Diversity and function of microbial communities in sediments from different deep-sea habitats

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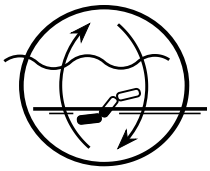
MAX-PLANCK-GESELLSCHAFT



MPI for Marine Microbiology

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Auf dem Deckblatt: Sedimente bedeckt mit weißen Matten an "site F", Logatchev Hydrothermalfeld, Mittel-Atlantischer Rücken. Copyright Woods Hole Oceanographic Institution.

Le rôle de l'infiniment petit dans la nature est infiniment grand.

*

In der Natur ist die Bedeutung des unendlich Kleinen unendlich groß.

Louis Pasteur (ca. 1880)

Summary

Deep-sea floors are diverse environments that range from permanently cold (desert-like plains) to hot systems (hydrothermal vents). In hot systems, primary productivity is performed by microbial communities which use chemical energy generated by geological processes (lithotrophy). This energy transfer from mantle to the ocean is as yet poorly understood, and the diversity and activity of microbes at these sites is therefore an interesting target for microbial ecologists. However, the vast majority of all globally distributed deep-sea sediments is permanently cold. The distribution of microorganisms in deep-sea floors and the factors controlling it at small and large scales are important for the understanding of the mechanisms that regulate biodiversity.

During this thesis, hydrothermally influenced sediments of the peridotite-hosted Logatchev hydrothermal vent field were investigated in an interdisciplinary study to reveal the diversity and activity of the associated microbial communities. *In situ* microprofiles showed that these sediments were controlled by diffusive transport, instead of previously reported advective processes. White mats on top of these sediments resemble *Beggiatoa*-mats from the basalt-hosted field in the Guaymas Basin. However, fluorescence *in situ* hybridization revealed that the overlying sulfur-mats were dominated by filamentous *Epsilonproteobacteria* or a vibrioid *Arcobacter*-type. The microbial community of the surface layer was predominantly composed of *Epsilonproteobacteria* (7-21%), *Deltaproteobacteria* (20-21%), and *Bacteroidetes* (19-20%). Comparative 16S rRNA gene sequence analyses identified various bacteria related to those found in basaltic systems.

The presence of an active microbial community in these sediment surface layers was confirmed by high oxygen consumption rates. Geochemical analyses detected metal-sulfides in the sediments, elemental sulfur in the mats and an intensive sulfide flux from below. *Ex situ* incubations and turnover rate experiments revealed that sulfide is consumed and that sulfate-reduction is performed by the surface sediment microbial community. This was consistent with the detection of *aprA*-genes and *soxB*-genes, which are both key genes of the sulfur cycle. Further metabolic capabilities such as denitrification and CO_2-fixation were indicated by primary analysis of metagenomic data retrieved by pyrosequencing. So far, our analyses suggest that sulfur cycling is one of the driving forces for primary production and biomass formation in surface sediments of the ultramafic-hosted Logatchev hydrothermal vent fields. Therefore, major differences in microbial composition between basalt- and peridotite-hosted fields were not detected.

Hydrothermally influenced sediments from the Mid-Atlantic Ridge and permanently cold sediments from three basins of the eastern South Atlantic Ocean were investigated to examine the ability of microorganisms to disperse in the deep-sea. Besides spatial distance, the structuring effect of the physical barrier Walvis Ridge, which separates the Cape Basin from the other two basins, was determined. The analysis of 16S rRNA gene sequences of the deep-sea sediments revealed phylotypes affiliated with *Gammaproteobacteria*, *Deltaproteobacteria* and *Acidobacteria,* which were present in all three basins. The distribution of these shared phylotypes seemed to be influenced neither by the Walvis Ridge nor by different deep water masses, suggesting a high dispersal capability, as also indicated by low distance–decay relationships. In contrast, the comparison of the total bacterial diversity of the cold sediments as well as of the hydrothermally influenced sediments revealed significant differences between the microbial communities. Within the Logatchev field and therefore for small distances (<10 km) microbial biogeography was primarily controlled by environmental heterogeneity. In contrast, the analysis of the permanently cold sediments revealed that at intermediate (10–3000 km) and large scales (>3000 km), both factors influenced bacterial diversity, indicating a complex interplay of local contemporary environmental effects and dispersal limitation.

