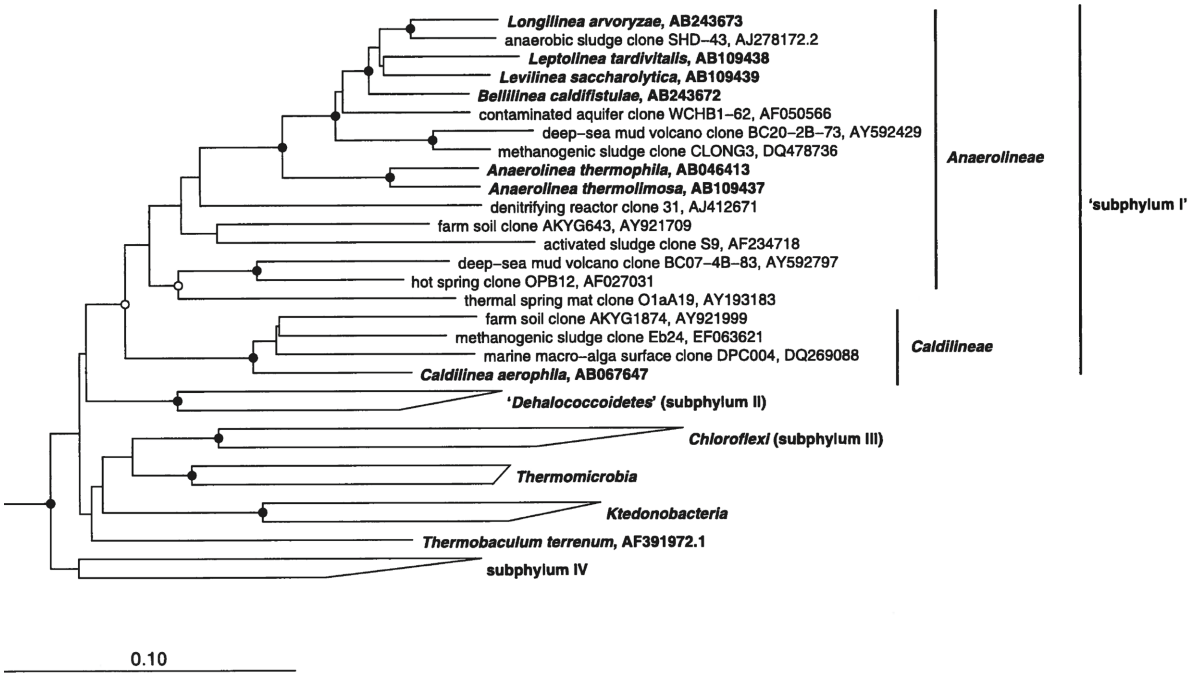

Figure 1-3 Evolutionary distance dendrogram of the bacterial phylum *Chloroflexi* derived from a comparative analyses of 16S rRNA gene sequences (from Yamada and Sekiguchi, 2009).

1.6 *In situ* detection and quantification of microbes in environmental samples

Unlike macroorganisms like animals and plants microorganisms can not be distinguished based on their morphology since it lacks diversity. It was actually the comparative sequence analysis of nucleic acids that has modernized the detection, identification and quantification of microorganisms (Amann et al., 1995).

The ribosomal rRNA based approach is currently still the most often used method in microbial autecology (Amann et al., 1995). Ribosomes are complexes of ribonucleic acids and structural proteins that catalyze protein synthesis in the cells. Virtually all organisms have this protein synthesis machinery. The small subunit of the ribosome contains the 16S rRNA, which consists of about 1500 nt. Significant parts of the 16S rRNA are quite strongly conserved in all organisms, however, some regions are variable enough to distinguish species, ultimately by a single base difference. Those characters allowed the use of rRNA molecule as taxonomic marker for identification of microbial cells, for example whole cell hybridization (Amann et al., 1995) including visualization.

Fluorescence in situ hybridization (FISH) is used for taxonomic identification and quantification of microbial cells (DeLong et al., 1989; Amann et al., 1990; Amann and Fuchs, 2008). Taking an advantage of the enormous collection of the 16S rRNA gene sequences

from both pure cultures and environmental samples in public databases (EMBL/DDBJ/GenBank) and curated databases like SILVA (Pruesse et al., 2007), one can design group-specific oligonucleotide probes based on an alignment with relevant numbers and quality of the sequences, and test it *in silico*. Fluorescently-labeled oligonucleotide probes hybridize to the rRNA in the microbial cells, and by washing off the excess probes, only the target group shows signals. Experimental optimization for cell permeabilization and denaturant concentration (formamide) is required for each sample and probe; however the entire protocol requires only a couple of hours. Since ribosomal content is known to be positively correlated with metabolic activity of cells (Schaechter et al., 1958; DeLong et al., 1989; Kemp et al., 1993; Wallner et al., 1993), it is possible to assess the activity of distinct target organisms in environmental samples by using FISH. The big advantage of the method is to visualize the cells under a microscope. Thus, FISH can be combined with direct cell counting. Even if the activities of microorganisms and their cellular rRNA contents are low, it is still possible to detect and enumerate cells by combining FISH with an amplification step such as catalyzed reporter deposition (CARD) (Pernthaler et al., 2002). In addition, the method can be applied to complex habitats like microbial mats to quantify unusually dense cell accumulations or long filamentous cells with the help of image analysis, and to visualize the localization of target microorganisms in mat structures (Amann et al., 1992; Treude et al., 2007).

Slot-blot hybridization is another method to quantify microorganisms (Stahl et al., 1988). In contrast to FISH, it is based on the quantification of the amount of extracted nucleic acids. In a commonly used set-up total RNA is blotted on a nylon membrane, immobilized, and hybridized with specifically designed rRNA-targeted oligonucleotide probes at stringent hybridization and washing conditions. By using multiple probes, one can calculate the total amount of specific rRNA in a sample. The advantage of the method is that there is no amplification process required such as PCR, and no bias by a lack of cell permeabilization (as possible for FISH). One main bias of slot blot hybridization is a variable efficiency of nucleic acid extraction from different microorganisms (e.g., from those with rigid cell walls). Furthermore, degradation of RNA during storage can occur; however, this is a problem for all other nucleic acid based quantification methods as well. The sensitivity of the slot-blot hybridization is high when using radioactively labeled oligonucleotide probes. A minimum of 0.1 ng of RNA can be detected. This method allows us to handle many samples at a time with high accuracy, even though the total amount of nucleic acid in the sample is low, for example in extractions from deep sea subsurface sediments.

Quantitative PCR (qPCR, real-time PCR) is one of the quantitative methods based on PCR amplification (Suzuki et al., 2000; Ponchel et al., 2003; Bustin et al., 2009). The principle of the method is a quantification of the copy numbers of a specific gene in a sample by measuring the amount of amplicons at every cycle of PCR, and compare it to the amplification behavior of known reference DNA under the same condition. Besides aware of some biases caused by the nucleic acid extraction, the amplification steps based on annealing temperature and the coverage of the primer sets, the method is quick and gives the possibility to handle many samples at one time. Especially for sediment samples the inhibition of PCR by co-extracted humic substances might be problematic. However, by applying appropriate dilutions of the template DNA, this effect could be neglected (Lloyd et al., 2010). It is known that the 16S rRNA gene copy numbers differ between taxonomic clades, and there is no chance to know the copy number for uncultured microorganisms until the genome sequence has been read.

1.7 Aims of this thesis

Massive 16S rRNA gene sequencing suggested a high abundance of MCG and *Chloroflexi* in marine sediments. However, there are no quantitative data available showing the distribution and abundance of these groups. The main aim of this doctoral thesis was to quantify uncultured MCG and *Chloroflexi* in marine sediments using cultivation-independent approaches, and investigate their distribution patterns in the environments. Since both groups of the microbes have not yet been isolated, visualization and quantification of them were done by a polyphasic approach including fluorescence *in situ* hybridization, rRNA slot blot hybridization and quantitative PCR to obtain validate data. Not only sediments, but also microbial mats were analyzed to study their distribution patterns within the mat.

2 General Discussion and Future Perspectives

In the following the results which I obtained in my doctoral thesis are discussed in a general context. The detailed results of the three manuscripts will not be repeated, yet I will focus on the methodological problems which had to be overcome during this thesis and on future perspectives.

2.1 Methodological aspects of the quantification and visualization of marine benthic *Crenarchaeota*

When I started my doctoral thesis in April 2008 there has been only circumstantial evidence that the Miscellaneous Crenarchaeotal Group is abundant in deep sea sediment. All the data were either obtained from 16S rRNA gene libraries (Biddle et al., 2006; Inagaki et al., 2006b; Sørensen and Teske, 2006), pyrosequencing of tagged 16S rRNA gene amplicons (Biddle et al., 2008), or lipid biomarkers (Lipp et al., 2008). In this thesis I analyzed eleven different habitats, and for most multiple depth layers. The quantification of MCG was done with three independent molecular methods, i.e. slot-blot hybridization, qPCR and FISH. Relative abundances of MCG were generally increasing with sediment depth. In methane-rich surface sediments MCG abundance was below 3% of total archaea, but on the other hand in subsurface sulfate-methane transition zones MCG were abundant as 15-100% in archaeal community. This indicates a major role in biogeochemical cycles in deep sediments.

2.1.1 Problems with slot blot hybridization

Slot blot hybridization is in theory a simple technique to quantify the rRNA of a distinct population, but in practice it turned out to be rather complicated. There were a couple of difficulties that I had to solve before the quantification of MCG could be performed in this study.

(1) Sufficient amounts of rRNA were difficult to extract from subsurface sediments. This had to be expected to a certain extent (Webster et al., 2003). However, in some cases the problem could not be solved and it was necessary to combine all the rRNA extracted from two different depth layers to have sufficient amounts for slot-blot hybridization. This should be

ideally avoided, because finally it makes the data interpretation difficult. Future applications of slot-blot hybridization for quantification of populations in subsurface environments can only be recommended if the efficiency of nucleic acid extraction can be improved or if the sensitivity for the quantification of bound probe can be increased.

(2) An unexpectedly high amount of MCG was detected in Hydrate Ridge sediments where ANME archaea dominate, when using the MCG-specific probe MCG717 (5'-ACA GCC TTC GCC ACT GGT-3', T_d = 55°C). Although the dissociation temperature was optimized and the probe coverage had been checked *in silico*, the results indicated an unspecific binding of the probe to non-target rRNA. When another probe (MCG493) was used, the results were more plausible and also comparable with qPCR data. This quantification error could be explained by an inappropriate T_d used for the hybridization with MCG717. Before future experiments with probe MCG717 are conducted it is strongly recommended to evaluate this probe in the context of the other two probes which have a similar target coverage.

(3) In general, the use of a universal probe such as UNIV1390 (Zheng et al., 1996) is recommended to quantify the total amount of rRNA. However, in this study the sum of archaeal rRNA (as detected by probe ARCH915) and bacterial rRNA (as detected by probe EUB338) had to be used instead, because of high background signals on some blots.

2.1.2 Problems with qPCR

qPCR is a rapid method to quantify the copy number of the target gene (in this case 16S rRNA gene). However, it requires a lot of technical considerations, thus many recommendation guidelines are published, for example The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009). In my study major considerations were the following: (1) Inhibition of amplification might be caused by co-extracted humic substances in the DNA. Humic acids which are abundant in soil or marine sediments are known to inhibit PCR reactions (Tsai and Olson, 1992). This has been solved to dilute the template DNA adequately (Lloyd et al., 2010). (2) A good coverage primer set is essential for the accurate quantification by qPCR. This was challenging for the highly diverse group MCG. The primers have been designed based on curated 16S rRNA sequences and showed almost complete coverage of MCG. However, in the meanwhile the 16S rRNA database has expanded so largely that the coverage of the MCG

primers was much lower (16%) than before. At least the mismatches were mostly central or towards the less important 5' end of the primer. Yet, future qPCR applications need to consider the exponential growth of databases and need to adjust primer set accordingly.

2.1.3 Visualization of MCG in marine sediments

When I started my doctoral study, MCG had not been visualized. Visualization and quantification of MCG cells were therefore a central goal of this study. Based on the observation of Biddle and colleagues (2006) using scanning electron microscopy, the morphology of the abundant *Archaea* in marine subsurface SMTZ, presumably MCG or MBGB, was coccoid. Based on quantitative results obtained in this study by slot-blot hybridization and qPCR, I selected sediment samples of MCG "hot spots", and applied specific probes for MCG. Initially, the visualization of the *Crenarchaeota* was difficult due to the following reasons: (1) there was no positive control such as pure culture of MCG, causing severe problems with the optimization of hybridization conditions, especially the cell permeabilization step. (2) DAPI staining does not work for most samples from deep subsurface. (3) There was a high background fluorescence caused by sediment particles.

After many optimization experiments, I noticed cells were too fragile to bear the normal condition of cell permeabilization such as SDS, proteinase K and lysozyme treatment. Those normal conditions were also tested on closest (but still far) cultured crenarchaeote *Nitrosopumilus maritimus* and it also showed blurry cell morphologies (data not shown). Thus, finally I came up to use relatively mild permeabilization condition as 0.01 M HCl containing 0.15% hydrogen peroxide for 10 minutes incubation. With this protocol I achieved to permeabilize and inactivate the endogeneous peroxidase at once, and visualize the MCG cells. MCG cell morphology was coccoid with a diameter of 0.4-0.5 μm. Infrequently, loose cell aggregates were observed which consisted of 4-5 cells. In rare cases, bigger cells with a diameter of 1 μm were also observed. Cell sizes known for *Thaumarchaeota*, which are relatives of MCG, were much bigger than those that I found for MCG. To confirm that the visualized cells are really belonging to MCG, I performed double hybridizations with MCG-specific probes and the archaeal probe ARCH915. The hybridization showed clear overlaps of both signals and DAPI staining, at least for White Oak River sediment samples. In contrast, the problem with DAPI staining of cells from deep subsurface sediment could not be solved in my doctoral thesis. This might be due to the small cell sizes and a low content of double stranded DNA. The cell abundance in subsurface sediments has conventionally been

evaluated by acridine orange direct cell counts (AODC, Cragg et al., 1995; Parkes et al., 2000). In the last decade, SYBR-I or SYBR-II have also been used due to its higher fluorescence intensity and sensitivity to nucleic acids (Weinbauer et al., 1998; Engelen et al., 2008). Recently, Morono and colleagues (2009) improved the SYBR Green I staining protocol by washing the sediment slurries with hydrofluoric acid which resulted in significantly reduced non-biological fluorescent signals such as amorphous silica and enhanced the efficiency of cell detachment from particles. Nevertheless, all these protocols for cell staining can not yet been combined with the CARD-FISH protocol. Future CARD-FISH experiments in subsurface sediments would certainly benefit from further systematic attempts to improve the visualization of single microbial cells by a better DNA staining protocol for counterstaining.

High background fluorescence from sediment particles was also a major problem. Some sediment particles showed a strong autofluorescence, and they were of a size as small as cells. In addition, the relatively weak FISH signals of MCG cells make any fluorescent background more difficult. For the yet best discrimination of cells from the rather reddish-brownish fluorescent background, I recommend to use green-fluorescent Alexa488-labeled tyramides for CARD-FISH. The fluorescence dye is bright and is clearly different from background fluorescence. In addition by using dual-hybridizations the reliability was improved.

With this protocol I counted cells and compared the results with those from other quantitative methods. In most cases the relative abundance of the cells was in the same range as that obtained by slot-blot hybridization and qPCR data. Discrepancy was observed for White Oak River sediments. With qPCR nearly 100% of archaea were assigned to MCG, while only 22-60% of archaeal cells were detected as MCG by FISH.

The CARD-FISH protocol developed in this study can be now used for monitoring of MCG in the environment. Another important future application could be the monitoring of MCG abundance in cultivation experiments. Furthermore, by combining this CARD-FISH protocol with nano-scale secondary ion mass spectrometry (NanoSIMS), it would be possible to assess whether MCG in fact are incorporating particular substrates more rapidly than others, thereby allowing to enter into studies of MCG ecophysiology.

2.2 Methodological aspects of the monitoring of sulfur-metabolizing bacterial population in a terrestrial hot spring microbial mat

Terrestrial hot springs share many common features with hydrothermal vent sites in marine systems. Temperature is high, and fluid usually contains reduced elements such as sulfide. The major difference is the availability of light and oxygen.

The Nakabusa Hot Spring in Japan is well known for its colorful and thick microbial mats developing in the stream of geothermally heated water (Nakagawa and Fukui, 2002 and 2003) There is a temperature gradient according to the flow of water, depending on the distance from the spring source, and different colored microbial mats distribute according to varying temperature. The water is slightly alkaline and contains sulfide. At 50-65°C, the microbial mat has been shown to contain cyanobacteria and filamentous anoxygenic phototrophic bacteria by spectrophotometry (Sugiura et al., 2001) and by cultivation (Hanada, 2003).

In this doctoral thesis, I examined the distribution patterns of photosynthetic *Chloroflexi* (*Chloroflexus aggregans*) and *Aquificae* (*Sulfurihydrogenibium* spp.) in microbial mats grown at 65°C of the Nakabusa Hot Spring. By CARD-FISH, I could determine the vertical distribution pattern of the two members of microbes in the microbial mat. Sulfur-oxidizing *Sulfurihydrogenibium* spp. were mostly distributed in the surface layer of the microbial mat while *Chloroflexi* were relatively homogeneously distributed in the entire mat. Other potential key players, sulfate-reducing bacteria, could not be detected by FISH but by cloning of 16S rRNA and *aprA* genes. According to the physiological experiments, active biological sulfide oxidation was observed under oxic conditions. Thus, I concluded that sulfur-oxidizing bacteria are actively scavenging oxygen in the surface layer of the microbial mat, and produce a favorable environment for other microbes like *Chloroflexi* and sulfate-reducing bacteria.

Again there were problems with FISH: (1) Thick huge rod-shaped cells were not stained by probes EUB338 I-III and (2) presence of *Thermodesulfobacterium*-like sulfate-reducing bacteria indicated by comparative sequence analysis could not be confirmed and visualized.

After testing some general probes on mat sections, I noticed there are many cells which are not stained by probes EUB338 I-III. These cells were consistently thick and long rods (10 μm). By an alignment of 16S rRNA gene sequences which I obtained from the clone library and DGGE analysis it became evident that there were at least two mismatches between the EUB338 I-III probes and the *Sulfurihydrogenibium*-like sequences. It has been reported that

the phylum *Aquificae* has strong mismatches against probes EUB338 I-III (Daims et al., 1999), thus I designed a new probe, AQI338, which is a modified EUB338 probe to cover the phylum *Aquificae*.

In contrast, the visualization of *Thermodesulfobacterium*-like sulfate-reducing bacteria in the microbial mat was not achieved in this thesis. Future attempts to visualize them should use alternative probes, since the probe used in this study was highly specific to a small clade, and most of sequences retrieved from the clone library fall in a clade not covered by this probe.

2.3 Distribution of marine *Chloroflexi*

In a side project of my doctoral studies I also investigated the distribution of *Chloroflexi* in marine sediments and microbial mats. Filamentous *Chloroflexi* could be observed in most of the habitats which I screened, including hydrothermal vents, cold seeps, and surface sediments. Numbers were <3% except for Black Sea microbial mats in which filamentous *Chloroflexi* accounted for up to 28% of total cells. Cells which hybridized with the *Chloroflexi*-specific probes were sorted by fluorescence activated cell sorting (FACS), and the filamentous cells were successfully sorted out. For further functional analysis, several PCR attemps to amplify the 16S rRNA gene in the sorted cell fractions were performed on about 100 filaments using different primer combinations. Unfortunately no reliable amplification of the genes of interest was achieved. There are several possibilities for the failure of PCR, e.g. inhibition by HRP-labeled probes and tyramide conjugates, suboptimal cell fixative or too high annealing temperatures. Further experiments are required to optimize PCR conditions.

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4 List of publications

1 **Kyoko Kubo, Karen Lloyd, Jennifer Biddle, Andreas Teske, Rudolf Amann, and Katrin Knittel.** Miscellaneous Crenarchaeotal Group (MCG), a dominant archaeal lineage in subsurface marine sediments.
Submitted to ISME J.

Concept was developed by K. Kubo and K. Knittel. Experiments were done by K. Kubo with help of J. Biddle and K. Lloyd. Data analysis was done by K. Kubo, R. Amann and K. Knittel. The Manuscript was written by K. Kubo and edited by K. Knittel and R. Amann.

2 **Kyoko Kubo, Katrin Knittel, Rudolf Amann, Manabu Fukui, and Katsumi Matsuura.** 2011. Sulfur-metabolizing bacterial populations in microbial mats of Nakabusa hot spring, Japan. Syst. Appl. Microbiol. **34:** 293-302

Concept was developed and conceived by K. Kubo and K. Matsuura. Experiments were done by K. Kubo. K. Knittel designed the FISH experiment and R. Amann the experiment for quantification of the biovolume. The Manuscript was written by K. Kubo and edited by K. Knittel and R. Amann. M. Fukui further developed the overall concept and commented on the manuscript.

3 **Kyoko Kubo, Julia Arnds, Juliane Wippler, Rudolf Amann, and Katrin Knittel.** *In situ* identification and quantification of *Chloroflexi* in marine sediments and microbial mats. In preparation.

K. Kubo, J. Arnds and K. Knittel developed the concept. Experiments were done by K. Kubo, J. Arnds, and J. Wippler. The Manuscript was written by K. Kubo and edited by K. Knittel and R. Amann.

5

Manuscripts

1

Miscellaneous Crenarchaeotal Group (MCG), a dominant archaeal lineage in subsurface marine sediments

Kyoko Kubo, Karen Lloyd, Jennifer Biddle, Andreas Teske,
Rudolf Amann, and Katrin Knittel

Manuscript has been submitted to ISME Journal

Miscellaneous Crenarchaeotal Group (MCG), a dominant archaeal lineage in subsurface marine sediments

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Members of the highly diverse Miscellaneous Crenarchaeotal Group (MCG) are globally distributed in various marine and continental habitats. Although massive 16S rRNA sequencing as well as intact lipid biomarker analysis suggested MCG as an abundant archaeal group in subsurface marine sediments, quantitative data are still lacking. In this study we applied a polyphasic approach (rRNA slot blot hybridization, quantitative PCR, and CARD-FISH) using newly developed probes and primers for the quantification of MCG Crenarchaeota in diverse types of marine sediments and microbial mats. In general, relative abundance of MCG strongly increased with sediment depth. While in methane-rich surface sediments MCG abundance was below 3% of total archaea, MCG constituted the major part of the archaeal community with 15-100% in subsurface sulphate methane transition zones thus indicating a major role in biogeochemical cycles. Furthermore, we provided an adapted CARD-FISH protocol for in situ visualization of MCG cells, to facilitate future studies addressing the metabolic capabilities of MCG.

Up until the 1990's members of the archaeal phylum *Crenarchaeota* were considered to be extremophiles. Cultivated strains were thermophilic or hyperthermophilic organisms utilizing sulfur for energy metabolism (Burggraf et al., 1997). Then DeLong (1992) and Fuhrman and colleagues (1992) discovered high numbers of pelagic crenarchaeota in the marine water column indicating the presence of mesophilic or psychrophilic species. In 2001, it was estimated that pelagic crenarchaeota represent one of the most abundant cell types with up to $1.3x10^{28}$ cells in the ocean (Karner et al., 2001), a number that was later confirmed in an independent study with $2.7x10^{28}$ cells in the world ocean (Schattenhofer et al., 2009). Mesophilic pelagic crenarchaeota have been assigned to Marine Group I (MG I), a sister group of thermophilic crenarchaeota. Recently, a third archaeal phylum, the *Thaumarchaeota*, was proposed for these organisms because of their distinct phylogeny and physiology (Brochier-Armanet et al., 2008). Cultivated strains are autotrophic ammonium-oxidizing archaea (Preston et al., 1996; Könneke et al., 2005; Muller et al., 2010; Tourna et al., 2011).

In marine sediments, two other distinct, phylum-level crenarchaeotal groups have been repeatedly found: the Marine Benthic Group B (MBGB) and Miscellaneous Crenarchaeotal Group (MCG). While the former is mostly restricted to marine habitats (a few sequences are from lakes), the latter is cosmopolitan and has been found at marine and continental sites. MCG comprises sequences from numerous marine or marine-influenced habitats such as deeply buried subsurface sediments at Peru Margin (Biddle et al., 2006; Inagaki et al., 2006; Sørensen and Teske, 2006), Cascadia Margin (Inagaki et al., 2006), Brazos Trinity and Ursa Basin in the Gulf of Mexico (Nunoura et al., 2009), hydrothermal vent sediments at Mariana Trough (Kato et al., 2009) and Lomonosov Ridge (Forschner et al., 2009), mud volcanoes in the Mediterranean Sea (Heijs et al., 2008; Kormas et al., 2008) or the Arctic Sea (Lösekann et al., 2007), brackish marine sediments at Aarhus Bay (Aquilina et al., 2010), salt marshes (Nelson et al., 2009), estuaries (Meng et al., 2009), mangrove soils (Lyimo et al., 2009). In addition, MCG comprises many sequences from continental habitats such as groundwater (Shimizu et al., 2007), hot springs (Barns et al., 1994; Meyer-Dombard et al., 2005), waste water sludges (Rivière et al., 2009), gold mine subsurface geothermal waters (Hirayama et al., acc.no. AB113635), gas fields (Mochimaru et al., 2007), freshwater lake sediments (Borrel, acc.no. GU135501, GU135490) or wetlands (Lai & Sun, acc.no. EU420699). All members of MCG and MBGB are uncultured.

MCG sequences were most frequently found near marine subsurface sulphate-methane transition zones (SMTZ). Biddle et al. (2006) showed that these crenarchaeota are heterotrophic using organic carbon derived from degradation of fossil organic matter. They

hypothesized that MCG and MBGB might perform "dissimilatory" methane oxidation (oxidation of methane without assimilation of its carbon) based on two facts: i) the lack of ^{13}C-depletion in archaeal biomass as determined by FISH-secondary ion mass spectrometry and ii) the high relatively fraction of total Gibbs free energy changes in SMTZs accounted for by anaerobic oxidation of methane (AOM). Despite the frequent observation of MCG Crenarchaeota in clone libraries there are no quantitative data for their in situ distribution available yet. Such data are needed for a better understanding of their ecology and ecophysiology.

In this study we used a polyphasic approach, including rRNA slot-blot hybridization, DNA-based quantitative PCR, and CARD-FISH, to investigate the distribution of MCG in eleven different habitats. Our data clearly showed that MCG crenarchaeota constitute a major part of the marine deep subsurface microbial community while they are rare in surface sediments. Furthermore, we provide a protocol for in situ visualization and enumeration of MCG cells.

Materials and methods

Study sites and sampling

Eleven sites were chosen for quantification of MCG on the basis of available biogeochemical and microbial diversity data. The sites are: (1) Smeerenburgfjorden, Svalbard, Arctic Ocean is a coastal, permanently cold silty sediment (Ravenschlag et al., 2000). (2) Janssand, North Sea is an intertidal flat located in the back barrier of the island of Spiekeroog (Gittel et al., 2008). (3) Haakon Mosby Mud Volcano (HMMV, Norwegian Sea) is an active submarine mud volcano (Niemann et al., 2006b). (4) Nyegga area, representing methane seeps in pockmarks located on the edge of the Norwegian continental slope. (5) Hydrate Ridge, Cascadia Margin, Pacific Ocean, is a cold seep characterized by discrete methane hydrate layers exposed at the seafloor (Boetius et al., 2000). (6) The Gulf of Mexico, is a cold seep characterized by hydrocarbon seepage of variable composition (Orcutt et al., 2010). (7) Peru Margin in the Pacific Ocean, ODP leg 201 sites 1227 and 1229, organic rich clayish and silty sediments (Parkes et al., 2005; Inagaki et al., 2006). (8) Equatorial Pacific Ocean and Peru Basin at open ocean ODP sites 1225 and 1231, organic poor sediments (D'Hondt et al., 2004; Meister et al., 2005). (9) Black Sea methane seeps, microbial mats cover massive carbonate chimneys (Michaelis et al., 2002) (10) White Oak River Estuary of North Carolina, USA, is a small coastal basin characterized by a high total organic carbon content and methane gas-rich

sediments (Lloyd et al., in press). (11) In Abu Dhabi on the Arabian Gulf, is an intertidal hypersaline microbial mat influenced by strong salinity fluctuations and high temperatures (Kohls, 2010). For details see Table 1 and Supplementary Information.

Table 1 Sampling sites characteristics

	Cruise	Site	Sample type	Latitude/ Longitude	PANGAEA event label[□]	Sampling Date	Water depth [m]	Chemosynthetic Communities and Geochemistry	References
Svalbard, Arctic Ocean		Smeerenburgfjorden station J	coastal surface sediments	79° 42.82' N 11° 05.19' E	not available	Jul. 1998	218	light grey until 2-4 cm, silt and very fine sand, rich in macrofauna, permanently cold (near 0°C)	Ravenschlag et al., 2000; Jørgensen et al., 2010
North Sea, German Wadden Sea		Janssand	intertidal flat surface and subsurface sediments	53° 44.18' N 7° 41.97' E	not available	Nov. 2004/ Apr 2005	-	sandy sediments, SMTZ at ca. 50 cmbsf and below 400 cmbsf	Gittel et al., 2008
Nyegga area, Norwegian Sea	Vicking	station 272-02	cold seep surface sediments	64° 39.79' N 5° 17.30' E	VKGD272/PC-2	May 2006	733	pockmarks in Nyegga area	Van Gaever et al., 2010
Haakon Mosby Mud Volcano (HMMV), Norwegian Sea	ARKXIX-3b	station 372	subsurface sediments	72° 0.26' N 14° 43.59' E	PS64/372-1	Jul. 2003	1250	mud volcano crater center site, high fluid flux, mainly aerobic oxidation of methane	DeBeer et al., 2006; Niemann et al., 2006
		station 371	subsurface sediments	72° 00.20' N 14° 43.88' E	PS64/371-1	Jul. 2003	1250	surrounding area covered by *Beggiatoa* mats	DeBeer et al., 2006; Niemann et al., 2006
		station 336	subsurface sediments	72° 0.02' N 14° 43.57' E	PS64/336-1	Jul. 2003	1250	sediments covered by siboglinid tube worms (*Pogonophora*)	DeBeer et al., 2006; Niemann et al., 2006
Cascadia Margin, Hydrate Ridge	SO148-1	station 19-2	cold seep surface sediments	44° 34.10' N 125° 08.81' W	SO148/1_19-2	Aug.2000	777	gas hydrate, high fluid flux, AOM and SRR, sediments covered by *Beggiatoa* mats	Knittel et al., 2005 and references therein
	SO143-2	station 105	cold seep surface sediments	44° 34.14' N 125 °08.81' W	SO143_105-1	Aug.1999	780	gas hydrate, high fluid flux, AOM and SRR, sediments covered by *Beggiatoa* mats	Knittel et al., 2005 and references therein
	SO148-1	station 38	cold seep surface sediments	44° 34.19' N 125 °08.85' W	SO148/1_38	Aug.2000	787	gas hydrate, high fluid flux, AOM and SRR, sediments populated by *Calyptogena*	Knittel et al., 2005 and references therein
	SO143-2	station 185	cold seep surface sediments	44° 34.19' N 125 °08.83' W	SO148_185-1	Aug.1999	785	gas hydrate, high fluid flux, AOM and SRR, sediments populated by *Calyptogena*	Knittel et al., 2005 and references therein
	ODP Leg 204	1245D	subsurface sediments	44° 35.17' N 125 °8.93'W	204-1245D	Aug. 2003	870	sand and silty clay, methane hydrate, high flux, SMTZ at 7mbsf	Tréhu et al., 2003
		1250D	subsurface sediments	44° 34.11' N 125 °9.02'W	204-1250D	Aug. 2002	796	high flux, no SMTZ in the first 20m only hydrates, high methane, low sulfate, high alkalinity because of upward fluid advection, rapid hydrate formation	Tréhu et al., 2003
Gulf of Mexico	SO174	GC234, station 87	cold seep surface sediments	27° 44.73' N 91° 13.33' W	SO174/1_87	Oct./ Nov. 2003	552	very little oily and carbonate, low particulate organic carbon content (2 wt%), high AOM-independent sulfate reduction rates, sediment covered by orange *Beggiatoa*	Orcutt et al., 2010
	SO174	GC185, station 156	cold seep surface sediments	27° 46.95' N 91° 30.47' W	SO174/2_156	Oct./ Nov. 2003	546	very gassy, near oily hydrate, high AOM rates, sediments covered by sulfide-oxidizing bacteria and tubeworms	Orcutt et al., 2010
Eastern Equatorial Pacific Ocean	ODP Leg 201	1225A	subsurface sediment	2° 46.25´N 110° 34.29´W	201-1225	Feb.2002	3761	deep open ocean site, low organic carbon content (<1% TOC)	D'Hondt et al., 2003, 2004
Peru Margin	ODP Leg 201	1227A	subsurface sediments	8° 59.50´S 79° 57.35´W	201-1227A	Mar.2002	427.5	SMTZ at ca. 40 mbsf, high organic carbon content (1-10% TOC)	D'Hondt et al., 2003, 2004
	ODP Leg 201	1229D	subsurface sediments	10° 58.57´S 77° 57.47´W	201-1229D	Mar.2002	152	SMTZ at ca. 30 and 88 mbsf, high organic carbon content (1-7% TOC)	D'Hondt et al., 2003, 2004
Peru Basin	ODP Leg 201	1231	subsurface sediment	12° 1.26´S 81° 54.24´W	201-1231	Mar.2002	4813	deep open ocean site, low organic carbon content (<1% TOC)	D'Hondt et al., 2003, 2004
White Oak River estuary, North Carolina, USA		Station H	anoxic estuarine sediments	34°44.49' N 77°07.44' W	not available	Jul 2008	ca. 1	tidally influenced brackish estuary, organic-rich, muddy sediments with diffusion-controlled AOM at stable SMTZ in ca. 30 to 40 cmbf; no advection or venting	Lloyd et al., in press
Black Sea, Dniepr area	P317/3	P822	cold seep microbial mat	44° 46.54′ N 31° 58.98′ E	PO317/3-822	Oct.2004	190	microbial mat at covering carbonate build-ups at methane seeps in anoxic waters, high AOM rates	Rossel et al., 2008
Arabian Gulf		Abu Dhabi	intertidal flat hypersaline mat, oxic part		not available	Dec.2006		temperature >50°C in the summer; salinity fluctuated from 6% to 22%	Kohls, 2010

TOC, Total organic carbon

□http://www.pangaea.de/

Phylogenetic analysis and probe design

The phylogeny of MCG sequences was inferred with the ARB software package (Ludwig et al., 2004) based on Release 104 of the ARB SILVA database (Pruesse et al., 2007). In total, 2827 nearly full-length crenarchaeotal sequences (>1250 bp) of which 385 belong to MCG were used for tree reconstruction. Phylogenetic trees were calculated by maximum likelihood analysis (PhyML, RAxML) and neighbor-joining algorithm. A 50% base frequency filter was used for 16S rRNA gene tree calculation to exclude highly variable positions. The resulting phylogenetic trees were manually compared and a consensus tree was constructed. Relevant partial sequences were subsequently added to the tree according to maximum parsimony criteria, without allowing changes in the overall tree topology.

Probes/primers MCG493, MCG528, and MCG732r were designed using the ARB probe design tool and evaluated by the Probe Match function, using the SILVA database release 92 (Sept. 2007, (Ludwig et al., 2004). Probes/primers all had perfect matches to the majority of marine MCG, and at least one mismatch to other *Crenarchaeota*, *Euryarchaeota*, *Bacteria*, and *Eukarya*.

Table 2 Oligonucleotide probes and primers used in this study

Probe/primer	Specificity	Sequence (5' - 3')	Target site[a]	Slot-blot T_d (°C) [b]	CARD-FISH FA conc[c]	Reference
ARCH915	most *Archaea*	GTGCTCCCCCGCCAATTCCT	915-934	56	35	Stahl and Amann, 1991
Arch806f	most *Archaea*	ATTAGATACCCSBGTAGTCC	787-806	NU	NU	Takai et al., 2000
EUB338 I-III	most Bacteria	GCTGCCTCCCGTAGGAGT	338-355	54	35	Amann et al., 1990
	supplement to EUB 338: *Planctomycetales*	GCAGCCACCCGTAGGTGT	338-355	NU	35	Daims et al., 1999
	supplement to EUB 338: *Verrucomicrobiales*	GCTGCCACCCGTAGGTGT	338-355	NU	35	Daims et al., 1999
NON338	Antisense of EUB338	ACTCCTACGGGAGGCAGC	338-355	NU	10	Wallner et al., 1993
MCG493	Miscellaneous Crenarchaeotal Group	CTTGCCCTCTCCTTATTCC	493-511	55	20-30	This study
MCG528	Miscellaneous Crenarchaeotal Group	CGGAGAGCTGGTATTACC	529-546	NU	30	This study
MCG528f	Miscellaneous Crenarchaeotal Group	CGGTAATACCAGCTCTCCGAG	528-548	NU	NU	This study
MCG732r	Miscellaneous Crenarchaeotal Group	CGCGTTCTAGCCGACAGC	731-749	NU	NU	This study

[a] Position in the 16S rRNA of E.coli
[b] T_d, dissociation temperature
[c] Formamide (FA) concentration in the hybridization buffer (%, vol/vol)
NU: Not used

Nucleic acids extraction

DNA and RNA were extracted from 1-6 g of frozen sediments using acidic phenol and bead beating based on protocols described previously (Stahl et al., 1988; MacGregor et al., 1997) with slight modifications. Detailed protocols are provided in the Supplementary Information.

Slot blot hybridization

Approximately 10-100 ng of RNA was blotted onto nylon membranes (MagnaCharge Membrane; GE Water & Process Tech., USA) in triplicate and hybridized with ^{33}P labeled oligonucleotides as described previously (Stahl et al., 1988). The probes and dissociation temperatures used in this study are given in Table 2. For probe MCG493, a dissociation temperature of 55°C was determined according the method described by Raskin et al. (1994). Hybridization intensity was measured with a blot imager Typhoon 9400 (GE Healthcare, Germany) and analyzed with ImageQuant software. Reference RNA from *Escherichia coli* served as standard for hybridizations with probe EUB338. As a standard rRNA for testing probe MCG493 and the general archaeal probe ARCH915 we used in vitro transcribed rRNA of clone HMMVCen-DS-Arch2653 (accession no FR852571). The MCG archaeal rRNA clone Aarhus Bay_Arch26 (accession no. FR852573) was used as the transcription template for generating rRNA with a single mismatch to probe MCG493, to evaluate this probe in melting curves. In vitro transcription was performed as described by Ravenschlag et al. (2001).

Quantitative PCR

DNA standards were prepared from TOPO 2.1 plasmids (Invitrogen) containing an insert of a nearly complete, PCR-amplified archaeal 16S rRNA gene (classified into MCG. The DNA concentration was determined with a NanoDrop 1000 Spectrophotometer (V.3.7.1, Thermo Scientific) and confirmed with PicoGreen (Invitrogen) fluorescence in a Roche LightCycler 480 Instrument. Primers Arc806f (Takai and Horikoshi, 2000) and ARCH915r (Stahl and Amann, 1991) were used to amplify total archaea, primer pair MCG528f/MCG732r was used to specifically amplify MCG crenarchaeota. Each 25 µl PCR reaction contained 1 µl DNA template at a ten-fold dilution, 12.5 µl QuantiFast SYBR®Green PCR master mix (Qiagen, Germany), 0.2 µl Arch806f (10 µM) and 0.2 µl Arch915r (10 µM). The qPCR protocol included the following steps: 95°C for 5 min initial denaturation of the template, 30 cycles at 95°C for 10 sec, at 60°C for 30 sec, and at 72°C for 5 sec. Melting curves were obtained from 95°C to 50°C at the speed of 0.11°C/s. All melt curves contained a large single peak at 86°C

with a small shoulder peak at 81°C. No low melting point primer dimers were detected. A Roche LightCycler® 480 and the integrated software Version 1.5 was used to determine the cycle threshold (*Ct*) of each reaction and the efficiency of amplification. Primers used are shown in Table 2.

Catalyzed reporter deposition fluorescence in situ hybridization

Sediment samples were fixed in 3% (w/vol) paraformaldehyde in phosphate-buffered saline (PBS; 130 mM NaCl, 10 mM sodium phosphate [pH 7.3]) for 2-4 hours at 4°C. The sediments were treated by mild sonication with a type MS73 probe (Sonopuls HD70; Bandelin, Germany) at a setting of 20 s, an amplitude of 42 µm, and <10 W. An aliquot was filtered onto a 0.2 µm pore-sized polycarbonate filter (Isopore; Millipore, Germany). Cells were embedded in 0.1% low melting point agarose (NuSieve® GTG® Agarose, Cambrex Bio Science Rockland Inc., ME, USA) and air-dried. Inactivation of endogenous peroxidases and permeabilization of cell walls was done by incubating the filters in 0.01 M HCl with 0.15% H_2O_2 for 10 min at room temperature. CARD-FISH and subsequent staining with DAPI followed a previous published protocol (Pernthaler et al., 2002). For dual CARD-FISH, the protocol was repeated on the same filters using a second probe and other fluorescently labeled tyramides after inactivation of peroxidases of initial hybridization as described above. The given CARD-FISH counts are means calculated from 10 to 150 randomly chosen microscopic fields corresponding to 100-800 total DAPI-stained cells. Images were taken at an epifluorescence microscope (Axioskop II; Zeiss, Germany). Oligonucleotide probes were purchased from Biomers (Germany). Probe sequences and formamide concentrations required for specific hybridization are given in Table 2. The specificity of new MCG probes was evaluated by Clone-FISH (Schramm et al., 2002).

Results

Phylogeny of benthic Crenarchaeota - basis of probe design

As a basis for MCG-specific probe design a phylogenetic tree of crenarchaeotal 16S rRNA was constructed based on the SILVA database release 104 (Oct. 2010). In 2006, four monophyletic subclusters of MCG have been distinguished by Sørensen & Teske (Sørensen and Teske, 2006) namely MCG-1, MCG-2, MCG-3, and MCG-4. Here, we show evidence for two further stable subclusters which we name MCG-5 and MCG-6 (Figure 1). Marine Benthic Group C (Vetriani et al., 1999) with its representative clone sequence CRA9-27is a

part of MCG-5. The yet smallest subclusters MCG-2 and MCG-4 are restricted to sequences retrieved from marine habitats while all others are mixed and contain sequences from both, marine and continental habitats. Overall MCG intragroup diversity was great with a similarity of only 77% between the most distant 16S rRNA gene sequences.

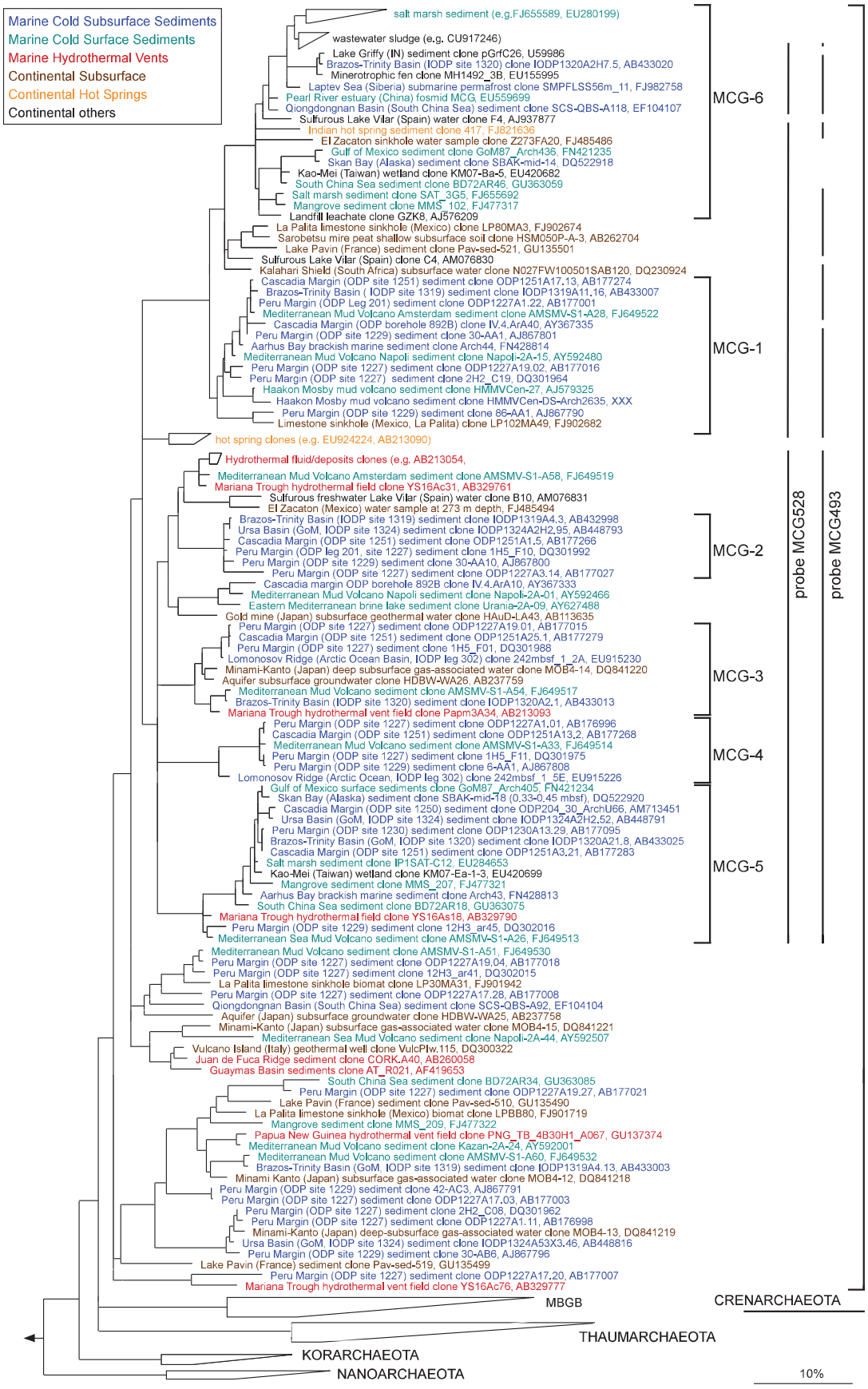
Marine Cold Subsurface Sediments
Marine Cold Surface Sediments
Marine Hydrothermal Vents
Continental Subsurface
Continental Hot Springs
Continental others
salt marsh sediment (e.g.FJ655589, EU280199)
wastewater sludge (e.g. CU917246)
Lake Griffy (IN) sediment clone pGrfC26, U59986
Brazos-Trinity Basin (IODP site 1320) clone IODP1320A2H7.5, AB433020
Minerotrophic fen clone MH1492_3B, EU155995
Laptev Sea (Siberia) submarine permafrost clone SMPFLSS56m_11, FJ982758
Pearl River estuary (China) fosmid MCG, EU559699
Qiongdongnan Basin (South China Sea) sediment clone SCS-QBS-A118, EF104107
Sulfurous Lake Vilar (Spain) water clone F4, AJ937877
Indian hot spring sediment clone 417, FJ821636
El Zacaton sinkhole water sample clone Z273FA20, FJ485486
Gulf of Mexico sediment clone GoM87_Arch436, FN421235
Skan Bay (Alaska) sediment clone SBAK-mid-14, DQ522918
Kao-Mei (Taiwan) wetland clone KM07-Ba-5, EU420682
South China Sea sediment clone BD72AR46, GU363059
Salt marsh sediment clone SAT_3G5, FJ655692
Mangrove sediment clone MMS_102, FJ477317
Landfill leachate clone GZK8, AJ576209
La Palita limestone sinkhole (Mexico) clone LP80MA3, FJ902674
Sarobetsu mire peat shallow subsurface soil clone HSM050P-A-3, AB262704
Lake Pavin (France) sediment clone Pav-sed-521, GU135501
Sulfurous Lake Vilar (Spain) clone C4, AM076830
Kalahari Shield (South Africa) subsurface water clone N027FW100501SAB120, DQ230924
Cascadia Margin (ODP site 1251) sediment clone ODP1251A17.13, AB177274
Brazos-Trinity Basin (IODP site 1319) sediment clone IODP1319A11.16, AB433007
Peru Margin (ODP Leg 201) sediment clone ODP1227A1.22, AB177001
Mediterranean Mud Volcano Amsterdam sediment clone AMSMV-S1-A28, FJ649522
Cascadia Margin (ODP borehole 892B) clone IV.4.ArA40, AY367335
Peru Margin (ODP site 1229) sediment clone 30-AA1, AJ867801
Aarhus Bay brackish marine sediment clone Arch44, FN428814
Mediterranean Mud Volcano Napoli sediment clone Napoli-2A-15, AY592480
Peru Margin (ODP site 1227) sediment clone ODP1227A19.02, AB177016
Peru Margin (ODP site 1227) sediment clone 2H2_C19, DQ301964
Haakon Mosby mud volcano sediment clone HMMVCen-27, AJ579325
Haakon Mosby mud volcano sediment clone HMMVCen-DS-Arch2635, XXX
Peru Margin (ODP site 1229) sediment clone 86-AA1, AJ867790
Limestone sinkhole (Mexico, La Palita) clone LP102MA49, FJ902682
hot spring clones (e.g. EU924224, AB213090)
Hydrothermal fluid/deposits clones (e.g. AB213054,
Mediterranean Mud Volcano Amsterdam sediment clone AMSMV-S1-A58, FJ649519
Mariana Trough hydrothermal field clone YS16Ac31, AB329761
Sulfurous freshwater Lake Vilar (Spain) water clone B10, AM076831
El Zacaton (Mexico) water sample at 273 m depth, FJ485494
Brazos-Trinity Basin (IODP site 1319) sediment clone IODP1319A4.3, AB432998
Ursa Basin (GoM, IODP site 1324) sediment clone IODP1324A2H2.95, AB448793
Cascadia Margin (ODP site 1251) sediment clone ODP1251A1.5, AB177266
Peru Margin (ODP leg 201, site 1227) sediment clone 1H5_F10, DQ301992
Peru Margin (ODP site 1229) sediment clone 30-AA10, AJ867800
Peru Margin (ODP site 1227) sediment clone ODP1227A3.14, AB177027
Cascadia margin ODP borehole 892B clone IV.4.ArA10, AY367333
Mediterranean Mud Volcano Napoli sediment clone Napoli-2A-01, AY592466
Eastern Mediterranean brine lake sediment clone Urania-2A-09, AY627488
Gold mine (Japan) subsurface geothermal water clone HAuD-LA43, AB113635
Peru Margin (ODP site 1227) sediment clone ODP1227A19.01, AB177015
Cascadia Margin (ODP site 1251) sediment clone ODP1251A25.1, AB177279
Peru Margin (ODP site 1227) sediment clone 1H5_F01, DQ301988
Lomonosov Ridge (Arctic Ocean Basin, IODP leg 302) clone 242mbsf_1_2A, EU915230
Minami-Kanto (Japan) deep subsurface gas-associated water clone MOB4-14, DQ841220
Aquifer subsurface groundwater clone HDBW-WA26, AB237759
Mediterranean Mud Volcano sediment clone AMSMV-S1-A54, FJ649517
Brazos-Trinity Basin (IODP site 1320) sediment clone IODP1320A2.1, AB433013
Mariana Trough hydrothermal vent field clone Papm3A34, AB213093
Peru Margin (ODP site 1227) sediment clone ODP1227A1.01, AB176996
Cascadia Margin (ODP site 1251) sediment clone ODP1251A13.2, AB177268
Mediterranean Mud Volcano sediment clone AMSMV-S1-A33, FJ649514
Peru Margin (ODP site 1227) sediment clone 1H5_F11, DQ301975
Peru Margin (ODP site 1229) sediment clone 6-AA1, AJ867808
Lomonosov Ridge (Arctic Ocean, IODP leg 302) clone 242mbsf_1_5E, EU915226
Gulf of Mexico surface sediments clone GoM87_Arch405, FN421234
Skan Bay (Alaska) sediment clone SBAK-mid-18 (0.33-0.45 mbsf), DQ522920
Cascadia Margin (ODP site 1250) sediment clone ODP204_30_ArchU66, AM713451
Ursa Basin (GoM, IODP site 1324) sediment clone IODP1324A2H2.52, AB448791
Peru Margin (ODP site 1230) sediment clone ODP1230A13.29, AB177095
Brazos-Trinity Basin (GoM, IODP site 1320) sediment clone IODP1320A21.8, AB433025
Cascadia Margin (ODP site 1251) sediment clone ODP1251A3.21, AB177283
Salt marsh sediment clone IP1SAT-C12, EU284653
Kao-Mei (Taiwan) wetland clone KM07-Ea-1-3, EU420699
Mangrove sediment clone MMS_207, FJ477321
Aarhus Bay brackish marine sediment clone Arch43, FN428813
South China Sea sediment clone BD72AR18, GU363075
Mariana Trough hydrothermal field clone YS16As18, AB329790
Peru Margin (ODP site 1229) sediment clone 12H3_ar45, DQ302016
Mediterranean Sea Mud Volcano sediment clone AMSMV-S1-A26, FJ649513
Mediterranean Mud Volcano sediment clone AMSMV-S1-A51, FJ649530
Peru Margin (ODP site 1227) sediment clone ODP1227A19.04, AB177018
Peru Margin (ODP site 1227) sediment clone 12H3_ar41, DQ302015
La Palita limestone sinkhole biomat clone LP30MA31, FJ901942
Peru Margin (ODP site 1227) sediment clone ODP1227A17.28, AB177008
Qiongdongnan Basin (South China Sea) sediment clone SCS-QBS-A92, EF104104
Aquifer (Japan) subsurface groundwater clone HDBW-WA25, AB237758
Minami-Kanto (Japan) subsurface gas-associated water clone MOB4-15, DQ841221
Mediterranean Sea Mud Volcano sediment clone Napoli-2A-44, AY592507
Vulcano Island (Italy) geothermal well clone VulcPlw.115, DQ300322
Juan de Fuca Ridge sediment clone CORK-A40, AB260058
Guaymas Basin sediments clone AT_R021, AF419653
South China Sea sediment clone BD72AR34, GU363085
Peru Margin (ODP site 1227) sediment clone ODP1227A19.27, AB177021
Lake Pavin (France) sediment clone Pav-sed-510, GU135490
La Palita limestone sinkhole (Mexico) biomat clone LPBB80, FJ901719
Mangrove sediment clone MMS_209, FJ477322
Papua New Guinea hydrothermal vent field clone PNG_TB_4B30H1_A067, GU137374
Mediterranean Mud Volcano sediment clone Kazan-2A-24, AY592001
Mediterranean Mud Volcano sediment clone AMSMV-S1-A60, FJ649532
Brazos-Trinity Basin (GoM, IODP site 1319) sediment clone IODP1319A4.13, AB433003
Minami-Kanto (Japan) subsurface gas-associated water clone MOB4-12, DQ841218
Peru Margin (ODP site 1229) sediment clone 42-AC3, AJ867791
Peru Margin (ODP site 1227) sediment clone ODP1227A17.03, AB177003
Peru Margin (ODP site 1227) sediment clone 2H2_C08, DQ301962
Peru Margin (ODP site 1227) sediment clone ODP1227A1.11, AB176998
Minami-Kanto (Japan) deep-subsurface gas-associated water clone MOB4-13, DQ841219
Ursa Basin (GoM, IODP site 1324) sediment clone IODP1324A53X3.46, AB448816
Peru Margin (ODP site 1229) sediment clone 30-AB6, AJ867796
Lake Pavin (France) sediment clone Pav-sed-519, GU135499
Peru Margin (ODP site 1227) sediment clone ODP1227A17.20, AB177007
Mariana Trough hydrothermal vent field clone YS16Ac76, AB329777
MCG-6
MCG-1
MCG-2
MCG-3
MCG-4
MCG-5
probe MCG528
probe MCG493
Miscellaneous Crenarchaeotal Group (MCG)
MBGB
THAUMARCHAEOTA
KORARCHAEOTA
NANOARCHAEOTA
CRENARCHAEOTA
10%