Zusammenfassung

Meeresböden in der Tiefsee sind sehr vielfältig und reichen von permanent kalten Sedimenten (Tiefseeebenen) bis hin zu hydrothermal beeinflusstem heißem Meeresgrund. Die Primärproduktion an Hydrothermalquellen wird von mikrobiellen Gemeinschaften übernommen, die in der Lage sind anorganische Verbindungen als Energiequellen zu nutzen (Lithotrophie). Dieser Energietransfer vom Erdmantel in den Ozean ist bisher noch wenig erforscht. Die Charakterisierung der an diesem Prozess beteiligten mikrobiellen Gemeinschaften und deren Aktivität ist daher von besonderem Interesse. Die meisten Tiefseeböden sind allerdings mit permanent kalten Sedimenten bedeckt. Die globale Verteilung als auch die Faktoren, die die Verbreitung von Mikroorganismen in der Tiefsee bestimmen, liefern wichtige Information über die Mechanismen, die für die Regulierung der Biodiversität verantwortlich sind.

Im Rahmen dieser Doktorarbeit wurde die Diversität und Aktivität von mikrobiellen Gemeinschaften in hydrothermal beeinflussten Sedimenten des Logatchev Feldes untersucht, das ultramafische Gesteine als wichtige Bestandteile aufwies. *In situ* Mikroprofile von den vorhandenen Sedimenten zeigten, dass Transportprozesse in diesen Sedimenten durch Diffusion bestimmt sind und nicht wie bereits bekannt durch advektive Prozesse. Weiße Matten auf der Sedimentoberfläche ähnelten *Beggiatoa*-Matten, die für Sedimente im Basalt-dominierten Guaymas Basin beschrieben wurden. Fluoreszenz *in situ* Hybridisierungen zeigten, dass filamentöse *Epsilonproteobacteria* oder vibrioförmige *Arcobacter* Spezies diese weiße Mattengemeinschaft dominierten. Die mikrobielle Gemeinschaft in der Oberflächenschicht setzte sich aus *Epsilonproteobacteria* (7-21%), *Deltaproteobacteria* (20-21%) und *Bacteroidetes* (19-20%) zusammen. Vergleichende 16S rRNA Gen-Analysen zeigten, dass viele nah verwandte bakterielle Sequenzen ebenfalls in Basalt-dominierten Hydrothermalquellen gefunden wurden.

Das Vorhandensein einer aktiven mikrobiellen Gemeinschaft in den hydrothermal beeinflussten Sedimenten wurde durch hohe Sauerstoffrespirationsraten bestätigt. Geochemische Analysen wiesen Metallsulfide in den Sedimenten, elementaren Schwefel in den Matten und eine intensiven Sulfideinfluss aus den tieferen Schichten nach. *Ex situ* Inkubationsversuche und Ratenmessungen wiesen auf Sulfid als Energiequelle, sowie auf dissimilatorische Sulfatreduktionsprozesse hin. Dies wurde unterstützt durch den Nachweis von Schlüsselgenen des Schwefelkreislaufs (*apr*A, *sox*B). Pyrosequenzierung des Metagenoms ergab Denitrifikation und CO_2-Fixierung als weitere mögliche metabolische Fähigkeiten der mikrobiellen Gemeinschaften. Die bisherigen Untersuchungen weisen darauf hin, dass schwefelumwandelnde Mikroorganismen die wichtigsten Primärproduzenten

darstellen und somit hauptsächlich für die Produktion von Biomasse in den hydrothermalen Sedimenten des ultramafischen Logatchev Feldes verantwortlich sind. Daher wurden keine bedeutenden Unterschiede in der mikrobiellen Zusammensetzung zwischen basaltischen und ultramafischen Systemen festgestellt.

Diese hydrothermal beeinflussten Sedimente des Mittelatlantischen Rückens, und permanent kalte Sedimente aus drei Tiefseebecken des östlichen Süd-Atlantiks wurden weiterhin untersucht, um die Verbreitung von Mikroorganismen in der Tiefsee zu erforschen. Dazu wurde der Einfluss der Entfernung wie auch der physikalischen Barriere "Walfischrücken" bestimmt, der das Kapbecken von den anderen beiden Tiefseebecken trennt. Die Analyse der 16S rRNA Gene aus den permanent kalten Sedimenten ergab Sequenzen von *Gammaproteobacteria*, *Deltaproteobacteria* und *Acidobacteria* für alle drei Tiefseebecken. Die Verteilung dieser Gruppen schien somit weder vom "Walfischrücken" noch von den daraus resultierenden verschiedenen Tiefenwassern beeinflusst zu sein. Dies wies auf eine weite Ausbreitung dieser Gruppen hin, die durch niedrige Distanz-Verteilungs-Faktoren (distance-decay) bestätigt wurde. Im Gegensatz dazu ergab der Vergleich der gesamten erhaltenen bakteriellen Gemeinschaft sowohl vom hydrothermalen also auch von den kalten Sedimenten signifikante Unterschiede in ihrer Zusammensetzung. Die Biogeographie der bakteriellen Gemeinschaft des Logatchev Feldes, und somit die Verteilung über geringe Distanzen (<10 km), wurde hauptsächlich bestimmt durch Umweltfaktoren. Dagegen zeigte die Analyse der kalten Sedimente, dass die Verteilung über intermediäre (10-3000 km) und große Distanzen (>3000 km) von Umweltfaktoren und der Distanz abhängt, welches auf ein komplexes Zusammenspiel von lokalen Umweltbedingungen und Verbreitungsgrenzen hindeutet.