Figure 1 Phylogenetic tree showing the affiliations of MCG 16S rRNA gene sequences with selected reference sequences of the domain *Archaea*. MCG comprises only sequences from uncultivated organisms. At least one representative sequence per habitat and group is included in the tree. The tree was constructed by using RaxML analysis in combination with a 50% filter excluding highly variable positions in a subset of >2800 nearly full-length sequences (>1250 bp). Partial sequences were inserted into the reconstructed tree by using parsimony criteria with global and local optimization, without allowing changes in the overall tree topology. Probe and primer specificities are indicated. Bar, 10% estimated sequence divergence.

Design and evaluation of MCG-specific oligonucleotide probes

The design of new probes always aims at a compromise of good target group coverage and high specificity. Due to the combination of a great MCG intragroup diversity with a relatively short stem separating MCG from other archaeal phyla it was impossible to design a single probe for all members of MCG. Thus, three new oligonucleotide probes, MCG493, MCG528, and MCG732r were developed for MCG-1 to -6 (Figure 1, Table 2). All probes were tested in silico for group coverage and specificity. Based on nearly full-length sequences (>1250 bp) present in SILVA 104 database release supplemented by several yet unpublished sequences, probes MCG493 and MCG528 covered 74% and 81%, respectively, of subclusters MCG-1 to MCG-6 (Figure 1). MCG-1 to MCG-6 sequences not targeted by these probes are mainly from terrestrial hot springs and salt marshes. Both probes showed 2-3 mismatches to other MCG sequences and are highly specific having only few outgroup hits for probe MCG493 (13 hits in total: 10x *Euryarchaeota*, 3x *Crenarchaeota*) and probe MCG528 (6 hits in total: 1x *Euryarchaeota*, 5x *Crenarchaeota*). Although both probes showed bright signals in Clone-FISH we recommend using probe MCG493 for in situ detection because in environmental samples the resulting fluorescence signal was much brighter than that of MCG528. Primer MCG732r has only been designed for the use in qPCR. Although having a lower in silico coverage of MCG-1 to MCG-6 (16%), this primer is still valuable because of a relaxed specificity of the qPCR technique. Most of the mismatches are in the center or near the 5' end of the sequence therefore expected not strongly discriminating in PCR.

Nucleic acids-based quantification of MCG in several types of sediments

Quantification of MCG was done in eleven different marine surface and subsurface sediments from coastal regions, intertidal flats, cold seeps, open ocean basins and estuaries as well as microbial mats. We defined the top 10 cm-layer of the seabed as surface sediments, deeper layers (>10 cm depth) as subsurface according to Whitman and colleagues (Whitman et al., 1998).

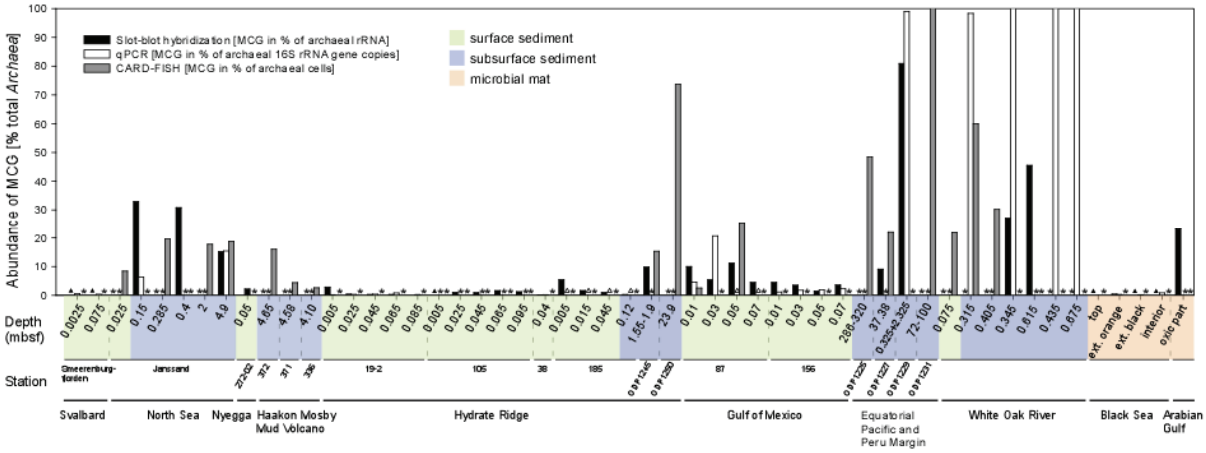

Figure 2 Relative abundance of MCG Crenarchaeota compared to total *Archaea* as determined for several distinct sedimentary habitats by rRNA slot blot hybridization, quantitative PCR, and CARD-FISH. Sediments retrieved from ≤ 0.1 mbsf are defined as surface sediments, sediments retrieved from > 0.1 mbsf as subsurface sediments. *, not analyzed; ▲, MCG rRNA not detected by slot blot hybridization; Δ, MCG rRNA genes not detected by qPCR.

Quantitative rRNA slot blot hybridization.

Archaeal rRNA yields greatly varied between habitats and sediment depths. In coastal surface sediments from Svalbard (Arctic Ocean) and Janssand (North Sea), low amounts of archaeal rRNA were detected, and ranged between 39 and 97 ng g^{-1} sediment (Table 3). From surface sediments at cold seeps archaeal rRNA recoveries were one to three orders of magnitude higher. Highest absolute archaeal rRNA amounts were detected in sediments covered by *Beggiatoa* mats at Hydrate Ridge (Cascadia Margin, station 19-2) at 4-5 cm sediment depth with 52 µg g^{-1}. These high values can mainly be explained by a high abundance of ANME archaea which made up, together with their sulphate-reducing partners, more than 90% of microbial biomass (Knittel et al., 2005). In Black Sea microbial mats archaeal rRNA detection was highest in the top and youngest part of the chimney with more than 100 µg g^{-1} mat wet weight.

For quantification of MCG rRNA the newly developed probe MCG493 was applied and resulted in easily visualized slot blot signals (Supplementary Figure 1). Except for surface sediments from Svalbard and most parts of Black Sea microbial mats, MCG rRNA could be detected in all types of habitats (Figure 2, Table 3). Highest MCG rRNA amounts were detected in sediments from station 19-2 at Hydrate Ridge with 178 ng g^{-1} in a depth of 4-5 cm. This was accompanied by the highest total archaeal rRNA amount detected.

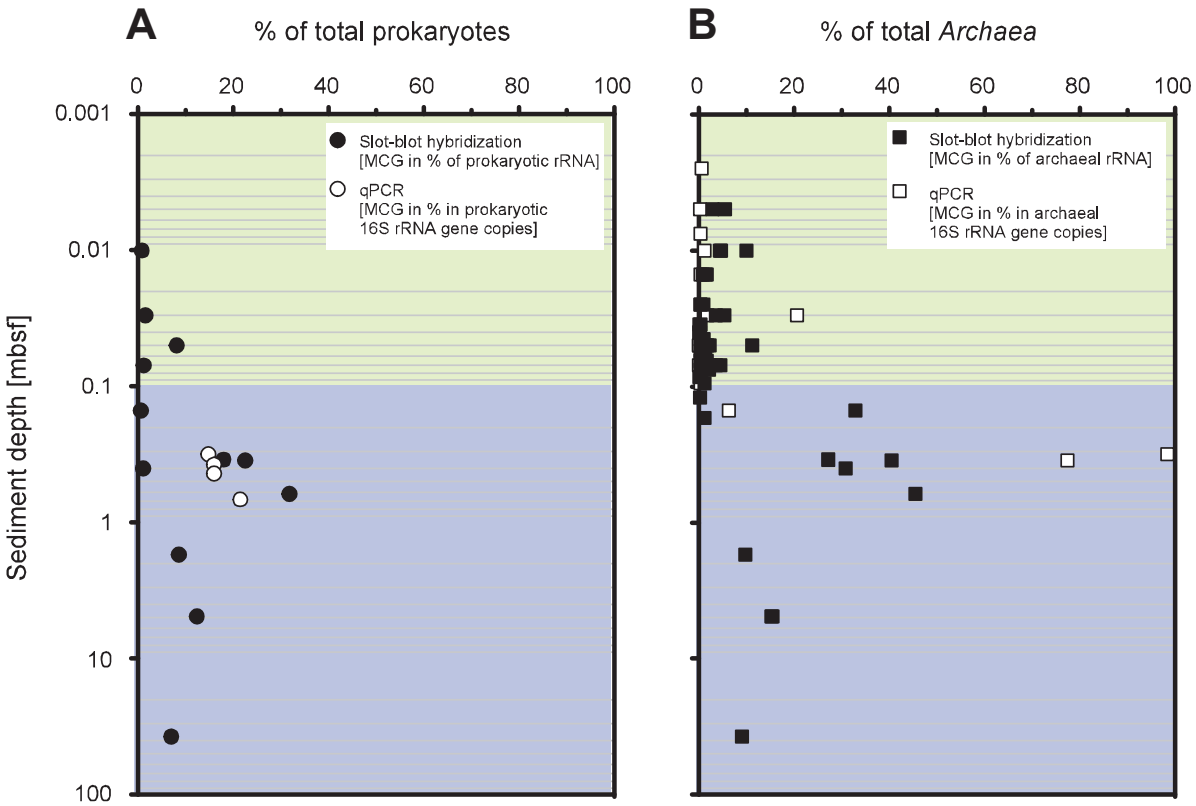

Figure 3 MCG abundance dependency on sediment depth as revealed by slot blot hybridization and quantitative PCR, plotted as % of total prokaryotes (A), and total archaea (B). The plotted data are given fully in Table 3.

Relative values of MCG calculated as a percentage of total archaeal rRNA varied greatly with depth and between sites (Figure 2). Data showed a general increase of MCG rRNA fraction with depth (Figure 3): 2.1 ± 2.7 % MCG rRNA of total archaeal rRNA in all samples from surface (0-0.1 mbsf) as compared to 28.1 ± 24.3% in all subsurface (> 0.1 mbsf) samples. In surface sediments from methane seeps at Hydrate Ridge and Haakon Mosby mud volcano MCG rRNA made up only a minor part of total archaeal rRNA with 0-2% MCG of total archaeal rRNA detected. In the subsurface, however, the portion of MCG rRNA increased to 10% (1.725 mbsf, Hydrate Ridge, ODP site 1245D). In subsurface sediments from Peru Margin MCG rRNA constituted the major part of total archaeal rRNA: greatest fraction was detected at ODP site 1229 with 80.9% in a mixed sediment from 0.325 + 2.325 mbsf depth. At ODP site 1227, 9.1% MCG rRNA was detected at 37.38 mbsf. Intertidal sand flat subsurface sediments from Janssand were dominated by MCG rRNA as well with 15.2% at 4.90 mbsf. Large fractions of MCG rRNA were also detected in White Oak River estuarine subsurface sediments with 27.1% and 45.5% of total archaeal rRNA at 0.345 mbsf

and 0.615 mbsf, respectively, and in hypersaline microbial mats from the Arabian Gulf with 23.2%.

Quantitative PCR.

As a complementary method to rRNA slot blot hybridization, qPCR on DNA was used for quantification of MCG abundance (Table 3, Figure 2). The highest archaeal 16S rRNA gene copy numbers were found in Black Sea microbial mats with $1.4x10^{10}$ copies g^{-1}. In Hydrate Ridge surface sediments between $1.4x10^{8}$ and $2.7x10^{9}$ copies g^{-1} (stations 19-2, 38) and quite low archaeal 16S rRNA gene copy numbers of $3.4x10^{5}$ and $2.3x10^{6}$ copies g^{-1} (station 105) were detected. These low numbers likely indicate an experimental problem since 10^{9}-10^{10} archaeal cells g^{-1} have been repeatedly visualized in these sediments (Boetius et al., 2000; Knittel et al., 2003). Most likely the bias was caused by insufficient cell lysis of AOM consortia which constituted more than 90% of microbial biomass. Yet, for many sites the MCG fraction of total archaeal 16S rRNA gene copy numbers was comparable to that detected at the rRNA level by slot blot hybridization (Figure 2). From all surface sediment samples MCG 16S rRNA gene copy numbers was 1.7 ± 4.4% of total archaeal gene copies as compared to 74.1 ± 43.3% from all subsurface samples. In detail, MCG 16S rRNA gene copy numbers were <1% of total archaeal gene copies in coastal surface sediments from Svalbard, Black Sea microbial mats, and at Hydrate Ridge. Fractions of MCG in intertidal flat sediments from Janssand (15.4% at 4.90 mbsf) and Gulf of Mexico surface sediments from station 87 (up to 20.6%) were similarly high as those detected by slot blot hybridization. In two habitats MCG were exceptionally abundant: at ODP site 1229 (99%, Peru Margin) and in the White Oak River estuary (98-121%). Values above 100% can be explained best by a reduced binding efficiency or insufficient coverage of used general archaeal primers.

Table 3 Quantification of MCG Crenarchaeota in diverse sediments and microbial mats.

			rRNA slot blot hybridization			qPCR		FISH		
Site	Station/sample	Depth [mbsf]	Bacteria rRNA ng‡	Archaea rRNA ng‡	MCG rRNA ng‡	Archaea gene copies°	MCG gene copies°	Total cells [cm^{-3}]	Archaea [cm^{-3}]	MCG [cm^{-3}]
Svalbard	Smeerenburgfjorden	0.0025	9.6E+03	7.7E+01	n.d.	2.0E+08	1.2E+06	n.a.	n.a.	n.a.
		0.0075	n.a.	9.7E+01	n.d.	1.8E+08	6.5E+05	n.a.	n.a.	n.a.
North Sea	Janssand	0.025	n.a.	n.a.	n.a.	n.a.	n.a.	2.8E+08	1.1E+08	9.7E+06
		0.15	4.4E+03	6.6E+01	2.2E+01	8.8E+06	5.5E+05	n.a.	n.a.	n.a.
		0.285	n.a.	n.a.	n.a.	n.a.	n.a.	4.6E+08	1.4E+08	2.7E+07
		0.40	6.5E+03	2.1E+02	6.6E+01	n.a.	n.a.	n.a.	n.a.	n.a.
		2.00	n.a.	n.a.	n.a.	n.a.	n.a.	1.9E+08	6.2E+07	1.1E+07
		4.9	2.2E+00	9.3E+00	1.4E+00	1.8E+08	2.8E+07	3.0E+08	8.4E+07	1.6E+07
Nyegga	272-02 (SOB mat)	0.05	n.a.	2.9E+02	6.4E+00	4.0E+09	1.6E+06	n.a.	n.a.	n.a.
HMMV	372 (Center)	4.65	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	3.3E+08	5.3E+07
	371 (*Beggiatoa*)	4.58	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	3.1E+09	1.4E+08
	336 (*Pogonophora*)	4.10	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	2.9E+09	7.6E+07
Cascadia Margin	19-2	0.005	n.a.	1.1E+03	3.1E+01	1.4E+08	3.1E+05	n.a.	n.a.	n.a.
Hydrate Ridge		0.015	n.a.	2.0E+03	1.6E+01	2.4E+08	9.8E+05	n.a.	n.a.	n.a.
		0.025	n.a.	8.1E+03	3.4E+01	5.4E+08	1.7E+06	n.a.	n.a.	n.a.
		0.035	n.a.	2.7E+04	8.6E+01	2.7E+09	5.1E+06	n.a.	n.a.	n.a.
		0.045	n.a.	5.2E+04	1.8E+02	1.7E+09	6.0E+06	n.a.	n.a.	n.a.
		0.055	n.a.	2.4E+04	7.7E+01	1.1E+09	6.9E+06	n.a.	n.a.	n.a.
		0.065	n.a.	2.7E+03	8.4E+00	4.4E+08	3.7E+06	n.a.	n.a.	n.a.
		0.075	n.a.	3.7E+03	1.2E+01	7.8E+08	5.1E+06	n.a.	n.a.	n.a.
		0.085	n.a.	1.4E+04	1.9E+01	5.8E+08	1.4E+06	n.a.	n.a.	n.a.
		0.095	n.a.	3.0E+03	n.d.	4.5E+08	1.6E+06	n.a.	n.a.	n.a.
	105	0.005	n.a.	6.9E+02	n.d.	5.1E+05	n.a.	n.a.	n.a.	n.a.
		0.015	n.a.	1.6E+03	2.2E+01	5.4E+05	n.a.	n.a.	n.a.	n.a.
		0.025	n.a.	1.4E+03	1.4E+01	4.2E+05	n.a.	n.a.	n.a.	n.a.
		0.035	n.a.	4.0E+02	n.d.	n.a.	n.a.	n.a.	n.a.	n.a.
		0.045	n.a.	1.0E+03	9.1E+00	1.6E+06	n.a.	n.a.	n.a.	n.a.
		0.055	n.a.	1.4E+03	1.8E+01	n.a.	n.a.	n.a.	n.a.	n.a.
		0.065	n.a.	2.0E+03	3.3E+01	3.4E+05	n.a.	n.a.	n.a.	n.a.
		0.075	n.a.	1.9E+03	4.0E+01	2.3E+06	n.a.	n.a.	n.a.	n.a.
		0.095	n.a.	1.7E+03	2.2E+01	n.a.	n.a.	n.a.	n.a.	n.a.
		0.17	n.a.	7.0E+02	8.0E+00	n.a.	n.a.	n.a.	n.a.	n.a.
	38	0.04	n.a.	2.8E+03	4.4E+00	2.2E+08	4.5E+05	n.a.	n.a.	n.a.
	185	0.005	n.a.	2.3E+02	1.2E+01	n.d.	n.d.	n.a.	n.a.	n.a.
		0.015	n.a.	6.1E+02	1.0E+01	n.d.	n.d.	n.a.	n.a.	n.a.
		0.045	n.a.	4.4E+02	4.3E+00	n.d.	n.d.	n.a.	n.a.	n.a.
	ODP1245D 1H2	1.55-1.90	5.0E+00	3.2E+01	3.1E+00	n.a.	n.a.	1.2E+08$^{\square\Delta}$	7.3E+06	1.1E+06
	ODP1250D 3H7	23.90	n.a.	n.a.	n.a.	n.a.	n.a.	1.1E+07$^{\square\Delta}$	6.2E+06	4.6E+06
Gulf of Mexico	87	0.01	7.3E+03	5.8E+02	5.8E+01	1.7E+07	7.9E+05	1.9E+09	1.0E+09	2.7E+07
		0.03	1.9E+02	6.8E+01	3.7E+00	2.8E+06	5.8E+05	n.a.	n.a.	n.a.
		0.05	6.1E+00	1.6E+01	1.8E+00	2.9E+06	n.d.	*	8.3E+08	2.1E+08
		0.07	1.3E+02	4.4E+01	2.0E+00	2.8E+06	n.d.	n.a.	n.a.	n.a.
	156	0.01	n.a.	1.1E+03	4.9E+01	3.6E+07	4.2E+05	n.a.	n.a.	n.a.
		0.03	n.a.	8.5E+02	3.0E+01	2.5E+07	4.4E+05	n.a.	n.a.	n.a.
		0.05	n.a.	6.7E+02	9.8E+00	2.9E+07	5.4E+05	n.a.	n.a.	n.a.
		0.07	n.a.	6.1E+02	2.2E+01	1.8E+07	4.1E+05	n.a.	.n.a.	n.a.
Equatorial Pacific	ODP1225A 32H3, 34H3, 35H5	286-320¨	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	2.6E+06	1.3E+06
Peru Margin	ODP1227A 5H3	37.38	3.3E+01	1.0E+02	9.4E+00	n.a.	n.a.	n.a.	1.5E+07	3.3E+06
	ODP1229D 1H1 + 1H2	0.325 + 2.325	8.5E+01	1.1E+02	8.6E+01	2.2E+08	2.2E+08	n.a.	n.a.	n.a.
Peru Basin	ODP1231B 9H2, 10H2, 11H5, 12H2	72-100§	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	2.48E+06	2.5E+06
White Oak River	Station H, core Jul 08-1	0.075	n.a.	n.a.	n.a.	n.a.	n.a.	1.0E+09	5.6E+08	1.2E+08
		0.315	n.a.	n.a.	n.a.	4.31E+08	4.23E+08	1.3E+08	5.0E+07	3.0E+07
		0.345	1.2E+01	2.4E+01	6.5E+00	4.07E+08	4.16E+08	n.a.	n.a.	n.a.
		0.405	n.a.	n.a.	n.a.	n.a.	n.a.	5.3E+08	3.7E+08	1.1E+08
		0.435	n.a.	n.a.	n.a.	4.37E+08	5.28E+08	n.a.	n.a.	n.a.
		0.615	8.8E+00	2.0E+01	9.1E+00	n.a.	n.a.	n.a.	n.a.	n.a.
		0.675	n.a.	n.a.	n.a.	2.81E+08	3.20E+08	n.a.	n.a.	n.a.
Black Sea	P822	top part	n.a.	1.0E+05	n.d.	1.4E+10	1.3E+07	n.a.	n.a.	n.a.
		exterior, orange	n.a.	1.2E+04	5.1E+01	2.4E+09	6.2E+06	n.a.	n.a.	n.a.
		exterior, black	n.a.	2.8E+04	n.d.	1.3E+10	2.4E+07	n.a.	n.a.	n.a.
		interior	n.a.	3.2E+04	n.d.	3.1E+09	2.6E+07	n.a.	n.a.	n.a.
Arabian Gulf	Abu Dhabi	oxic part	1.7E+03	2.6E+02	6.0E+01	n.a.	n.a.	n.a.	n.a.	n.a.

n.a., not analyzed,; n.d., not detected
* too weak signals for counting
$^{\square}$ acridine orange direct cell counts
$^{\Delta}$ Orcutt, 2007
° 16S rRNA gene copy number g^{-1} sediment or mat
‡ 16S rRNA [ng] g^{-1} sediment or mat
§ sediments from 72 mbsf (9H2), 81 mbsf (10H2), 96 mbsf (11H5), and 100 mbsf (12H2) were mixed in a ratio of 1 :1.3 : 1.7 : 1.6
¨ sediments from 286 mbsf (32H3), 307 mbsf (34H3), 320 mbsf (35H5) were mixed in a ratio of 1.6 : 1 : 1

Ratio Bacteria:Archaea

Based on the size of MCG rRNA fraction some samples were selected for quantification of total bacterial 16S rRNA (Table 3, Figure 4). In general, the relative percentage of bacterial

rRNA decreased with sediment depth. For example, surface sediments from Janssand intertidal sand flat was strongly dominated by bacterial rRNA (97-98% bacterial rRNA : 2-3% archaeal rRNA) at a depth of 0-0.4 mbsf, but by archaeal rRNA in a deeper sediment horizon at 4.90 mbsf (19% bacterial rRNA : 81% archaeal rRNA). The ratio of bacterial to archaeal rRNA percentages was comparably low for other deep surface and subsurface sediments investigated in this study, i.e. 14%:86% at ODP site 1245 at Hydrate Ridge, 24%:76% at ODP site 1227, 45%:55% at ODP site 1229, and 34%:66% at White Oak River.

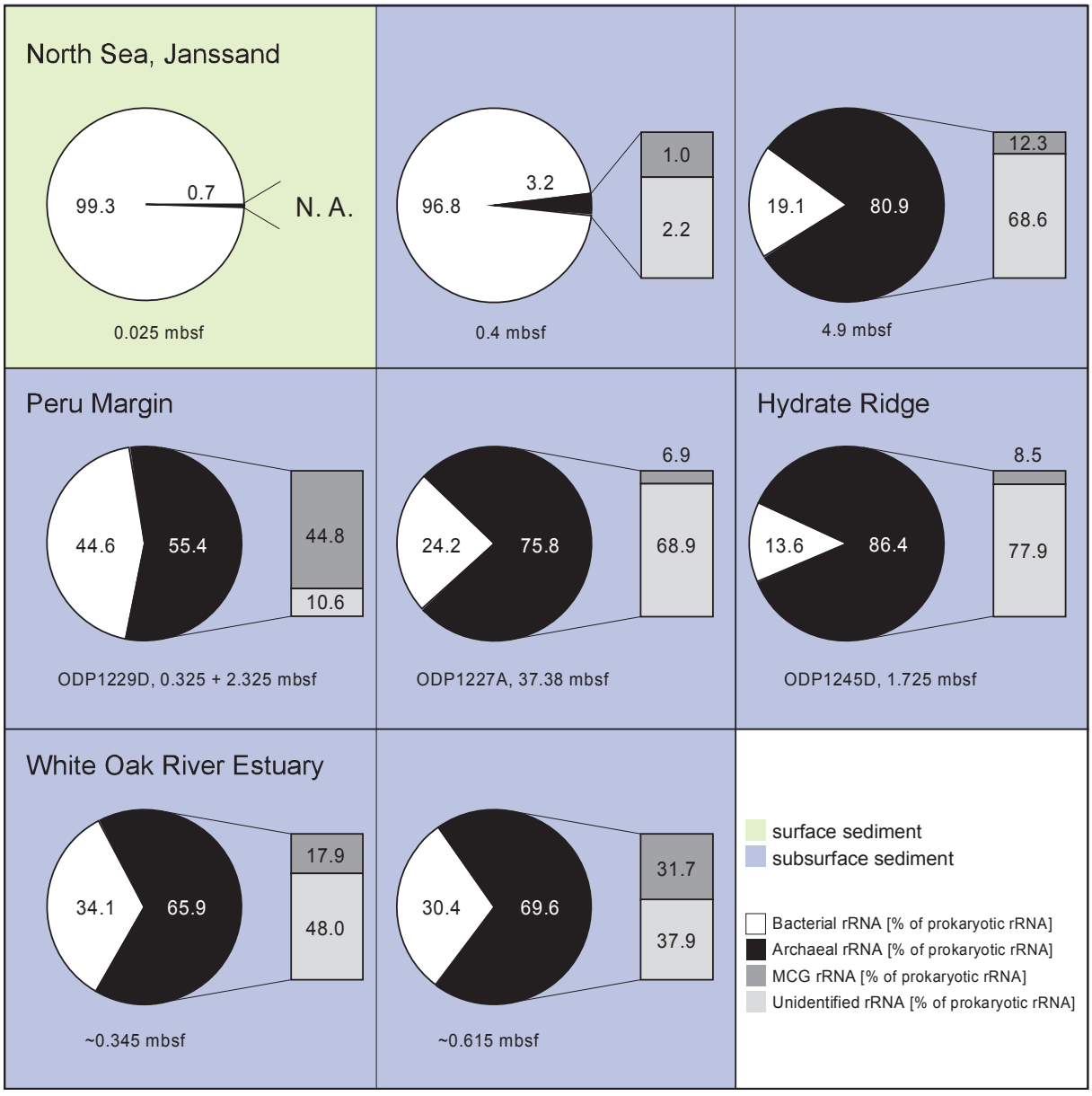

Figure 4 Slot blot-based determination of the ratio *Archaea*:*Bacteria* 16S rRNA in selected surface and subsurface sediments. The sum of detected archaeal and bacterial 16S rRNA was set as 100% of prokaryotic rRNA. The archaeal fraction is further resolved in the column which shows the proportion of MCG and other, yet unidentified *Archaea*.

Development of a CARD-FISH protocol for in situ identification and quantification of MCG

White Oak River sediments were selected as a MCG hot spot to adapt the standard CARD-FISH protocol (Pernthaler et al., 2002) for in situ detection of MCG cells. The crucial step in MCG visualization was the permeabilization of the cell walls. Archaeal cell walls are usually permeabilized either by a treatment with 10-15 µg ml^{-1} proteinase K for 1-5 min at room temperature or at 37°C, 0.5% SDS for 10 min at room temperature or with 60 U ml^{-1} achromopeptidase at 37°C (Teira et al., 2004; Herndl et al., 2005; Knittel and Boetius, 2009; Labrenz et al., 2010). However, none of these methods was successful for the visualization of MCG. Instead, we used 0.01 M HCl for 10 min for permeabilization of crenarchaeotal cell walls. Higher HCl concentrations (> 0.1 M) did not increase the fraction of hybridized cells, but rather caused a visible disintegration of cells. We recommend the use of Alexa488-labelled tyramides, which resulted in highest probe signals and lowest background fluorescence.

MCG cells could be visualized in White Oak River sediments using probe MCG498. About 90% of the detected MCG cells were small and coccoid (Figure 5) with a cell size of 0.4-0.5 µm. A minor part of the MCG community had a much larger cell size with up to 1 µm in diameter. In most cases MCG were detected as single cells; however, they were also found to form aggregates of 2-5 cells. DAPI signal of MCG was clearly visible in most habitats except for all ODP sites investigated where DAPI staining did not work at all. To corroborate the identification of detected MCG cells, dual hybridizations were performed with probe MCG493 and the general archaeal probe ARCH915. MCG493 signals were always co-localized with ARCH915 signals (Figure 4). In negative controls hybridized with anti-sense probe NON338, no signals were observed (data not shown).

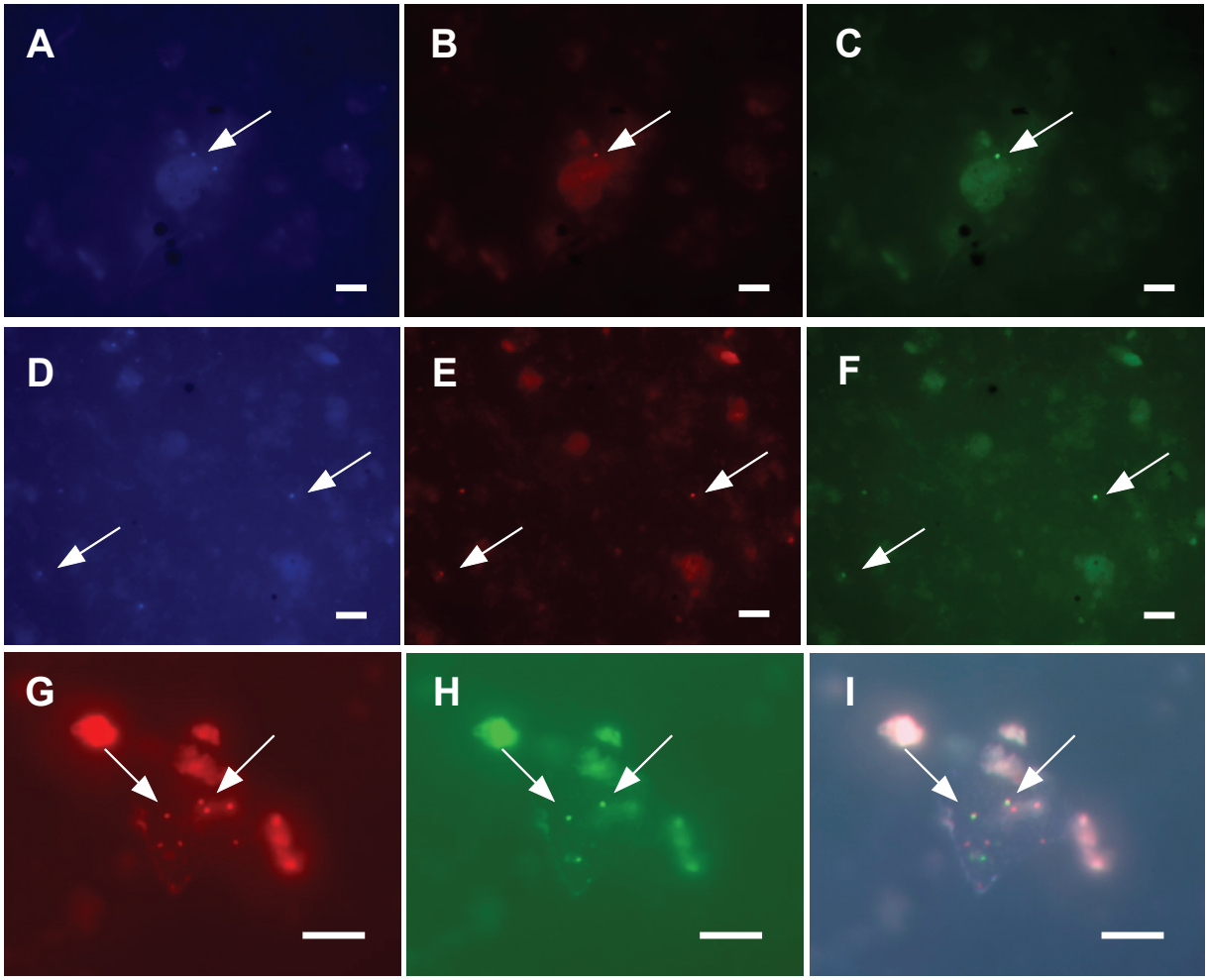

Figure 5 Single cells of MCG Crenarchaeota in subsurface sediments from the sulphate-methane transition zone of the White Oak River estuary (0.4 mbsf; panels A-F) and ODP site 1227 at Peru Margin (5H3, 37.38 mbsf; panels G-I), visualized by CARD-FISH. Panels A and D show DAPI staining (blue); other panels show the corresponding FISH signals obtained by dual hybridization with the general archaeal probe ARCH915 (B, E, G; red) and MCG-specific probe MCG493 (C, F, H; green). Arrows point to MCG cell signals. Scale bars, 5 µm.

In White Oak River sediments 1.2 x 10^8 cells cm^{-3} were detected at shallow depth (12% of total cell counts, 22% of total *Archaea*). Within the subsurface absolute MCG abundance was comparably high but the relative fraction of MCG of total archaea increased to 30% and 60%. In other subsurface sediments, MCG Crenarchaeota were as dominant as in White Oak River sediments; they contributed 18-20% of total *Archaea* in Janssand intertidal sand flats, 15-74% at ODP sites 1245 and 1250 at Hydrate Ridge, 22% at Peru Margin ODP site 1229, 100% at Peru Basin ODP site 1231, and 48% at eastern equatorial Pacific ODP site 1225. The two latter sites showed very low microbial activities and low organic matter content. In deep sediments (4.65 mbsf) from Haakon Mosby mud volcano center, a site which is strongly

dominated by MCG 16S rRNA sequences (Lösekann-Behrens & Knittel, unpublished), MCG cells accounted for 16% of total *Archaea*.

Discussion

Diversity and distribution of MCG

MCG crenarchaeota are cosmopolitan. Sequences were retrieved from diverse marine and continental environments indicating a presumably high ecophysiological flexibility. MCG crenarchaeota can cope with high temperature at hot springs or vents (Barns et al., 1994; Meyer-Dombard et al., 2005; Kato et al., 2009), but also with rather cold temperatures at Arctic mud volcanoes (Lösekann et al., 2007) or in deep sea sediments (Inagaki et al., 2003; Parkes et al., 2005; Biddle et al., 2006; Inagaki et al., 2006; Sørensen and Teske, 2006; Teske, 2006). They seem also to have adapted to different salinities. There are sequences from freshwater lakes or groundwater (Mochimaru et al., 2007), brackish sediments (Aquilina et al., 2010), salt marshes (Nelson et al., 2009), estuaries (Meng et al., 2009) as well as many retrievals from marine sediments. This broad distribution is reflected in a great MCG intragroup diversity with some 16S rRNA similarity values as low as 77% which is close to the cut-off value of 77.5% proposed for bacterial classes (Yarza, 2011).

Establishment of a protocol for visualization of uncultured marine benthic crenarchaeota

Here, we provide a CARD-FISH protocol optimized for permeabilization of MCG cell walls, to enable the in situ detection of MCG cells. Previous estimates of crenarchaeotal contribution to biomass in marine subsurface sediments were based on qPCR (Schippers et al., 2005; Lipp et al., 2008), slot blot analysis (Schippers et al., 2005; Lipp et al., 2008) and lipid biomarkers (Biddle et al., 2006; Lipp et al., 2008). The CARD-FISH protocol presented here allows direct cell counting independent of gene copy numbers or DNA/RNA extraction efficiencies. We clearly showed the presence of intact MCG crenarchaeotal cells in several marine surface and subsurface sediments. Since the probe targets rRNA, which is often rapidly degraded in inactive cells (Kemp et al., 1993) the hybridization signal is indicative of currently or recently active MCG cells. Based on this protocol, functional studies, as e.g. Nano-SIMS analysis (Musat et al., 2008), will enable future studies to address the metabolism of MCG.

Most deep sea MCG cells were very small (0.4-0.5 μm); they have a comparable size to that reported for their sister group Marine Benthic Group B (MBGB) Crenarchaeota (0.2-

0.4 µm (Knittel et al., 2005). The detection of a second, larger morphotype (up to 1 µm) of MCG cells might reflects the observed large MCG intragroup 16S rRNA sequence diversity.

MCG crenarchaeota constituted a major part of Archaea in subsurface sediments

Marine Group I archaea account for up to 40% of total microbes in meso- and bathypelagic deep ocean waters (Karner et al., 2001). In analogy to this dominance of Marine Group I, rRNA gene surveys and intact lipid biomarker analysis have indicated a dominance of other crenarchaeotal clades, including MCG, in marine subsurface environments (Parkes et al., 2005; Biddle et al., 2006; Inagaki et al., 2006; Sørensen and Teske, 2006; Lipp et al., 2008). Here, we present the first quantitative data for the abundance of MCG crenarchaeota in marine environments, based on a combination of slot blot hybridization, qPCR, and CARD-FISH.

All methods suggest a higher abundance in deeper sediments. In addition, MCG abundance might be negatively correlated with methane concentration and the abundance of ANME clades. For example, MCG Crenarchaeota contributed only <1% to the archaeal community in gas hydrate-bearing surface layers at Hydrate Ridge methane seeps. These layers are characterized by high methane concentrations in the range of 10 to 50 mM (Torres et al., 2002) and high methane fluxes (30-50 mmol m^{-2} d^{-1}), and are strongly dominated by AOM-mediating consortia previously shown to constitute >90% of microbial biomass at these sites (Boetius et al., 2000; Knittel et al., 2003; Knittel et al., 2005). Sulphate-dependent AOM rates are extremely high and fuel microbial life. Similar values were observed for Haakon Mosby mud volcano and microbial mats from Black Sea methane seeps which are dominated by similar aerobic or anaerobic methanotrophic communities as Hydrate Ridge sediments (Michaelis et al., 2002; Treude et al., 2005; Niemann et al., 2006a), Arnds et al., unpublished data). In contrast, in subsurface sulphate-methane transition zones and near-surface sediments with low methane fluxes in the lower µmol range m^{-2} d^{-1}, low AOM rates and ANME communities below detection limits were reported (Inagaki et al., 2003; Biddle et al., 2006; Inagaki et al., 2006; Lipp et al., 2008; Orcutt et al., 2010), MCG crenarchaeota constitute the major part of the archaeal community. The dominance of MCG in subsurface sediments was independent of water depths, temperature or total carbon contents (organic-poor ODP sites 1225 and 1231 versus organic-rich ODP sites 1227 and 1229). Currently, we can only speculate about their physiological properties. Biddle and colleagues (Biddle et al., 2006) proposed that MCG Crenarchaeota assimilate sedimentary organic compounds other than methane and did not exclude an oxidation of methane without assimilation of methane

carbon. Further support for heterotrophy in MCG comes from a DNA stable isotope probing study where MCG incorporated ^{13}C-labelled acetate into biomass (Webster et al., 2010). In the present study we showed the dominance of MCG in subsurface SMTZ, a result that is compatible with the hypothesis that MCG might be involved in oxidation of methane, albeit possibly without measurable methane assimilation. Methane was present and consumed at all sites even in open-ocean sediments from site 1225 and 1231 where sulphate reduction is minimal (D'Hondt et al., 2004). In seep surface layers characterized by high methane concentration or gas hydrates, MCG might be outcompeted by the anaerobic methanotrophs of the ANME clades.

In SMTZ layers of White Oak River estuarine sediments, MCG is highly abundant as well. Almost all archaeal gene copies as detected by qPCR could be assigned to MCG. These numbers are either overestimated or numbers obtained by CARD-FISH are underestimating the MCG population, since only 22-60% of total archaeal cells were identified as MCG. Sequences of ANME-1 were found in much smaller abundances than MCG in general archaeal clone libraries (Lloyd et al., in press).

Dominance of Archaea versus Bacteria in subsurface sediments

We further aimed to contribute to the ongoing discussion of archaeal versus bacterial dominance in subsurface sediments (Inagaki et al., 2003; Mauclaire et al., 2004; Schippers et al., 2005; Biddle et al., 2006; Lipp et al., 2008). Our data showed bacteria to archaea rRNA ratios that ranged from approximately one-third *Bacteria* versus two-third *Archaea* (ODP site 1227 and White Oak River) to one-fifth *Bacteria* versus four-fifth *Archaea* (ODP site 1245D and Janssand). Thus, the data obtained in this study suggest that *Archaea* constitute the major part of subsurface life.

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Supplementary information

Study sites and sampling

In this study we investigated the following sites: (1) Smeerenburgfjorden, Svalbard, Arctic Ocean is a coastal, permanently cold silty sediment (Ravenschlag et al., 2000). (2) Janssand, North Sea is an intertidal flat located in the back barrier of the island of Spiekeroog characterized by sandy sediments, high input of organic carbons and steep geochemical gradients (Gittel et al., 2008). (3) Haakon Mosby Mud Volcano (HMMV, Norwegian Sea) is an active submarine mud volcano. The emitted gas consists of >99% methane and fluids are depleted in sulphate (Niemann et al., 2006). The site is characterized by three main concentric habitats above the gassy sediments, these are the centre of the HMMV, which was devoid of visible epifauna; thiotrophic bacterial mats dominated by *Beggiatoa* species; and surrounding fields at the rim which are populated by siboglinid tubeworms. MCG crenarchaeotal 16S rRNA gene sequences dominated clone libraries obtained from deep sediments at the crater center (Lösekann-Behrens, unpubl. data). (4) The Nyegga area represents methane seeps in pockmarks located on the edge of the Norwegian continental slope. (5) Hydrate Ridge, Cascadia Margin, Pacific Ocean, is a cold seep characterized by discrete methane hydrate layers exposed at the seafloor. The crest of Hydrate Ridge is populated by thick bacterial mats of *Beggiatoa* species and large fields of the clam *Calyptogena* both of which indicative of active gas seepage. Sulphate reduction is clearly fuelled by methane with extremely high rates in the μmol cm^{-3} d^{-1} range (Boetius et al., 2000). Hydrate Ridge subsurface sediments from ODP sites 1245D and 1250D were both characterized by massive methane hydrates and carbonates. From site 1245 sediments at 0-25 mbsf solely MBGB/DSAG sequences were retrieved; in contrast at 200 mbsf, only MCG sequences were found (Inagaki et al., 2006) (6) In the Gulf of Mexico, cold seep sediments were characterized by hydrocarbon seepage of variable composition (Orcutt et al., 2010). Station 87 is little oily and showed high rates of methane-independent sulphate reduction and a relatively low particulate organic carbon content while station 156 is very gassy, near oily and showed high rates of AOM (Orcutt et al., 2010). Archaeal 16S rRNA clone libraries were dominated by anaerobic methanotrophs at both sites, no MCG sequences have been detected. (7) At the Peru Margin in the Pacific Ocean, ODP leg 201 sites 1227 and 1229 are characterized by organic rich clayish and silty sediments containing diatom ooze without detectable methane (Parkes et al., 2005; Inagaki et al., 2006). Total organic carbon (TOC) content is relatively high compared to open ocean sites and scattered in the range of 1-10% (Meister et al., 2005). At site 1227, MCG sequences constituted up to 90% of an archaeal

clone library (Inagaki et al., 2006) (8) In the Equatorial Pacific Ocean and Peru Basin at open ocean ODP sites 1225 and 1231, TOC contents are about a hundred-fold lower than at the Peru margin sites, resulting in at least one order of magnitude lower total cell numbers (D'Hondt et al., 2004; Meister et al., 2005). No methane was detected (Inagaki et al., 2006). (9) At Black Sea methane seeps, microbial mats covering massive carbonate chimneys perform AOM coupled to sulphate reduction (Michaelis et al., 2002) (10) The White Oak River Estuary of North Carolina, USA, is a small coastal basin characterized by a high total organic carbon content and methane gas-rich sediments (up to 0.8 mM, (Martens et al., 1998; Lloyd et al., in press). The temperature is fluctuating seasonally from ca. 11 to 28°C. Microbial diversity analysis suggested a predominance of MCG crenarchaeota in the sulphate-methane transition zone (SMTZ; Biddle and Lloyd, unpublished data). (11) In Abu Dhabi on the Arabian Gulf, intertidal hypersaline microbial mats are influenced by strong salinity fluctuations (6-20%), and exposed to intense light intensities and high temperatures of up to 55°C (Kohls, 2010).

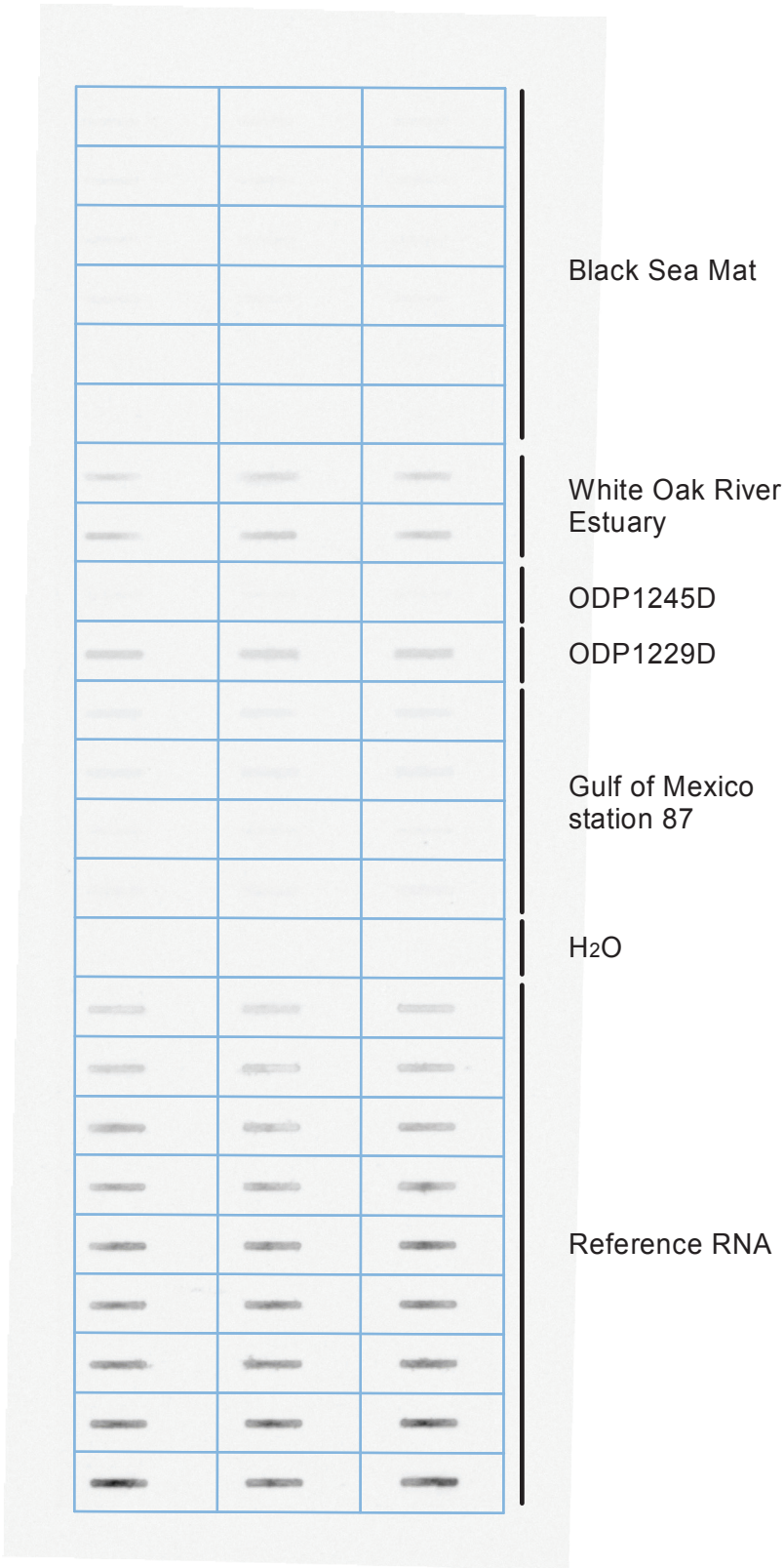
All sediment samples were fixed or frozen at the sampling site and stored at -80°C until further analysis, except for sediments from Janssand and the Gulf of Mexico which were stored at -20°C and sediments from Nyegga site 272-02 which were kept anoxically with methane in the headspace at 4°C for two years.

Nucleic acid extraction

In 50 ml sterile screw-cap conical tubes, 1-2 g of sediment were mixed with 2.5 ml of phenol (pH 7.8, Roth), 175 µl of 20% sodium dodecyl sulfate (SDS), 1.5 ml of extraction buffer (250 mM sodium acetate, 50 mM EDTA, pH 8.0), 2 g of 0.1 mm glass beads, and 0.5 g of 0.4-0.6 mm glass beads. The tubes were subjected to bead beating (FastPrep®-24, MP Biomedicals, OH, USA) at a speed of 6.5 m/s twice for 45 sec with intermitted cooling on ice. Tubes were centrifuged for 15 min at 4511 x g at 4°C. The nucleic acids in the supernatant were extracted twice with phenol:chloroform:isoamylalcohol (25:24:1, v/v/v; pH 7.8; Roth) and twice with chloroform:isoamylalcohol (24:1, v/v). Between each extraction step, the tubes were vigorously vortexed for 10 sec and centrifuged for 25 min at 5411 x g at 4°C. Finally nucleic acids were precipitated with one volume of 7.5 M ammonium acetate (pH 8.0) and two volumes of isopropanol at -20°C over night. Pellets of nucleic acids were washed with 80% ethanol and dissolved into RNase-free water. For rRNA slot blot hybridization, extracted nucleic acids were treated with DNase (TURBO DNase, Ambion, Germany), following the manufacturer's instructions. RNA was checked for DNA contamination on a

polyacrylamide gel. For quantitative PCR, nucleic acids were further purified using the RNA PowerSoil Total RNA Isolation Kit (Mo Bio Laboratories, CA, USA) and RNA PowerSoil DNA Elution Accessory Kit (Mo Bio Laboratories), following the manufacturer's instructions, and precipitating nucleic acids for 30 minutes at room temperature to prevent the crystallization of residual phenol. DNA yield was too low for samples from Hydrate Ridge station 38 and 19-2. Therefore, these samples were extracted using the protocols described above.

For microbial mats, nucleic acids were extracted using the guanidinium-salt method (Chomczynski and Sacchi, 2006) with slight modifications. Approximately 5 mm^3 of microbial mat was washed with 1x PBS. The mat was homogenized in 400 µl of D-solution and transferred to a 2 ml bead-tube (Lysing matrix B, MP Biomedicals) with 40 µl of 2 M sodium acetate and water-saturated phenol (pH 5.1, Roth). The tube was bead-beaten twice (FastPrep-24, MP biomedicals, OH, USA) at speed 6 m/s for 20 sec. Tubes were centrifuged for 10 min at 16,100× g at 4°C. RNA was extracted from the supernatant with phenol-chloroform-isoamylalcohol and precipitated with isopropanol. The RNA pellet was washed with ethanol and dissolved in RNase free water. The quality of the RNA was checked by agarose gel electrophoresis.



Supplementary figure 1. Image of a rRNA slot blot for quantification of MCG crenarchaeota. RNA was extracted from sediment samples, blotted on a nylon membrane and finally hybridized by 33P-labelled probe MCG493. As reference, different amounts of in vitro transcribed rRNA were blotted and used for standard curve construction.

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2

Sulfur-metabolizing bacterial populations in microbial mats of Nakabusa hot spring, Japan

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Sulfur-metabolizing bacterial populations in microbial mats of the Nakabusa hot spring, Japan

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ABSTRACT

At the Nakabusa hot spring, Japan, dense olive-green microbial mats develop in regions where the slightly alkaline, sulfidic effluent has cooled to 65 °C. The microbial community of such mats was analyzed by focusing on the diversity, as well as the *in situ* distribution and function of bacteria involved in sulfur cycling. Analyses of 16S rRNA and functional genes (*aprA*, *pufM*) suggested the importance of three thermophilic bacterial groups: aerobic chemolithotrophic sulfide-oxidizing species of the genus *Sulfurihydrogenibium* (Aquificae), anaerobic sulfate-reducing species of the genera *Thermodesulfobacterium*/*Thermodesulfatator*, and filamentous anoxygenic photosynthetic species of the genus *Chloroflexus*. A new oligonucleotide probe specific for *Sulfurihydrogenibium* was designed and optimized for catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH). *In situ* hybridizations of thin mat sections showed a heterogeneous vertical distribution of *Sulfurihydrogenibium* and *Chloroflexus*. *Sulfurihydrogenibium* dominated near the mat surface (50% of the total mat biovolume), while *Chloroflexus* dominated in deeper layers (up to 64% of the total mat biovolume). Physiological experiments monitoring *in vitro* changes of sulfide concentration indicated slight sulfide production by sulfate-reducing bacteria under anoxic-dark conditions, sulfide consumption by photosynthetic bacteria under anoxic-light conditions and strong sulfide oxidation by chemolithotrophic members of Aquificae under oxic-dark condition. We therefore propose that *Sulfurihydrogenibium* spp. act as highly efficient scavengers of oxygen from the spring water, thus creating a favorable, anoxic environment for *Chloroflexus* and *Thermodesulfobacterium*/*Thermodesulfatator* in deeper layers.

Introduction

Microbial mats develop in a wide range of aquatic habitats, such as geothermal hot springs, hypersaline ponds, marine cold seeps or hydrothermal vents. On the deep sea floor, light is absent and filamentous mat-forming chemoautotrophic sulfur bacteria develop, while microbial mats from terrestrial hot springs are also often composed of phototrophic bacteria [9]. In this environment, two types of phototrophs contribute to the formation of mats: (i) oxygenic phototrophs (cyanobacteria) growing autotrophically with water and carbon dioxide as the sole electron donor and carbon source, respectively, and (ii) anoxygenic phototrophs growing by photosynthesis without producing oxygen.

One of the best investigated hot springs is the slightly alkaline, sulfidic hot spring at Nakabusa, Nagano Prefecture, Japan. This site is well known for the formation of dense, colorful microbial mats (Fig. 1). Due to high temperatures of up to 70 °C, the thermophilic microorganisms in the mats are protected from grazing by higher organisms like insects (Matsuura, personal communication) [4]. The spring water is of volcanic origin and contains various reduced sulfur compounds in high concentrations, which can be used as electron donors for microbial growth [27]. The temperature and sulfide concentration in Nakabusa spring water are the key factors structuring the microbial community [27]. Close to the source, the temperature is approximately 75 °C, which is beyond the tolerance of any cyanobacteria. However, at this point, streamers extend from gray-colored mats, and 16S rRNA gene sequences from sulfide-oxidizing (*Aquifex* spp., *Sulfurihydrogenibium* spp.) and sulfate-reducing bacteria (*Thermodesulfobacterium*-affiliated species) have been retrieved from the streamers [27]. At low sulfide concentrations (<0.1 mM), filamentous *Aquifex*-like bacteria dominated, while at high sulfide concentrations (>0.1 mM) large sausage-shaped *Sulfurihydrogenibium*-like bacteria dominated the

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Fig. 1. Sampling site. Nakabusa hot spring in Nagano prefecture, Japan. Microbial mats from two contrasting sites were sampled. Site 1 consisted of grayish mats which developed close to the spring source under flowing water of about 75 °C, whereas at site 2 olive-green mats developed on an almost vertical concrete wall overflowing with spring water of 65 °C. Mats were growing to a thickness of approximately 4 mm.

microbial mat community, as determined by DGGE analysis [27]. Fluorescence *in situ* hybridization (FISH) of the streamer confirmed the presence of *Aquifex* spp., a dominance of sulfate-reducing *Thermodesulfobacterium*-related species (82% of the total DAPI counts) at high sulfide concentration [27] and the presence of numerous sausage-shaped bacteria. Thus, Nakagawa and Fukui [27] proposed active sulfur cycling in the streamers. Species of *Sulfurihydrogenibium* were also assumed to dominate the so called sulfur-turf microbial mats in many other neutral to alkaline hot springs [13,18,19,41].

A major change in the microbial community structure of Nakabusa mats and streamers occurs further down when the spring water flowing down a wall has cooled to a temperature of approximately 70 °C [26]. At this point, the mats turn olive-green, indicating the growth of photosynthetic organisms. Based on pigment analysis, Sugiura et al. [36] suggested a dominance of anoxygenic photosynthetic bacteria related to the green non-sulfur bacterial group of Chloroflexi. Similar mats were also observed in several other alkaline hot springs in Japan [9], Italy [29], in Yellowstone National Park, USA [28], and Iceland [34]. For Icelandic hot springs, Skirnisdottir et al. [34] reported that *Chloroflexus* spp. were the dominant mat organisms in a low-sulfide spring (0.030 mM) below 70 °C, whereas Aquificae were dominant in a high-sulfide spring (0.364 mM) of a similar temperature.

In this study, the olive-green microbial mat of the Nakabusa hot spring was analyzed in more detail. This is the first study linking diversity and community structure with the function of key microbial populations. The focus was on sulfur cycling inside the mat using a combination of molecular methods (DGGE, comparative 16S rRNA gene sequence analysis, FISH) and physiological experiments. The diversity of sulfur-metabolizing bacteria and photosynthetic bacteria was further studied by the analysis of the key genes for dissimilatory adenosine 5′-phosphosulfate reductase (*aprA*) and subunit M of the photosynthesis reaction center (*pufM*). Using newly developed specific oligonucleotide FISH probes, the spatial distribution and interactions of key populations were shown for the first time in intact mat sections. Furthermore, the biomass of key populations was estimated.

Materials and methods

Study site and microbial mat sampling

The Nakabusa hot spring is located in the Nagano Prefecture, Japan (36°23′ 15″N, 137°45′ 00″E). The pH of the spring water was slightly alkaline (pH 8.5–9.0). Earlier studies reported a sulfate concentration of 0.019–0.246 mM, a total organic carbon content of 0.393–0.415 mg L^{-1}, and moderate sulfide concentrations of 0.046–0.123 mM [26,27]. However, these chemical characteristics can vary between different sampling sites and seasons.

In September 2006 and October 2007, mats from two contrasting sites were sampled. Site 1 consisted of grayish mats which developed close to the spring source under flowing water with a temperature of approximately 75 °C (called "microbial streamer"), whereas at site 2 olive-green mats developed on an almost vertical concrete wall with overflowing spring water of 65 °C (Fig. 1). All mats had grown to approximately 4 mm thickness.

Microbial mat samples were kept in sterile plastic tubes (for molecular analysis) or glass bottles completely filled with hot spring water to avoid oxidation. Samples were transferred to the laboratory on ice and used for physiological experiments within 8 h.

Sulfide consumption measurements

Artificial hot spring water (~0.5 mM S^{2-}, 1 mM Cl^-, 1 mM PO_4^{3-}, 0.6 mM SO_4^{2-}, 2 mM K^+, ~4.1 mM Na^+, pH 8.5) amended with 1 mM HCO_3^- was used to measure the CO_2-dependent change of the sulfide concentration. An aliquot (60 mL) of spring water was added to a clean sterilized glass bottle (70 mL), bubbled with H_2O-vapor-saturated N_2 gas for 20 min, and preheated to 65 °C in a water bath. Approximately 1 g of the mat from site 2 grown at 65 °C was placed in the bottle, which was subsequently sealed with a butyl rubber stopper, under a headspace of N_2 gas and incubated at 65 °C. Bottles were wrapped in aluminum foil for dark treatment, and were illuminated with incandescent light (approximately 200 W/m^2). For oxic incubations the butyl rubber stopper was removed and the water was stirred at approximately 500–600 rpm to incorporate air. Subsamples of 250 μL of spring water were collected with a gas-tight syringe (Hamilton, Nevada, USA) in several minute intervals. The subsamples were immediately fixed with 500 μL 0.4% zinc acetate and alkalized with 500 μL 0.04 N NaOH. Dissolved sulfide concentrations were measured colorimetrically by the methylene blue formation method [5].

DNA extraction

Approximately 0.5 g (wet weight) of the mat pieces were placed in a 1.5 mL tube containing 1 mL of extraction buffer (100 mM Tris–HCl, 10 mM EDTA, 100 mM NaCl, 0.5% SDS [pH 8.0]). Initially, three different protocols for mechanical cell lysis were applied: (i) homogenization with a pestle, (ii) homogenization with a pestle

and bead beating at 2700 rpm for 45 s (Disruptor Genie, Scientific Industries, Inc., NY, USA), and, (iii) homogenization with a pestle and bead beating combined with an additional two cycles of freezing–thawing using liquid nitrogen and a 65 °C water bath. For clone library construction extended protocol (iii) was used.

After centrifugation at 19,600 × *g* for 2 min at 4 °C, the supernatant was transferred to a new tube. Two steps of chemical cell lysis were subsequently applied to the mats. First, the mats were incubated in 60 μL lysozyme solution (100 mg mL^{-1}) at 37 °C for 10 min and finally in 60 μL proteinase K (1 mg mL^{-1}; both in 100 mM Tris–HCl, 10 mM EDTA [pH 8.0]) at 37 °C for 30 min. The DNA was finally extracted according to the protocol by Wilson [40] based on CTAB and phenol–chloroform–isoamyl alcohol (25:24:1; v/v/v) purification.

PCR

For amplification of bacterial 16S rRNA genes, primer pair Eub27F [15]/Uni1390R [42], primers APS7-F/APS8-R for the adenosine 5′-phosphosulfate reductase gene (*aprA*) [8], and primers pufLM-F/pufLM-R for the *pufLM* genes of the photosynthesis gene cluster [25] were used. The primers targeted *pufLM* genes of purple sulfur bacteria, purple non-sulfur bacteria and green non-sulfur bacteria (e.g. *Chloroflexus* spp.). For DGGE analysis, bacterial 16S rRNA genes were amplified using primer set 341F/907R [24] in which the GC-clamp was attached to the 5′ end of primer 341F.

PCR conditions were as follows: an initial denaturation step at 94 °C for 1 min, followed by 25 cycles of denaturation at 94 °C for 2 min, annealing at 45 °C for 1.5 min, and elongation at 72 °C for 2 min, and a final extension step at 72 °C for 10 min. PCRs were performed in a 2720 Thermal Cycler (Applied Biosystems, Tokyo, Japan). For amplification of *aprA*, the following conditions were used: 94 °C for 30 s, 48 °C for 55 s, and 72 °C for 1 min. Conditions for *pufLM* were: 98 °C for 30 s, 55 °C for 1 min and 72 °C for 1.5 min.

DGGE analysis

DGGE was performed using the D-code system (Bio-Rad, Hercules, CA, USA), as described by Muyzer et al. [23]. PCR products were applied onto a 1.5 mm thick, 6% (w/v) polyacrylamide gel in 0.5× TAE (20 mM Tris–acetate, 10 mM acetate, 0.5 mM EDTA, pH 8.3) with a gradient that contained 20–50% denaturant (100% denaturant was 7 M urea and 40% [v/v] deionized formamide). Electrophoresis was run in 0.5× TAE electrophoresis buffer at a constant voltage of 200 V and a temperature of 60 °C for 4 h. After electrophoresis, the gels were stained with ethidium bromide (1 μg mL^{-1}). DGGE bands were excised from the gels, and reamplified by PCR using the same primers as described above but applying 19 cycles.

Clone library construction

For molecular cloning of 16S rRNA, *aprA*, and *pufLM* genes the TOPO® XL cloning kit (Invitrogen, Tokyo, Japan) was used according to manufacturer's recommendation.

Sequencing and phylogenetic analysis

Obtained clones were checked for the presence and size of inserts by PCR. Amplification products of the correct size were purified using the Rapid PCR Purification System (Marligen Biosciences, MD, USA). Partial sequence analyses were performed by using the DYEnamic ET Terminator Kit (GE Healthcare) and an ABI PRISM 310 capillary sequencer (Applied Biosystems, Tokyo, Japan). Subunit M was partially sequenced from cloned *pufLM* genes. Sequence affiliations were obtained using BLASTX [1] searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The phylogenetic affiliation of *aprA* and 16S rRNA genes was inferred with the ARB software package [17] based on Release 102 of the SILVA database [32]. Trees were calculated by maximum likelihood analysis (RAxML, PHYML) and the neighbor-joining algorithm. A 50% base frequency filter was used for 16S rRNA tree calculation to exclude highly variable positions. The phylogenetic tree of the protein-encoding gene *aprA* was generated from deduced amino acid sequences by PhyML and neighbor-joining analysis with a 30% amino acid frequency filter. Relevant partial sequences were subsequently added to the tree according to maximum parsimony criteria, without allowing changes in the overall tree topology.

Probe design

Oligonucleotide probes were designed using the probe tool in the ARB software package [17]. The probes were tested for coverage (target group hits) and specificity (outgroup hits) *in silico* with the ARB probe match tool [17]. For evaluation of probe coverage, only sequences that possessed sequence information at the probe binding site were considered. Probe specificity was based on 410,427 prokaryotic sequences of the SILVA SSU Ref dataset Release 102 [32]. Specific FISH conditions were determined by applying elevated formamide concentrations on cultured reference organisms having no or one mismatch to the probe [20]. For the newly designed probe SFH646, *Sulfurihydrogenibium azorense* (DSM15241) was used as the perfect match strain and *Persephonella guaymasensis* (DSM14351) as a control having a single mismatch.

Catalyzed reporter deposition fluorescence in situ hybridization

Microbial mat samples were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; 130 mM NaCl, 10 mM sodium phosphate [pH 7.3]) for 1 h at 4 °C. The fixed microbial mat was then washed twice in PBS and stored in a 1:1 mixture of PBS/ethanol at −20 °C. The fixed mat was embedded into Tissue Tek O.C.T. compound (Sakura Finetek U.S.A., Inc., Torrance, USA) after removing excess liquid and was then frozen at −80 °C. The embedded sample was cross-sectioned into 10 μm thick sections using a cryostat microtome at −30 °C (MICROM HM505E, Zeiss, Germany).

Endogenous peroxidases were inactivated by incubating the sections in 0.01 M HCl for 10 min at room temperature. Cell walls were permeabilized by incubating the sections in 15 μg mL^{-1} proteinase K (dissolved in 0.1 M Tris–HCl, 0.05 M EDTA [pH 8.0]) for 5 min at 37 °C [38]. CARD-FISH was performed according to the protocol by Pernthaler et al. [30]. Catalyzed reporter deposition was performed using tyramides labeled with the fluorochromes Alexa Fluor 488. For dual hybridization, a Cy3-monolabeled oligonucleotide probe was added according to the general FISH protocol [35]. Finally, samples were stained with 4′,6′-diamidino-2-phenylindole (DAPI). Probes and formamide concentrations used in this study are given in Table 1. Oligonucleotide probes were purchased from Biomers (Germany).

Confocal laser scanning microscopy

Hybridized samples were examined with an epifluorescence microscope (Axioskop II; Zeiss, Germany). Micrographs of mat sections were obtained by confocal laser scanning microscopy (LSM510; Zeiss, Germany).

Biovolume determination

Quantification of cell biovolumes was carried out based on images of mat sections hybridized with *Sulfurihydrogenibium*- and Chloroflexi-specific probes, and stained with DAPI. Confocal laser

Table 1
Oligonucleotide probes used in this study.

Probe	Target organisms	Probe sequence (5′–3′)	Group coverage[a]	Group coverage [%]	FA conc.[b]	Reference
EUB338-I	Bacteria	GCTGCCTCCCGTAGGAGT	355789/391178			[2]
EUB338-II	Supplement to EUB338: order Planctomycetales	GCAGCCACCCGTAGGTGT	2175/391178	93.5	35	[6]
EUB338-III	Supplement to EUB338: order Verrucomicrobiales	GCTGCCACCCGTAGGTGT	7436/391178			[6]
NON338	Control probe complementary to EUB338	ACTCCTACGGGAGGCAGC	0/391178	0	35	[39]
AQI338	Phylum Aquificae (except *Desulfurobacteriaceae*)	GCTGCCCCCCGTAGGGGT	777/851	91.3	40	This study
SFH646	*Sulfurihydrogenibium* spp. (except *S. yellowstonense*) including Nakabusa microbial mat sequences	CTCCCTGCCTCAAGTCCA	25/40	62.5	60	This study
Tdes830	*Thermodesulfobacteriaceae* (except *Thermodesulfobacterium*)	GTTAGCTTCGGCCCAGAGAG	49/98	50.0	20	[27]
CFX1238	*Chloroflexus* spp.	CGCATTGTCGTGGCCATT	20/32	62.5	20	[28]

[a] No. of targeted sequences/total sequences within the target group.
[b] Formamide concentrations in the hybridization buffer (v/v).

scanning micrographs were taken with an image area of 8489 μm^2 and an optical slice thickness of 0.9 μm. Images were converted to single tiff-images. The area of the signals was quantified after manual setting of thresholds with the software ImageJ (version 1.43u, http://rsb.info.nih.gov/ij/, Rasband 1997–2009). Three replicate images were analyzed per layer and the mean biovolume was calculated. DAPI stained sections were used for calculation of the total biovolume.

Nucleotide sequence accession numbers

The sequences from this study will appear in the EMBL, GenBank and DDBJ databases under the following accession numbers: 16S rRNA genes (FR691779–FR691803), *aprA* (FR691858), and *pufM* (FR691857).

Results

Microbial diversity in olive-green microbial mats based on 16S rRNA

First insights into the composition of the Nakabusa hot spring microbial mat communities were obtained by DGGE. Numerous bands were detected in the profiles of three individual pieces of a mat grown at 65 °C and of a microbial streamer collected from the 75 °C site, respectively (Fig. 2). Some of the bands were present in all profiles (e.g. bands NKB9, -12, -18, -19), while others were unique for 65 °C (NKB50, -69, -70) or 75 °C (NKB5). Representative DGGE bands were selected for sequencing. All sequences obtained were closely related to thermophilic microorganisms which have been frequently isolated from other hot springs. Among these, species involved in the sulfur cycle were preferentially retrieved. At least three different phylotypes (bands NKB5, -9, -18, -19) were closely affiliated with members of the genus *Sulfurihydrogenibium* (98–99% sequence similarity). They belonged to the order Aquificales of the phylum Aquificae, which is a group of metabolically versatile chemolithoautotrophic thermophiles with the shared ability to oxidize hydrogen and sulfur. A second phylotype (band NKB12, 93% sequence similarity) was distantly related not only to *Thermodesulfobacterium* and *Thermodesulfatator* spp., which are both sulfate-reducing bacteria completely oxidizing acetate and fatty acids to CO_2, but also to *Caldimicrobium* spp. which are thiosulfate and sulfur-reducing organisms. A third phylotype (band NKB70, 98–99% sequence similarity) was closely related to *Chloroflexus aggregans*, which is a filamentous anoxygenic phototroph using reduced sulfur compounds. The band was intense and unique to the olive-green microbial mats but was not observed in the DGGE profile of the microbial streamer. Further sequences obtained from the DGGE bands were related to Spirochaetes (band NKB48), Chlorobi (band NKB50), Acidobacteria (band NKB46), and OP10 (band NKB69).

As a basis for probe design, 21 clones were also sequenced from a 16S rRNA gene library obtained from the olive-green microbial mat.

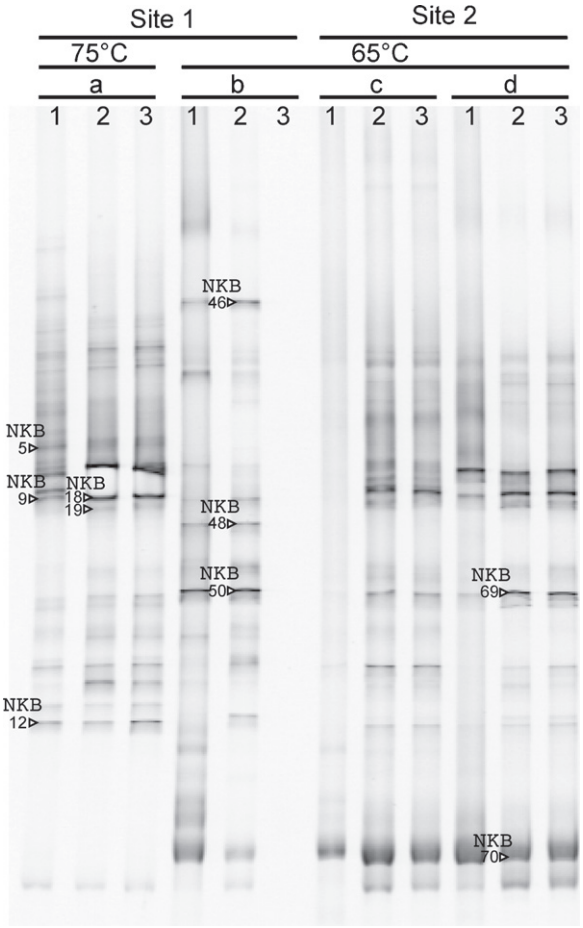

Fig. 2. DGGE profiles of 16S rRNA genes obtained from streamers and mats grown in spring water at 75 °C and 65 °C, respectively. Numbers 1–3 refer to different protocols used for mechanical cell lysis during DNA extraction. Numbered bands were sequenced.

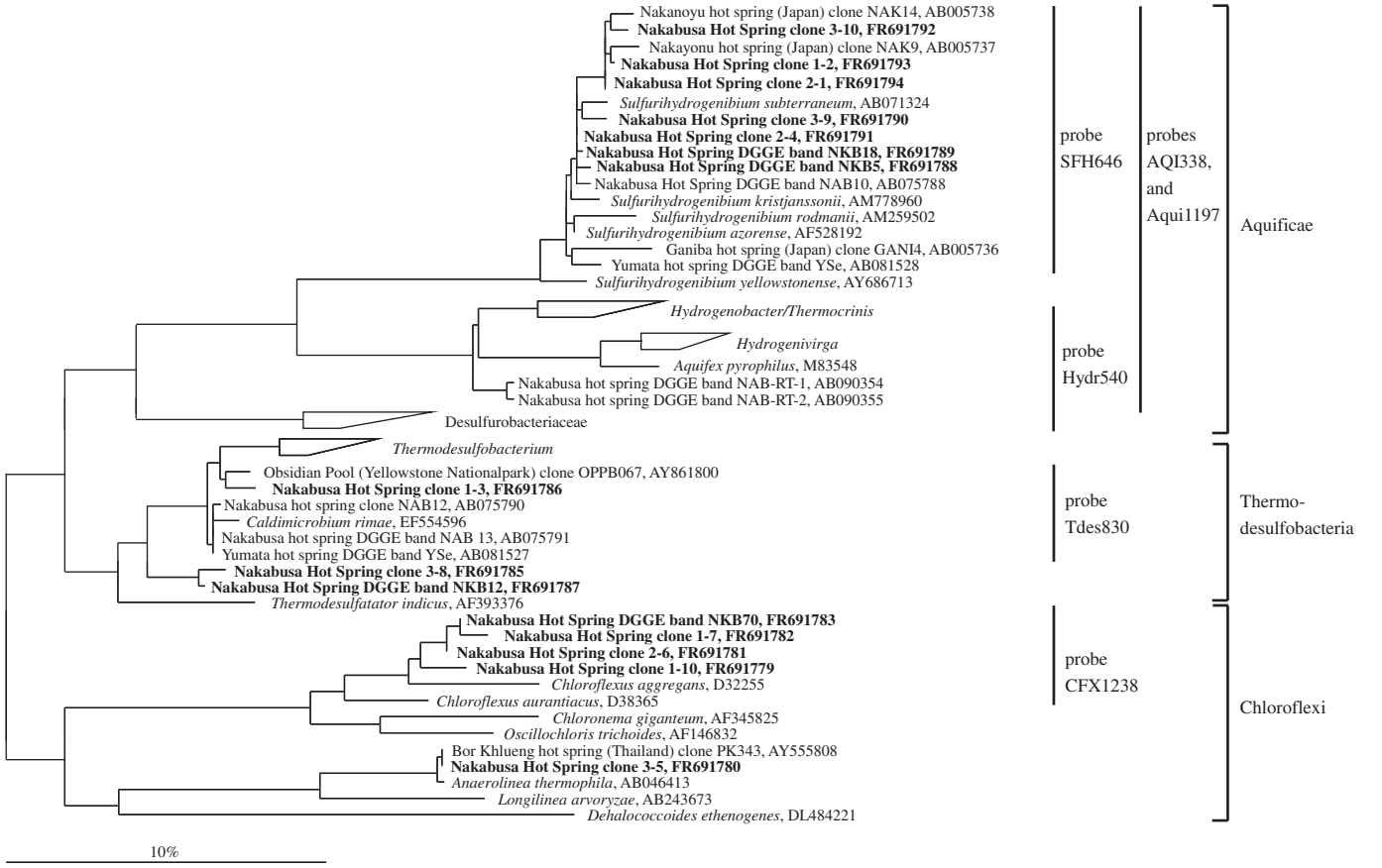

Fig. 3. 16S rRNA gene-based phylogenetic tree showing the affiliations of selected Nakabusa sequences obtained by cloning and DGGE analysis with bacterial reference sequences. Sequences from Nakabusa microbial mats are highlighted in boldface type. The tree was constructed by using maximum likelihood analysis in combination with filters that considered only 50% conserved regions of the 16S rRNA. Partial sequences were inserted into the reconstructed tree by using parsimony criteria with global–local optimization, without allowing changes in the overall tree topology. Probe specificity is indicated by the vertical lines. The scale bar gives 10% estimated sequence divergence.

Sequence similarities to cultured species were often high (>98%) and similarly high to sequences obtained from the DGGE bands, for instance, Aquificae (9 sequences), Chloroflexi (5 sequences), Thermodesulfobacteria (2 sequences), Thermotogae (3 sequences), Dictyoglomi (1 sequence), and candidate division Termite Group-1 (TG-1, 1 sequence, Fig. 3).

Metabolic genes

The dissimilatory adenosine 5′-phosphosulfate (APS) reductase encoded by the gene *apr* is a key enzyme in microbial sulfate reduction. Homologues have been found in some anaerobic photo- and chemotrophic sulfur-oxidizing bacteria [21], in which they are postulated to operate in the reverse direction, oxidizing sulfite to APS. Subunit A is highly conserved among sulfate reducers, thus it is often used to study the microbial community of the sulfur cycle [8,21]. The primer set used in this study (APS7-F/APS8-R) was restricted to the amplification of sulfate reducers. Sequencing of three representative clones from an *aprA* clone library resulted in a single phylotype most closely affiliated with *aprA* genes from *Thermodesulfobacterium* species (87% amino acid identity; Fig. 4).

The diversity of photosynthetic bacteria in the mats was further studied by comparative sequence analysis of the gene encoding subunit M of the photosynthesis reaction center, *pufM* [25]. Sequencing of three representative clones retrieved from the olive-green microbial mats at 65 °C resulted in highly similar (>99% sequence similarity) sequences that clustered with those of *C. aggregans* and showed amino acid identities of 98%.

Probe design

Probe target site analysis of the 16S rRNA gene sequences retrieved from the mat showed that most sequences were covered by the general bacterial probe EUB338 I-III, except for the *Sulfurihydrogenibium*-related sequences which had two mismatches [6]. Thus, a probe complementary to the modified EUB338 target site was designed and named AQI338. When compared to a curated 16S rRNA database (SILVA Release 102), AQI338 showed high coverage (91.3%) of the phylum Aquificae (Table 1). The deep-branching family *Desulfurobacteriaceae* was not targeted by AQI338 (2 mismatches) but excluding this group from the analysis would still have given a probe coverage of 94.8%. Probe AQI338 had 132 outgroup hits. Of these, 105 hits were within the candidate division OD1, whereas others were single hits within Proteobacteria, other uncultured bacterial candidate divisions, and archaeal Halobacteria. This new probe can be applied together with the other EUB338 probes to obtain an improved coverage of the domain Bacteria. We are aware of a previously published phylum-specific FISH probe for Aquificae, Aqui1197 [33], with almost identical group coverage (92.1%) as AQI338 and only two outgroup hits. However, this probe requires the application of a helper oligonucleotide which we tried to avoid in order to simplify the application.

Additionally, probe SFH646 was designed to target *Sulfurihydrogenibium* spp., including the sequences obtained from the Nakabusa microbial mat. Experiments showed that probe SFH646 gave a bright CARD-FISH signal with *S. azorense*, which was used as the target organism. *P. guaymasensis* was used as the reference for one

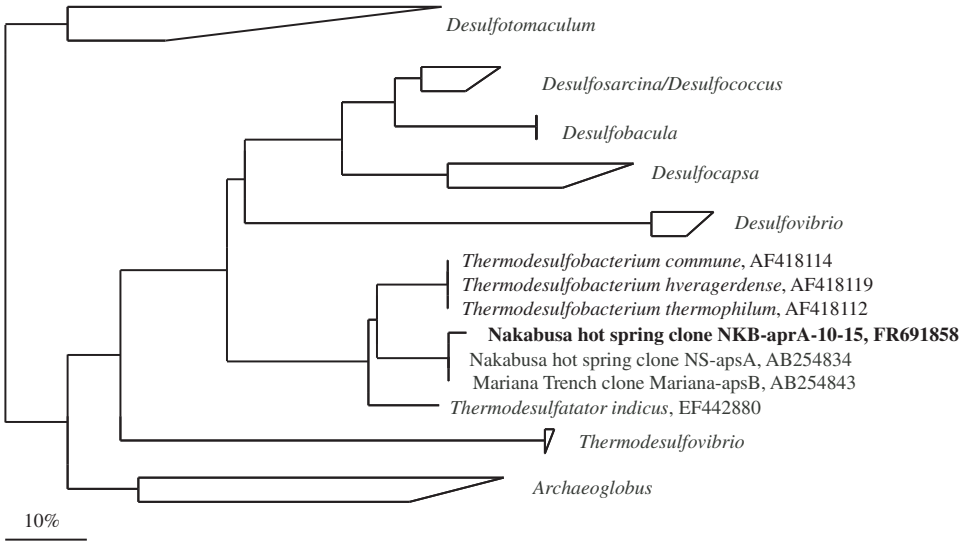

Fig. 4. Phylogenetic tree based on comparative analysis of 291 amino acid positions of adenosine 5′-phosphosulfate reductase gene (*aprA*) sequences. The representative sequence from Nakabusa microbial mats is highlighted in boldface type and shown together with selected reference sequences from cultivated bacteria. The tree was generated from deduced amino acid sequences by PhyML and neighbor-joining analysis with a 30% amino acid frequency filter. The scale bar gives 10% estimated sequence divergence.

mismatch to the probe. Stringent conditions were achieved using 60% formamide in the hybridization buffer.

In situ localization of main populations by CARD-FISH

Microscopic analysis of cross sections of the microbial mat grown at 65 °C revealed that the mat was laminated in 20–30 μm thick layers (Fig. 5). The layers seemed to be separated by thin channels which might enable sulfidic hot spring water to be transported and distributed throughout the mats. Total cell biovolumes, as determined by image analysis of DAPI stained mat sections, varied between 15% (mat surface) and 45% of the mat volume (Table 2).

Members of the *Sulfurihydrogenibium* and Chloroflexi were the dominant microbes in Nakabusa olive-green microbial mats. Their vertical distribution was heterogeneous (Fig. 5). *In situ* hybridization with both the Aquificae-specific probe AQI338 and the newly designed highly specific probe SFH646 for *Sulfurihydrogenibium* spp. and Nakabusa clone sequences revealed that these cells were mainly located near the mat surface (uppermost 150 μm) which was whitish and covered with a gelatinous layer. Stained *Sulfurihydrogenibium* cells were sausage-like, ca. 10 μm in length and 2 μm in width. They were either loosely aggregated or dispersed. In this top 150 μm layer, *Chloroflexus* filaments were less abundant. Quantification of the *Chloroflexus* and *Sulfurihydrogenibium* biovolumes resulted in a ratio of 0.7 (Table 2), and the latter biovolume made up approximately 50% of the total biovolume. Below the surface layer dominated by *Sulfurihydrogenibium*, there was a thin mixed layer of approximately 100 μm that contained both *Chloroflexus* filaments and *Sulfurihydrogenibium* in almost equal biovolumes (ratio 1.1). Deeper than 250 μm, *Sulfurihydrogenibium* cells were only rarely observed and *Chloroflexus* related filaments dominated, shifting the biovolume ratio to 51–3619. The *Chloroflexus* biovolume was up to 64% of the total cell biovolumes (Table 2). In the deep layers, there was background fluorescence originating from cells resembling the sausage-like *Sulfurihydrogenibium*. Since these cells were not detected by CARD-FISH, the background fluorescence might originate from decaying *Sulfurihydrogenibium* that had been overgrown. *Thermodesulfobacterium*-related cells were not detected by CARD-FISH using probe Tdes830, although two sequences of this group were retrieved.

Physiological studies addressing sulfur cycling in the microbial mats

Pieces of the olive-green microbial mat growing at 65 °C were incubated with artificial spring water under oxic and anoxic conditions, and with or without light in order to follow CO_2-dependent chemolithoautotrophic sulfide oxidation. Under oxic-dark conditions, rapid consumption of sulfide was observed (Fig. 6). The initial concentration of sulfide (ca. 400 μM) decreased in the presence and in the absence of HCO_3^- to almost zero within 20 and 70 min, respectively. As a control for abiotic oxidation of sulfide, a mat sample was sterilized by boiling for 10 min prior to the oxic-dark incubation. No oxidation of sulfide was observed (data not shown).

Under anoxic-light conditions, the sulfide concentration remained stable in the absence of HCO_3^-, however, it decreased

Table 2
Biovolume of *Chloroflexus* spp. and *Sulfurihydrogenibium* spp. in a Nakabusa microbial mat developed in 65 °C spring water.

Depth [mm]	Biovolume			
	Total cell biovolumes[a] [% of mat volume]	*Chloroflexus* [% of total cell biovolumes]	*Sulfurihydrogenibium* [% of total cell biovolumes]	Ratio *Chloroflexus*/*Sulfurihydrogenibium*
0.15	15	34	50	0.7
0.25	44	45	43	1.1
0.7	43	64	1	51
1.35	43	39	0.2	200
2.2	45	38	0.01	3619
2.4	34	9	0.02	560

[a] Determined by quantification of DAPI-stained cells.

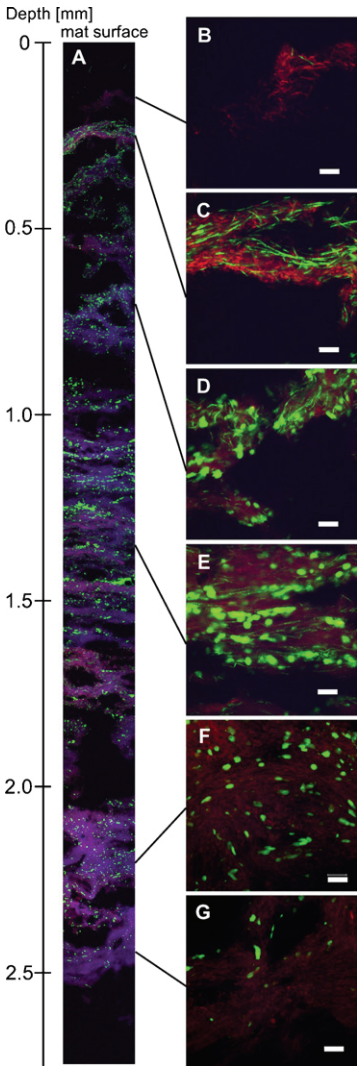

Fig. 5. Confocal laser scanning micrographs of a Nakabusa microbial mat thin section hybridized with probe SFH646 specific for *Sulfurihydrogenibium* (red) and probe CFX1238 specific for *Chloroflexus* spp. (green). DAPI staining of total cells is shown in blue. (A) Profile through the mat. The depth below the mat surface is indicated on the left. (B)–(G) Close-ups of *Sulfurihydrogenibium* and *Chloroflexus* distributions and aggregations at different depths of the mat. Scale bar, 10 μm.

when the medium was amended with HCO_3^-. Under anoxic-dark conditions, a slight increase of sulfide concentration was observed in the presence of HCO_3^-, indicating sulfide production. Similar results were obtained using natural spring water (data not shown).

Discussion

A combination of physiological experiments and nucleic acid based molecular studies was used to analyze the structure and function of the olive-green microbial mat that developed at 65 °C in sulfidic spring water. Comparative 16S rRNA sequence analysis suggested that all the bacteria found in the clone library (except for termite group 1) used sulfur compounds in their metabolism. Therefore, in the following discussion the localization, abundance and potential metabolism of members of *Sulfurihydrogenibium*, *Chloroflexus*, and *Thermodesulfobacterium/Thermodesulfatator/Caldimicrobium* in sulfur conversions catalyzed inside the microbial mat will be considered.

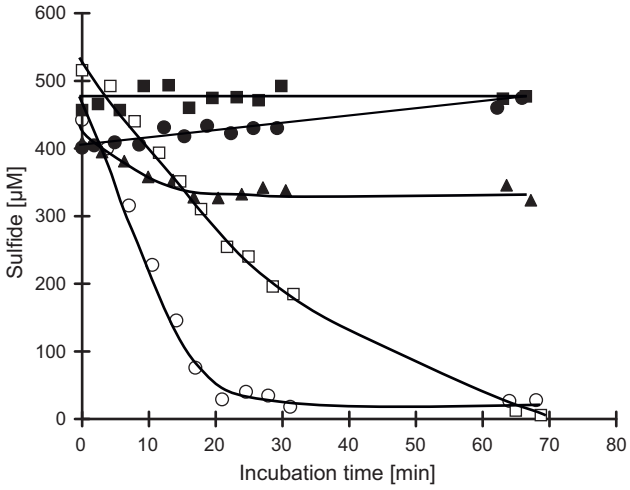

Fig. 6. Sulfide consumption by the microbial mat grown in artificial hot spring water at 65 °C. Conditions: ■, $-HCO_3^-$ and anoxic-light; ●, $+HCO_3^-$ and anoxic-dark; ▲, $+HCO_3^-$ and anoxic-light; □, $-HCO_3^-$ and oxic-dark; ○, $+HCO_3^-$ and oxic-dark.

Aerobic chemoautotrophic sulfur oxidation

Physiological experiments revealed a rapid consumption of sulfide in the microbial mat during oxic-dark conditions. Under identical incubation conditions but without the addition of HCO_3^-, the consumption rate of sulfide decreased to approximately 50% (Fig. 6), suggesting that the aerobic sulfide oxidation in the microbial mat was linked to chemoautotrophy. 16S rRNA analysis showed the presence of diverse *Sulfurihydrogenibium* phylotypes of the phylum Aquificae having the closest affiliation (98.9% similarity) to *S. subterraneum* and *S. kristjanssonii*. Vertical CARD-FISH profiling confirmed the molecular data and showed a heterogeneous distribution of Aquificae with highest abundances close to the mat surface. Both *S. subterraneum* and *S. kristjanssonii* are strictly chemolithoautotrophic, thermophilic organisms that have been isolated from terrestrial hot springs. They show growth solely with molecular hydrogen or sulfur/thiosulfate as an electron donor [7,37]. While *S. subterraneum* is a facultative anaerobic organism capable of using electron acceptors other than oxygen (e.g. Fe(III) or nitrate), *S. kristjanssonii* is strictly aerobic. Thus, the presence of *Sulfurihydrogenibium* species near the surface of Nakabusa mats (0–300 μm) suggests oxic to suboxic conditions in this region. *Sulfurihydrogenibium* was also found to dominate the microbial community of microbial streamers that formed in 72–80 °C spring water at Nakabusa [27], indicating a broad temperature range for this group. Nakagawa and Fukui [27] proposed that changes in the Aquificae community were related to changes in the concentration of dissolved sulfide in the hot spring water: *Aquifex*-like phylotypes were dominant at sulfide concentrations below 0.1 mM, whereas *Sulfurihydrogenibium* phylotypes were dominant when sulfide was >0.1 mM. However, our results were slightly different from earlier observations: *Aquifex* sequences were not detected, and *Sulfurihydrogenibium* dominated at moderate sulfide concentrations in the spring water, where the availability of oxygen seemed to structure the mat rather than the sulfide concentrations. Although the biovolume of Aquificae was much smaller than that of Chloroflexi, Aquificae seemed to contribute to the high productivity of the microbial mat with a fast chemoautotrophic sulfur-oxidation.

Anaerobic phototrophic sulfur oxidation

Under anoxic conditions in the absence of HCO_3^- no sulfide consumption could be detected. In the presence of HCO_3^- and light, however, a decrease in the sulfide concentration was detected that

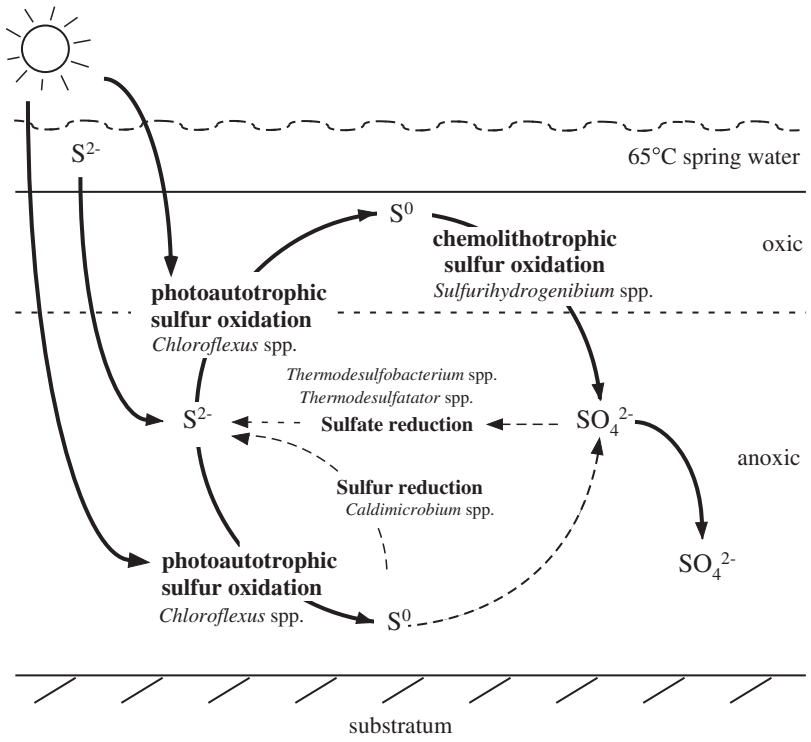

Fig. 7. Proposed simplified sulfur cycle within Nakabusa hot spring microbial mats.

indicated photoautotrophic sulfur oxidation. The only phototrophs detected in the mats were members of the genus *Chloroflexus* which are filamentous anoxygenic phototrophs. Comparative sequence analysis of both 16S rRNA gene and *pufM* showed the closest relationship with *C. aggregans* (99% and 94% sequence identities, respectively). *C. aggregans* has been described as a thermophilic bacterium that grows anaerobically as a photoheterotroph or aerobically as a chemoheterotroph [10]. Although autotrophic photosynthesis has not yet been observed in *C. aggregans*, there are some indications for possible autotrophic growth of a close relative, *Chloroflexus aurantiacus* [31]. Comparative genomics of *C. aurantiacus*, *C. aggregans* and *Roseiflexus* sp. provided evidence for the presence of genes coding for the 3-hydroxypropionate autotrophic pathway [14], suggesting that *C. aggregans* is capable of fixing bicarbonate.

In this study we showed a wide distribution of filamentous Chloroflexi throughout the mat with the highest abundance (64% of the total cell volume) in the deeper layers. This pattern can be explained by high metabolic versatility. As described above, *Chloroflexus* cells obtain their energy mainly through photosynthesis in the anoxic interior of the mat, while *Chloroflexus* cells near the oxic surface region of the mat coexist with *Sulfurihydrogenibium* as chemoorganotrophs. The presence of *Chloroflexus* in the Nakabusa mat was previously described by Sugiura et al. [36] based on the detection of photosynthetic pigments.

Sulfate reduction

During incubations of olive-green mats under anoxic-dark conditions, a slight sulfide production was observed. Few 16S rRNA gene sequences obtained from the mat affiliated with sulfate-reducing bacteria and they were distantly related to the genera *Thermodesulfobacterium* and *Thermodesulfatator*. These findings confirm previous studies in which *Thermodesulfobacterium*-related DGGE bands and FISH analysis pointed towards the importance of this group [26,27]. The only *aprA* gene phylotype obtained in this study was also affiliated with *Thermodesulfobacterium*. This genus includes thermophilic, obligate anaerobic autotrophic or heterotrophic sulfate-reducing bacteria, as described by Jeanthon et al. [12] and references therein. *In situ* hybridizations with a FISH probe specific for some of the obtained *Thermodesulfobacterium/Thermodesulfatator*-related Nakabusa sequences did not reveal signals which might be explained by a low abundance of this group in the 65 °C mat. Based on our data we can currently only speculate about sulfate reduction and a complete sulfur cycle in the mat. Further experiments (e.g. sulfate reduction measurements) with fresh mat samples or the application of other FISH probes, such as probe TDSBM652 [16], are needed to fully address the relevance of this process.

Chemoautotrophic sulfur reduction

Comparative 16S rRNA sequence analysis revealed that the group of sequences related to *Thermodesulfobacterium/Thermodesulfatator* was closely related to *Caldimicrobium rimae* (96% sequence similarity), a thermophilic, strictly anaerobic, chemolithoautotrophic thiosulfate or elemental sulfur-reducing bacterium [22]. Thus, chemoautotrophic sulfur reduction might also contribute to the reduction of sulfur compounds in Nakabusa mats.

Conclusions

In this study, ample evidence was obtained for microbial sulfur cycling in the olive-green mats growing at 65 °C. A sketch of the proposed microbial sulfur cycle in this part of the Nakabusa mats is shown in Fig. 7. The microbial mat community is dependent on sulfide which is continuously supplied from hot spring water, and the biomass is sustained mainly by two ways of autotrophic oxidation of sulfide, photoautotrophy and chemoautotrophy. Rapid oxidation of sulfide with oxygen by *Sulfurihydrogenibium* in surface layers likely causes a strong oxygen gradient in the mat. The anoxic conditions in the deeper layers favor filamentous anoxygenic photosynthetic *Chloroflexus* and sulfate-reducing bacteria.

A second important outcome of this study was the design of two new probes for members of the bacterial phylum Aquificae. The more general probe AQI338 had an Aquificae coverage of >90%, which was as good as that of the previously described Aqui1197. When applied in dual hybridizations these two probes can be used for a highly confident identification of Aquificae members [3]. Furthermore, AQI338 can be added to the general bacterial probe mixture EUB338 I-III [6] so that it also targets most members of Aquificae. The genus-specific probe SFH646 will in the future facilitate the identification, quantification and localization of members of the genus *Sulfurihydrogenibium* in thermophilic microbial mats and other habitats. If used together with probe Hydr540 [11] almost all Aquificae members could be covered.

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3

In situ identification and quantification of *Chloroflexi* in marine sediments and microbial mats

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In preparation

In situ identification and quantification of *Chloroflexi* in marine sediments and microbial mats

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In this study, we analyzed the distribution of marine uncultured *Chloroflexi* in various aquatic habitats. Mainly marine sediments and microbial mats were screened by fluorescence in situ hybridization (FISH) with 16S rRNA-targeted oligonucleotide probes. We frequently found *Chloroflexi* in the surface layer of sediments, yet, in low numbers of <1% of total cells. The benthic *Chloroflexi* had most often a filamentous morphology, but sometimes also coccoid cells were detected. In the Black Sea microbial mat, well known for its anaerobic oxidation of methane catalyzed by a microbial community composed mainly of methanotrophic archaea of the ANME-1 and ANME-2 type and the deltaproteobacterial DSS clade, high abundance of filamentous *Chloroflexi* were counted (up to 28% of all cells). Thin sections of the mat showed that filamentous *Chloroflexi* cells were surrounding ANME-2/DSS aggregates. Dominance of filamentous *Chloroflexi* had been reported before for microbial mats in wastewater treatment and hot springs, thus they seem to be particularly adapted to heterotrophic growth on mats. The network of filamentous *Chloroflexi* might support the mat structure.

The phylum *Chloroflexi* forms a deep branch within the domain *Bacteria*. It is phylogenetically as diverse as the phylum *Proteobacteria* and currently divided into six major classes, *Anaerolineae* (Yamada et al., 2006), *Caldilineae* (Yamada et al., 2006), "Chloroflexi", "Dehalococcoidetes", *Ktedonobacteria* (Cavaletti et al., 2006), and *Thermomicrobia* (Hugenholtz and Stackebrandt, 2004). Based on comparative 16S rRNA sequence analysis Rappé and Giovannoni (2003) divided the phylum in eight subgroups including uncultured clades, although it was not entirely consistent with the mostly used classification based on (Hugenholtz et al., 1998). The phylogenetic diversity of *Chloroflexi* reflects their metabolic diversity. Members of this phylum can perform respiration, fermentation, dehalorespiration (Maymó-Gatell et al., 1997) as well as anoxygenic photosynthesis (Pierson and Castenholz, 1974; Pierson et al., 1985; Hanada et al., 2002).

Chloroflexi seem to be widely distributed. They have been found in microbial mats formed at hot springs (Kubo et al., 2011), on granules occurring in wastewater treatment reactors (Yamada et al., 2005), in cyanobacterial mats (Klappenbach and Pierson, 2004), in soil (Chandler et al., 1998; Dunbar et al., 2002), in fresh water lakes (Urbach et al., 2001; Zwart et al., 2002),in meromictic lakes (Bowman et al., 2000; Koizumi et al., 2004) and there are numerous PCR-based hints that they might be widely distributed in marine sediments. Cultivated representatives are often thermophilic, and have filamentous or coccoid cell-shapes. Recently, the cultivation of new filamentous *Chloroflexi* from class *Anaerolineae* has been reported (Yamada et al., 2006). Also this group is moderately thermophilic (Yamada and Sekiguchi, 2009).

Sequences of uncultured *Chloroflexi* have also been detected in the marine water column (Morris et al., 2004; Varela et al., 2008). The so-called SAR202 clade is a component of open ocean bacterioplankton (Giovannoni et al., 1996; Schattenhofer et al., 2009). "Dehalococcoidetes" related uncultured *Chloroflexi* were reported for marine sediments (Hunter et al., 2006; Dang et al., 2009), tidal flat sediments (Wilms et al., 2006) , or as symbionts in marine sponges (Hentschel et al., 2002; Ahn et al., 2003; Siegl and Hentschel, 2010). *Anaerolineae* related sequences were reported from various marine sediments as well. Furthermore, 16S rRNA gene sequences of *Chloroflexi* were frequently retrieved from deep sea subsurface sediments where they often dominate clone libraries obtained by using general bacterial primer sets (Parkes et al., 2005; Inagaki et al., 2006; Teske, 2006; Webster et al., 2006; Li et al., 2008; Blazejak and Schippers, 2010) . In strong contrast to the numerous reports on PCR-based detection, few data are available on the abundance and metabolic functions of marine *Chloroflexi*.

In this study we used oligonucleotide probes targeting the 16S rRNA of *Chloroflexi* to identify and quantify this group in various marine sediments. In addition, we report here on the high abundance and spatial distribution of filamentous *Chloroflexi* in Black Sea microbial mats

Materials and methods

Samples

The sampling sites and type of samples analyzed in this study are listed in Table 2.

Microbial mats from the Black Sea were obtained from two different stations: (1) station P780 from expedition PO317/3, R/V Poseidon in 2004 from Danube area (43° 50' 34.80" N, 30° 27' 39.60" E). The mat is dominated with ANME-2 aggregates. (2) station 268-146 from expedition M72-2, R/V Meteor in 2007 (44° 46' 30.072" N, 31° 59' 32.352" E). Pink-colored mat, associated to carbonate parts was collected. Both mats were stored at 4°C under anoxic condition, amended with CH_4.

Probe design

The 16S rRNA-targeted oligonucleotide probes used in this study are listed in Table 1. The probe reported in this study was designed using the ARB software package (http://www.arb-home.de/) (Ludwig et al., 2004). The probes were tested for coverage (target group hits) and specificity (outgroup hits) *in silico* with probe match tool of ARB (Ludwig et al., 2004). This analysis considered 555,585 prokaryotic sequences of the SILVA SSU Ref dataset Release 106 (Pruesse et al., 2007). Specific FISH conditions were determined by Clone-FISH (Schramm et al., 2002) applying elevated formamide concentrations (Manz et al., 1992) on clones having no mismatch to the probe.

Table 1 Oligonucleotide probes used in this study

Probe	Target organisms	Probe sequence (5'-3')	Position [a]	FA concn. [b]	Reference
EUB338 I	most Bacteria	GCTGCCTCCCGTAGGAGT	338-355	35	Amann et al. 1990
EUB338 II	*Planctomycetales*	GCAGCCACCCGTAGGTGT	338-356	35	Daims et al. 1999
EUB338 III	*Verrucomicrobiales*	GCTGCCACCCGTAGGTGT	338-357	35	Daims et al. 1999
NON338	control probe complementary to EUB338	ACTCCTACGGGAGGCAGC	-	35	Wallner et al. 1993
GNSB941	all *Chloroflexi*	AAACCACACGCTCCGCT	941-957	35	Gich et al. 2001, Björnsson et al. 2002
CFX665	Black sea microbial mat *Chloroflexi*	CCCGAAATTCCACCTCCC	665-682	30	This study
DSS658	*Desulfosarcina/Desulfococcus* (including ANME-2 partners)	TCCACTTCCCTCTCCCAT	658-675	50	Manz et al. 1998
ARCH915	domain Archaea	GTGCTCCCCCGCCAATTCCT	915-934	35	Stahl et al. 1991
ANME 2c 760	ANME 2c Euryarchaeota	CGCCCCCAGCTTTCGTCC	760-777	60	Knittel et al. 2005
ANME 1 350	ANME 1 Euryarchaeota	AGTTTTCGCGCCTGATGC	350-367	40	Boetius et al. 2000

[a] Position in the 16S rRNA of *E. coli*

[b] Formamide (FA) concentration in the hybridization buffer (%, vol/vol)

Sample fixation and fluorescence in situ hybridization

Mat samples obtained from station P780 and station 268-146 from the Black Sea were fixed in 3% paraformaldehyde in phosphate-buffered saline (PBS; 130 mM NaCl, 10 mM sodium phosphate [pH 7.3]) for 11 hours at 4°C. The fixed microbial mat was then washed twice in filter-sterilized PBS and stored in a 1:1 mixture of PBS and 96% ethanol at -20°C. The fixed mat was embedded into Tissue Tek OCT compound (Sakura Finetek USA Inc., Torrance, USA) after replacing the PBS:EtOH in the sample with serial dilution of the OCT compound in PBS and frozen at -30°C. The embedded sample was sectioned into 10 µm-thick layers using a cryostat microtome (MICROM HM505E; Zeiss, Germany) according to Knittel et al. (2005).

Endogenous peroxidases were inactivated by incubating the sections in 0.3% H_2O_2 in methanol for 30 min at room temperature and washing with 50 ml of MilliQ water. For permeabilization of bacterial cell walls, sections on slides were incubated in 10 mg/ml lysozyme (dissolved in 0.1 M Tris-HCl, 0.05 M EDTA [pH 8.0]) for 30 min at 37°C, washed in MilliQ water twice (Teira et al., 2004) and dehydrated by absolute ethanol. CARD-FISH was performed according to the protocol of Pernthaler et al. (2002). For dual hybridizations with two probes, the CARD-FISH protocol was repeated on the same sections using a second probe and fluorescently labeled tyramide. Inactivation of HRP was done by incubation with methanol containing 0.3% H_2O_2 for 30 min and washing the sections with 50 ml of MilliQ water. The third hybridization was done with a Cy3-labeled oligonucleotide probe according to protocol for fluorescently labeled probes (Snaidr et al., 1997). The formamide concentrations used in this study are listed in Table 2. Oligonucleotides were purchased from Biomers (Ulm, Germany).

Fluorescence activated cell sorting (FACS) of Chloroflexi from the Black Sea microbial mat

An in-solution FISH protocol (Wallner et al., 1993; Kalyuzhnaya et al., 2006) was used with slight modifications for FACS of *Chloroflexi* from the Black Sea microbial mat. Prior to the CARD-FISH, the microbial mat was mechanically homogenized in PBS:EtOH (1:1, v/v) and ultrasonicated by a type MS73 probe (Sonopuls HD70; Bandelin, Germany) at a setting of 20 s, an amplitude of 42 µm, and <10 W. The cell suspension was hybridized for 3.5 h at 46°C, and washed for 10 min at 48°C. After CARD-FISH large aggregates which had from during the hybridization of the cell suspension were removed by 4 min of centrifugation at 2000 × g. Subsequently, cells were sorted with a MoFlo flow cytometer (Beckman Coulter; Brea, CA,

USA) into sterile 1.5 ml tubes using a 488 nm Argon-ion laser (400 mW) (INNOVA 306C; Coherent, Germany). Side angle light scatter (SSC) was detected through a 488 ± 5 nm band-pass filter and the fluorescence from Alexa488 stained cells (excitation at 499 nm, emission at 520 nm) was detected with a 530 ± 20 nm filter. As a sheath fluid, sterile and particle-free (<0.2µm) 0.1% NaCl (wt/vol) was used. The sorting results were analyzed by Summit® v.3.1. software package (Beckman Coulter). Sorting of probe-positive cells was performed at 106,770 Hz at an amplitude of 10.8 V and a delay of 45 10/16 droplets by using an nozzle with 70 µm. Sorted cell fraction was used for further metabolic gene analysis.

PCR on sorted cells

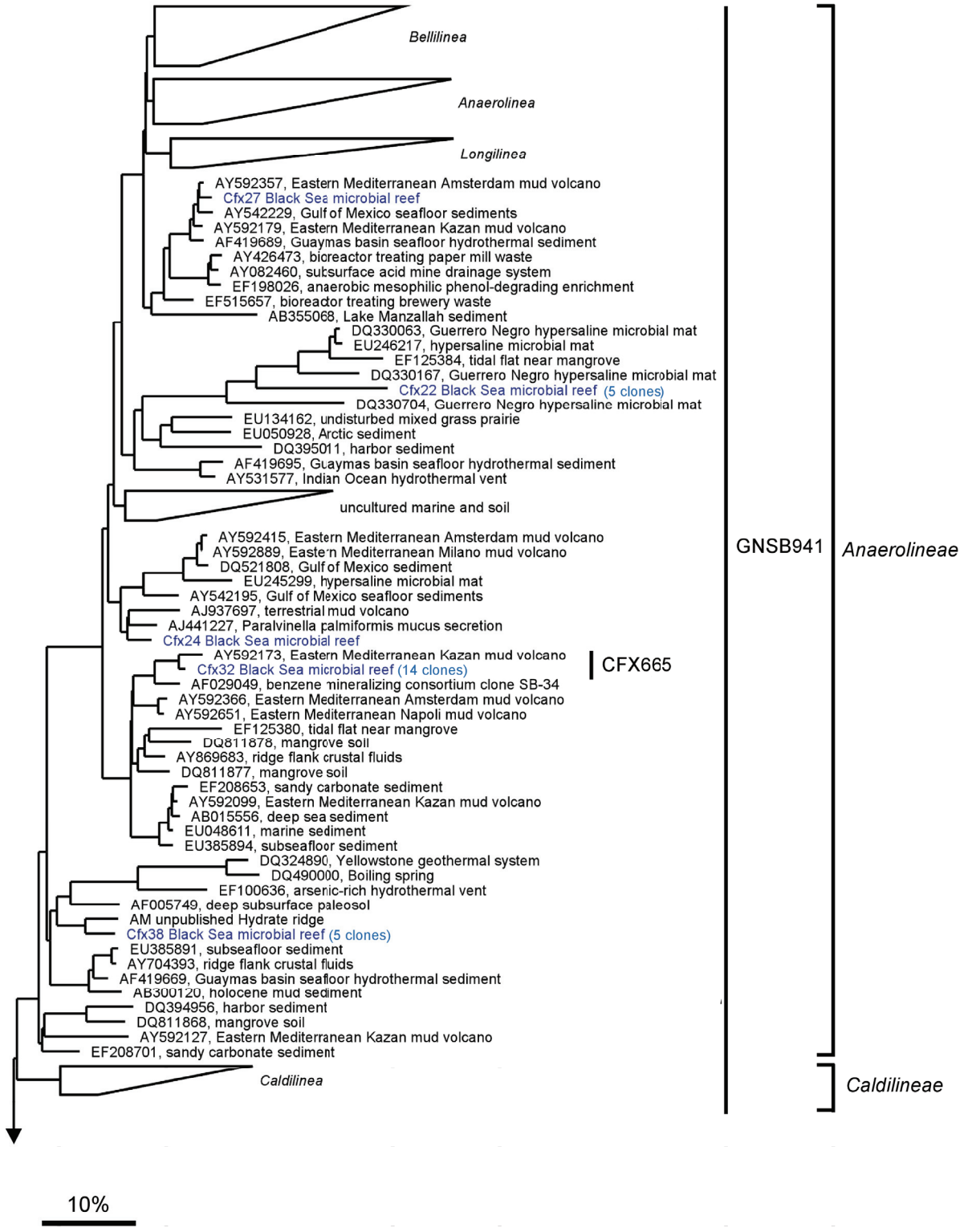
For amplification of bacterial 16S rRNA genes from sorted cells, primer pair GM3F/GM4R (Muyzer et al., 1995), GM3F/GNSB941R (Gich et al., 2001) and GM3F/CFX665R were used. PCR consisted of an initial denaturation step at 96°C for 4 min, followed by 25 cycles of denaturation at 96°C for 1 min, annealing at 58°C for 1 min, and elongation at 72°C for 2 min, and a final extension step at 72°C for 10 min. PCR products were checked on agarose electrophoresis and the amount of amplicon were quantified photometrically (NanoDrop 1000 Spectrophotometer, V.3.7.1, Thermo Scientific). Selected samples were sequenced.

Microscopy

Hybridized samples were examined with an Axioskop II epifluorescence microscope (Zeiss, Jena, Germany). Micrographs of mat sections were obtained by confocal laser scanning microscopy (LSM510 and LSM780; Zeiss, Jena, Germany).

Results

In this study we used two probes targeting *Chloroflexi* for in situ identification and quantification by FISH. We developed the oligonucleotide probe CFX665 which is highly specific for *Chloroflexi* sequences retrieved from Black Sea microbial mats (Fig. 2). The sequences for which the probe was designed group within *Anaerolineae* (Fig. 1). This specific probe was combined with a general *Chloroflexi* probe, GNSB941, published before by Gich and colleagues (2001). GNSB941 covers 94% (8251 hits within 9026 sequences) of total phylum *Chloroflexi*, while CFX665 targeted 18 sequences in all sequences from *Chloroflexi* and among those, 15 sequences were from our clone library from the Black Sea microbial mat.


Bellilinea
Anaerolinea
Longilinea
AY592357, Eastern Mediterranean Amsterdam mud volcano
Cfx27 Black Sea microbial reef
AY542229, Gulf of Mexico seafloor sediments
AY592179, Eastern Mediterranean Kazan mud volcano
AF419689, Guaymas basin seafloor hydrothermal sediment
AY426473, bioreactor treating paper mill waste
AY082460, subsurface acid mine drainage system
EF198026, anaerobic mesophilic phenol-degrading enrichment
EF515657, bioreactor treating brewery waste
AB355068, Lake Manzallah sediment
DQ330063, Guerrero Negro hypersaline microbial mat
EU246217, hypersaline microbial mat
EF125384, tidal flat near mangrove
DQ330167, Guerrero Negro hypersaline microbial mat
Cfx22 Black Sea microbial reef (5 clones)
DQ330704, Guerrero Negro hypersaline microbial mat
EU134162, undisturbed mixed grass prairie
EU050928, Arctic sediment
DQ395011, harbor sediment
AF419695, Guaymas basin seafloor hydrothermal sediment
AY531577, Indian Ocean hydrothermal vent
uncultured marine and soil
AY592415, Eastern Mediterranean Amsterdam mud volcano
AY592889, Eastern Mediterranean Milano mud volcano
DQ521808, Gulf of Mexico sediment
EU245299, hypersaline microbial mat
AY542195, Gulf of Mexico seafloor sediments
AJ937697, terrestrial mud volcano
AJ441227, Paralvinella palmiformis mucus secretion
Cfx24 Black Sea microbial reef
AY592173, Eastern Mediterranean Kazan mud volcano
Cfx32 Black Sea microbial reef (14 clones)
AF029049, benzene mineralizing consortium clone SB-34
AY592366, Eastern Mediterranean Amsterdam mud volcano
AY592651, Eastern Mediterranean Napoli mud volcano
EF125380, tidal flat near mangrove
DQ811878, mangrove soil
AY869683, ridge flank crustal fluids
DQ811877, mangrove soil
EF208653, sandy carbonate sediment
AY592099, Eastern Mediterranean Kazan mud volcano
AB015556, deep sea sediment
EU048611, marine sediment
EU385894, subseafloor sediment
DQ324890, Yellowstone geothermal system
DQ490000, Boiling spring
EF100636, arsenic-rich hydrothermal vent
AF005749, deep subsurface paleosol
AM unpublished Hydrate ridge
Cfx38 Black Sea microbial reef (5 clones)
EU385891, subseafloor sediment
AY704393, ridge flank crustal fluids
AF419669, Guaymas basin seafloor hydrothermal sediment
AB300120, holocene mud sediment
DQ394956, harbor sediment
DQ811868, mangrove soil
AY592127, Eastern Mediterranean Kazan mud volcano
EF208701, sandy carbonate sediment
Caldilinea
CFX665
GNSB941
Anaerolineae
Caldilineae
10%

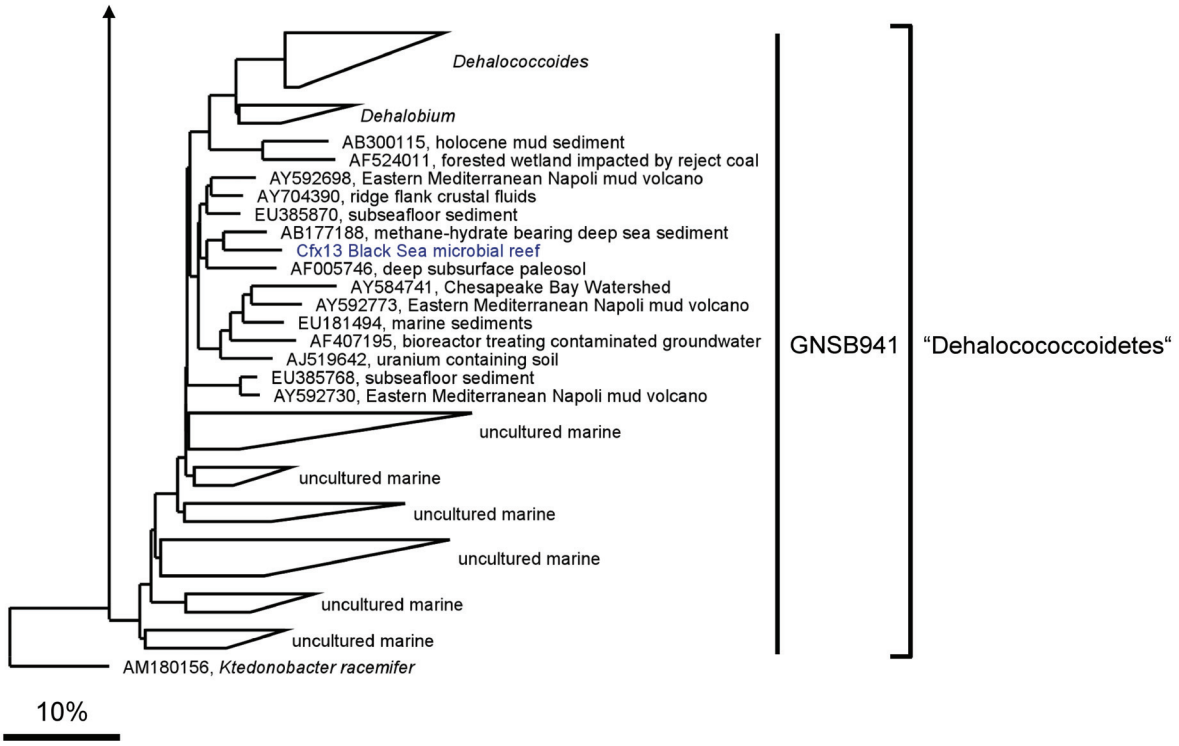


Figure 1 16S rRNA gene-based phylogenetic tree showing the affiliations of selected the Black Sea microbial mats sequences obtained by cloning with reference *Chloroflexi* sequences. Sequences from Black Sea mats are highlighted in blue. Number of similar clones (99% sequence similarity) is given in brackets. Probe specificity is indicated by vertical lines. The scale bar gives 10% estimated sequence divergence. (modified after Arnds, 2009)

Distribution of Chloroflexi in marine sediments

Chloroflexi were found almost everywhere in the shallow sediments with probe GNSB941 (Table 2). In Hot Lake, at the shallow hydrothermal vent site, in the surface layer (0-1 cm), total cell counts were ca. 8 x 10^8 cells/ml (Huang et al. unpublished). We detected filaments with GNSB941 probe in the surface sediment. In another hydrothermal vent site of Quepos slide, Costa Rica, we observed many filamentous cells in the surface sediments covered by sulfur-oxidizing bacteria of the genus *Beggiatoa*. The same trend was also observed in Guaymas Basin, where the sediment is also covered with *Beggiatoa* spp. (Teske et al., 2002) In cold seep habitats, the filamentous *Chloroflexi* were observed, thus the temperature does not seem to be the key factor of the distribution of filamentous *Chloroflexi*. The cell numbers detected were usually below 1% (Table 3). Coccoid *Chloroflexi* cells were, however, detected in one Gulf of Mexico sediment layer (11.25 cmbsf) in high abundance of 12%. The coccoid cells were mostly found below 10 cm depth.

Table 2 CARD-FISH screening for Chloroflexi in various habitats

Sites	Station	Depth [cmbsf]	GNSB941	EUB338I-III	Total cell number (DAPI)	*Chloroflexi* morphology	Habitats	Location
Gulf of Mexico	87	0-2	-	+	+++		little oily, low POC, high AOM independent SRR, sediment covered by orange Beggiatoa.	27°44.73' N 91°13.33' W
		6-8	-	+	+++			
		14-16	+	+	+++	cocci		
	161	surface	++	+++	+++	filamentous, rods	Oily hydrate, carbonate	27°33.48' N 90°58.86' N
		bottom	++	+++	+++	filamentous		
	140	0-2	-	+++	+		Oily, hydrate, carboonate nodules	21°54.00' N 93°26.40' W
		6-8	-	+++	+			
		10-12	-	+++	+			
	156	0-2	-	+	++		very gassy, near oily hydrate, high AOM rates, sediemns covered by SOB and tubeworms.	27°46.95' N 91°30.47' W
		6-8	-	+	++			
		10-12	-	+	+++			
Gulf of Mexico (Asphalt Volcano)	19-6	0-2.5	-	+++	+++		Asphalt Volcano	93°26.18' N 21°53.99' W
		5-7.5	++	+++	+++	filamentous		
		10-12.5	++	+++	+++	filamentous, rods, cocci		
	25-9	0-2.5	++	+++	++	rods, cocci	Asphalt Volcano	93°26.20' N 21°53.9' W
		5-7.5	++	+++	++	rods, cocci		
		10-12.5	++	++	++	rods, cocci		
Guaymas Basin	4489-1	0-1	++	+++	+++	filamentous, cocci, rods	hydrothermal vent, sediment covered with Beggiatoa mat, temperature around 90°C-115°C.	
		4-5	-	++	+			
		8-9	-	++	+			
	4487-1	0-1	++	+++	+++	filamentous, rods	hydrothermal vent, below Beggiatoa mat, oily.	
		4-5	-	++	+			
		7-9	-	+	+			
Japan Trench, off Kamaishi	957	0-2	++	++	++	filamentous, cocci	cold seep	39°6.35' N 143°53.56' E
		4-6	-	++	+			
		8-10	+	++	+	filamentous, cocci		
Hydrate Ridge	19-2	0-1	++	+++	+++	filamentous	gas hydrate, high fluid flux. AOM and SRR is high, sediments covered by *Beggiatoa*	44°34.10' N 125°08.81' W
		4-5	-	+++	++			
		8-9	-	++	++			
	38	0-1	+	+++	++	rods	gas hydrate, high fluid flux. AOM and SRR is high, sediments populated by	44°34.19' N 125°08.85' W
		4-5	+	+++	+++	rods		
		8-9	-	+++	++			
White Oak River estuary		6-9	-	+	+		estuary sediment, high concentration of CH_4	34°44.49' N 77°07.44'W
		39-42	-	+	+			
Gulf of Mexico	deep sediment		-	+	+			
Amon Mud Volcano	760	0-1	+++	+++	+++	filamentous		32°22.13' N 31°42.66' E
		4-5	-	++	+			
Amon Mud Volcano	825	0-1	++	+++	++	filamentous	cold seep	32°22.13' N 31°42.67' E
		4-5	+	+	+	filamentous		
		8-9	+	++	+	filamentous		
Tommeliten Oil Field	K1	0-3	+	+++	+++	rods	Gas seep, carbonate	56°29.90' N 02°59.80' E
	K2	0-3	+	+++	+++	rods		
	K3	0-3	+	+++	+++	filamentous		
	K4	3-6	+	+++	+++	filamentous, rods		
	K5	6-10	+	+++	+++	rods		
Hakon Mosby Mud Vulcano	ATL-19	0-1	+	+++	+++	filaments	cold seep	72°00.19' N 14°43.67' E
		4-5	-	+	+			
		8-9	-	+	+			
Hakon Mosby Mud Vulcano	ATL-22	0-1	++	+++	++	filamentous, rods	cold seep	72°00.08' N 14°43.39' E
		3-4	++	+++	++	filamentous, cocci, rods		
		8-9	++	+++	++	filamentous		
Costa Rica, Quepos slide	68, white mat	0-1	+++	+++	+++	filamentous	hydrothermal vent	
	68, orange mat	0-2	+	+++	+++	filamentous, cocci		
	81, orange mat	0-2	+++	+++	+++	filamentous		
Hot Lake		0-1	+	++	+++	filamentous	hydrothermal vent	38° 38.43' N 15° 6.60' E

Table 3 Quantification of single Bacteria and Chloroflexi in selected habitats by CARD-FISH.

Sites	Depth [cmbsf]*	Total cells (DAPI) [10^8 cm^{-3}]	Bacteria (EUB I-III) [% in total cells]	Chloroflexi (GNSB941) [% in total cells]	*Chloroflexi* [% in total Bacteria]
Costa Rica, Quepos Slide	1	1.0	36.4	0.08	0.21
station 81	3	1.3	38.1	0.01	0.03
(sediment covered with orange	11	1.6	15.4	0.00	0.03
Beggiatoa spp. mat)	13	0.6	15.4	n.d.	n.d.
	15	0.6	10.3	n.d.	n.d.
	17	0.6	6.9	n.d.	n.d.
Costa Rica, Quepos Slide	0.5	1.4	57.0	0.01	0.01
station 68	1.5	1.6	50.3	n.d.	n.d.
(sediment covered with white	2.5	1.6	26.8	n.d.	n.d.
Beggiatoa spp. mat)	3.5	2.0	25.3	n.d.	n.d.
	15	1.1	8.5	n.d.	n.d.
Gulf of Mexico, Asphalt Volcano	1.25	5.8	72.2	0.08	0.11
station 19-6	3.75	2.5	79.3	0.18	0.22
	6.25	4.2	58.7	0.06	0.11
	8.75	3.2	71.7	0.14	0.19
	11.25	6.1	87.2	12.39	14.21
	13.75	7.1	78.5	0.09	0.12
	16.25	7.0	64.5	0.06	0.09

* cmbsf: cm below sea floor
n.d.: Not detected.
Probe names are in parenthesis.

Distribution of Chloroflexi in Black Sea microbial mats

With probe CFX665 specific for a subgroup of *Anaerolineae*, it was demonstrated that the microbial mats in the Black Sea contained a high relative abundances of mostly filamentous *Chloroflexi* which accounted for up to 28% of total cells. By triple–FISH of mat sections we showed that these multicellular filaments were often surrounding the tight ANME-2/DSS aggregates (Fig. 2). There, the filaments were in contact with the mucus layer embedding the aggregates. ANME1 chains frequently observed in other Black Sea microbial mats (Michaelis et al., 2002; Knittel et al., 2005; Treude et al., 2007) were not associated with *Chloroflexi* filaments. From cell counts, relative abundance of GNSB941 hybridized cells were 8.5-9.9% in microbial mat from P882, 0.4% in P795, 4.7% in pink mat at P787, 6.4% in brown mat at P784, and 4.5-27.8% in microbial mat at P780.

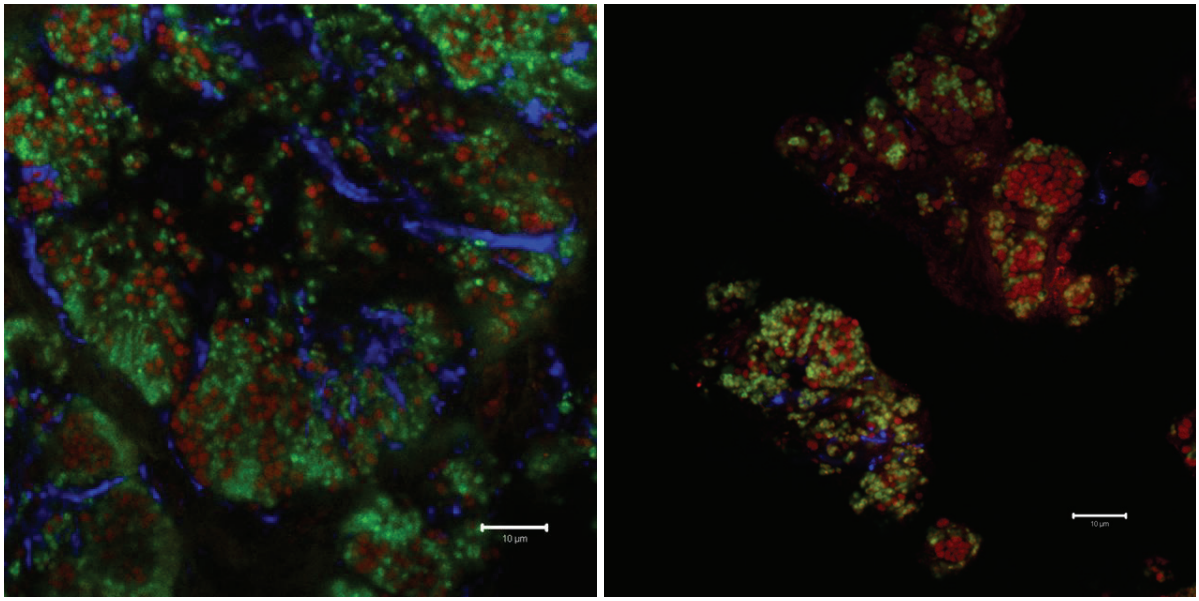


Figure 2 Laser scanning microscopic images of the Black Sea microbial mat sections. Green, DSS; Red, ANME2; Blue, *Chloroflexi*. Scale bars = 10 µm.

Flow cell sorting of Chloroflexi filaments from mat

Based on the CARD-FISH signals conferred by hybridization with probe CFX665 approximately 2,500,000 filamentous *Chloroflexi* were successfully sorted by flow cytometry from 0.5 g of homogenized microbial mat from station P822 (Fig. 3). The microbial mat piece was tightly packed and dense, therefore detachment of the filaments was problematic. Even after the homogenization, small aggregates bigger than the nodule size (>70 µm) were observed. Mild centrifugation used after the homogenization step allowed the removal of those aggregates from supernatant, but keeping dispersed filaments. The sorted cells showed a very homogeneous morphology with cell diameters of about 1.5 µm and a length of 30-50 µm (Fig. 4). It has been shown that the microbial mat contains long filaments (>100 µm) as well as short filaments (Arnds, 2009), thus only short filaments might be remained in the supernatant. The purity of the sorted fraction is higher than 90% based on microscopic counting. Attempts to amplify 16S rRNA genes from 100 sorted filaments by PCR failed.

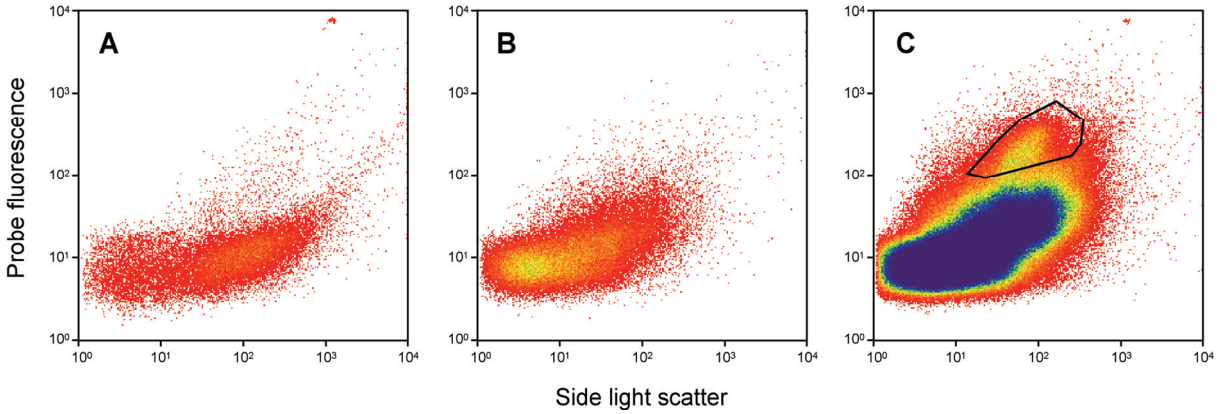


Figure 3 Flow cytometric sorting of prokaryotic cells in Black Sea microbial mat sample after CARD-FISH and resuspension. A: Non-hybridized cells, B: Probe NON338, C: Probe CFX665. The dot plots are side light scatter versus probe fluorescence (Alexa488). Cells within the gate indicated were sorted for subsequent analysis.

Discussions

In this study we examined a total of 20 surface sediment samples from the marine sea floor by CARD-FISH. It became obvious that, although we detected *Chloroflexi* in most sediments, they seem to be only a minor component of the benthic microbial community with relative cell numbers below 1% of total microbial cells. Only in one layer of Gulf of Mexico sediment, we detected up to 12% of coccoid *Chloroflexi* cells at a depth of 11.25 cm. Since only about 0.1% of total cells were GNSB941-positive in the other layers of this sediment core this singular quantification should be considered with care. Additional experiments need to be conducted in the future to corroborate this finding. Overall, our data are not supporting a high abundance of *Chloroflexi* in marine surface sediments which had been assumed by others based on high amount of 16S rRNA in surface sediment measured by membrane hybridization (Koizumi et al., 2004) and frequencies of 16S rRNA genes of *Chloroflexi* retrieval related to by PCR with general bacterial primers (e.g. Inagaki et al., 2006). We suggest this might have been a primer bias favoring amplification of 16S rRNA genes of *Chloroflexi* and by efficient retrieval of DNA from members of this group.

Most often the *Chloroflexi* detected in the surface layers of marine sediments were filamentous, for example the cells identified in the Hot Lake sediment sample taken at a hydrothermal vent site. Total cell counts in the top first cm were ca. 8 x 10^8 cells/ml, and the habitat seemed to be dominated by epsilonproteobacterial sulfur-oxidizers (Huang et al., personal communication). In this shallow habitat which was characterized by absence of oxygen, low pH and high concentrations of reduced sulfur compound (Italiano and Nuccio,

1991) light is still available. We, therefore, suggest that the filaments detected in Hot Lake surface sediments are anoxygenic phototrophs related to the family *Chloroflexaceae*.

In the contrast, the filamentous *Chloroflexi* cells highly abundant in the microbial mat in the Black Sea are likely organoheterotrophs. Neither light nor oxygen is available in the mats. The mats have been shown to provide a clearly structured system with extracellular polymeric substances (EPS) building the framework of the mats (Krüger et al., 2008). Spectrophotometric analyses revealed that the extracted EPS contained 10.9% of neutral sugars, 27.4% of proteins and 2.3% of uronic acids. The remaining unidentified part of the EPS might consist of organic compounds, like nucleic and fatty acids, or of detritus and inorganic compounds (Krüger et al., 2008). Our data suggest that *Chloroflexi* is likely one group of organisms which utilize organic matter produced by the ANME-2/DSS consortia. The filamentous *Chloroflexi* could be classified as commensals if their activity is neutral for the consortia. However, we can at this time also not rule out that they are parasites with a negative effect or even mutualistic symbionts. Beneficial effect of the filaments might be a stabilization the mats by the network structure they might drill through the dense matrix by the gliding mobility known for some strains of *Chloroflexi* (Pierson and Castenholz, 1974; Hanada et al., 1995) thereby improving the access of the consortia to the substrates methane and sulfate.

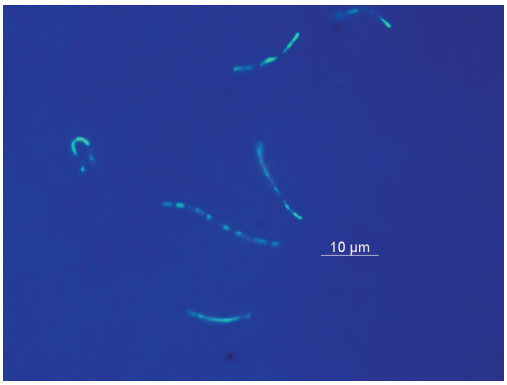


Figure 4 Sorted *Chloroflexi* cells from the microbial mat of the Black Sea. Cells were hybridized with specific *Chloroflexi* probe (CFX665). Scale bar = 10 µm.

Filaments had been reported before as significant components of Black Sea microbial mats. Based on their morphology two different types of filaments were discriminated: Firstly, there are carbonate-associated thick filaments with strong autofluorescence which are 10-20 µm in diameter and up to 900 µm in length (Peckmann et al., 2001). Secondly, thinner filaments associated with gelatinous microbial mats have been noticed (Pimenov et al., 1998).

The filamentous cells hybridizing with the *Chloroflexi* probe CFX665 were of the second morphotype (Fig. 3) proving that filaments in the Black Sea microbial mats belong to at least two different microbial clades.

In an attempt to further characterize the function of the *Chloroflexi* identified in the Black Sea microbial mat we successfully developed a sorting protocol. Unfortunately, amplification of 16S rRNA genes from the sorted cells failed although using 100 filaments. Assuming an average cell number per filament of 20 this is equivalent to approximately 2,000 cells. This number should be more than sufficient for the amplification of fixed cells subjected to FISH (Wallner et al., 1997). We attribute the repeated failure of PCR amplification to the fact that we performed CARD-FISH. Cells might after CARD-FISH no longer be suited for PCR amplification. Alternatively, our problems might also have been due to other factors (e.g. presence of PCR inhibitors or a particular dense cell wall of the *Chloroflexi* cells. Yet, we recommend using a well-established protocol based on ethanol fixation and regular FISH for FACS of cell populations of interest (Lösekann et al., 2007).

We detected *Chloroflexi* in almost all screened cold seep habitats. These seeps usually contain high concentrations of methane and sulfide. Orphan and colleagues (2002) indicated that filamentous bacteria of a very similar diameter (2-3 μm) and length like our filamentous *Chloroflexi* are using methane in Eel River sediment. Alternatively, the seep *Chloroflexi* might use acetate which has been shown to taken up by group I *Chloroflexi* (*Anaerolineae* and *Cardiliniae*) (Yamada et al., 2006). Anyhow, based on the small number of *Chloroflexi* identified in cold seep and other sediments they do not seem to play a major role in biogeochemical cycles.

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6 Appendix

6.1 Curriculum vitae

Kyoko Kubo

Education:

Apr. 2008 - present (June 2011)

Ph.D. study at Dept. Molecular Ecology, Max Planck Institute for Marine Microbiology, student of the International Max Planck Research School of Marine Microbiology (MarMic), Bremen, Germany.

Stipend of Deutscher Akademischer Austausch Dienst (DAAD) (Apr. 2008 – Sept. 2011)

Apr. 2006 - Mar. 2008

M.Sc. study at Dept. Biological Sciences, graduate school of Science and Engineering, Tokyo Metropolitan University, Japan.

Master thesis entitled "Involvement of photosynthetic bacteria in sulfur cycling in hot spring microbial mats"

Scolarship of Japan Student Services Organization (JASSO) (Apr. 2006 – Mar. 2008)

Apr. 2003 - Mar. 2006

Studies of Science/Biology at the Tokyo Metropolitan University, Japan.

Apr. 2000 - Mar. 2003

Tokyo Metropolitan Kokusai High School (International course)

Poster presentations:

Jan. 2009 ASLO Aquatic Science Meeting in Nice, France, 654. **Kubo, K.**; Knittel, K.; Boetius, A.; Amann, R.: Quantification of Crenarchaeota at marine methane seep site.

July 2009 Gordon Research Conference on Applied & Environmental Microbiology in Mount Holyoke, MA, USA. **K. Kubo**, K. Knittel, A. Boetius, K. Lloyd, A. Teske and R. Amann. Quantification of Crenarchaeota from marine methane seeps.

Others:

Apr. 2008 Attendance of "International Workshop on ribosomal RNA Technology" in Bremen, Germany

Sept. 2010 Attendance of "Absolventenseminar für DAAD Stipendiaten" in Berlin, Germany

7 List of abbreviations

ANME	anaerobic methanotrophic archaea
AOM	anaerobic oxidation of methane
aprA	adenosine 5'-phosphosulfate reductase gene
bp	base pairs
CARD-FISH	catalyzed-reporter-deposition fluorescence *in situ* hybridization
DAPI	4',6-diamidino-2-phenylindole
DDBJ	DNA Data Bank of Japan
DNA	deoxyribonucleic acid
DSS	sulfate-reducing bacteria of the *Desulfosarcina/Desulfococcus* branch of Deltaproteobacteria
EMBL	European Molecular Biology Laboratory
FA	formamide
FISH	fluorescence *in situ* hybridization
GenBank	the NIH genetic sequence database
HRP	horseradish peroxidase
MBGB	Marine Benthic Group B
mbsf	meter below sea floor
MCG	Miscellaneous Crenarchaeotal Group
nt	nucleotides
PBS	phosphate buffered saline
PCR	Polymerase Chain Reaction
pufLM	gene for photosynthesis reaction center subunit L and M gene
qPCR	quantitative PCR
RNA	ribonucleic acid
rRNA	ribosomal RNA
SDS	sodium dodecyl sulfate
SMTZ	sulfate-methane transition zone
SOB	sulfur-oxidizing bacteria
SRB	sulfate-reducing bacteria
SSC	saline-sodium-citrate
T_d	dissociation temperature
TE	Tris-EDTA

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