Abbreviations

AOM	anaerobic oxidation of methane
ANME	anaerobic methanotrophs
ANOSIM	analysis of similarity
ATP	adenosine triphosphate
CPDW	Circumpolar Deep Water
DNA	deoxyribonucleic acid
DGGE	denaturing gradient gel electrophoresis
DSR	dissimilatory sulphate reduction
EPR	East Pacific Rise
EH	environmental heterogeneity
et al.	and others
FISH	fluorescence *in situ* hybridization
GOLD	Genomes OnLine Database
HGT	horizontal gene transfer
LHV	Logatchev hydrothermal vent
MAR	Mid-Atlantic Ridge
MLSA	multilocus sequencing analysis
NADW	North Atlantic Deep Water
nMDS	non-metric multidimensional scaling
ORF	open reading frame
OTU	operational taxonomic units
PCR	polymerase chain reaction
RNA	ribonucleic acid
rTCA	reductive tricarboxylic acid
SD	spatial distance
spp.	species
SRR	sulfate-reduction rate
Sox	sulfur oxidation
T-RFLP	terminal restriction fragment length polymorphism

I Introduction

1 Deep-sea floor

The deep-sea floor (>1000 m water depth) covers 67% of the Earth's surface. The seabed is a highly diverse and dynamic environment. Besides the vast desert-like plains of deep-sea mud there are also diverse landscapes of canyons, cold seeps, deep-water coral reefs, mud volcanoes, carbonate mounds, brine pools, gas hydrates, hot vents, seamounts, ridges, fractures and trenches (Figure 1).

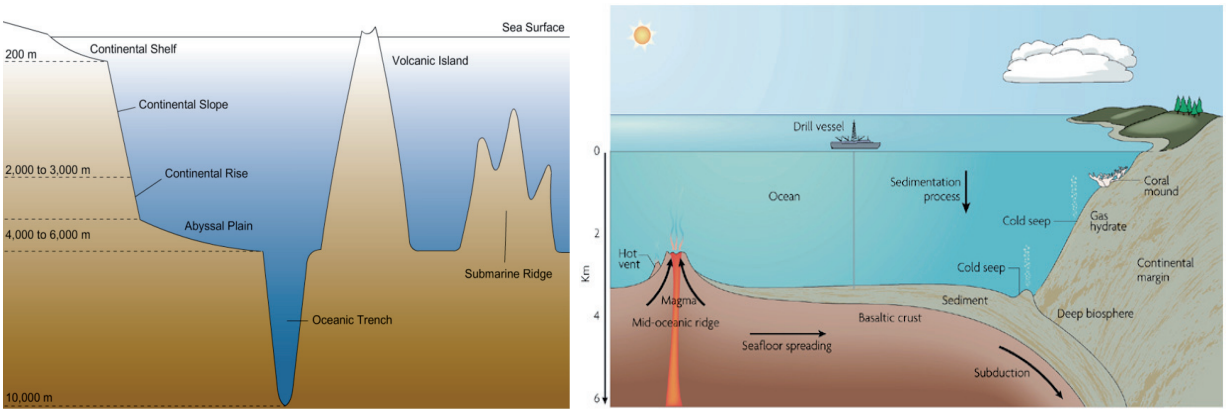

Figure 1: Vertical section of the seabed and seafloor structures (Jørgensen and Boetius, 2007).

The most unique physical parameter in the deep-sea is hydrostatic pressure. The pressure increases by ca. 1 bar for every 10 meters, reaching a maximum of ~1100 bar at the bottom of Challenger Deep in the Mariana Trench. The deep-sea floor can be divided in the surface and subsurface seabed. The surface layer is mixed by burrowing animals at sediment-covered seafloors, providing exchange with the ocean water. The process in which living organism actively transport bottom water through the sediment and therefore through their habitat is known as bioirrigation (Glud et al., 1994; Hughes and Gage, 2004; Quéric and Soltwedel, 2007). The seafloor of the deep sea is however not completely sedimented (Figure 2). Near ridge axes, where the crust is young, bare unsedimented rock is exposed and interacts directly with the seawater. Therefore, for hard grounds, such as those found at the mid-ocean ridges and in the central Pacific, it is more difficult to define the extension of surface versus subsurface seabed. In the subsurface, temperature is the only environmental variable that appears to set the ultimate limit for life, limiting life at a depth of 2-4 km below the seafloor (Wilhelms et al., 2001).

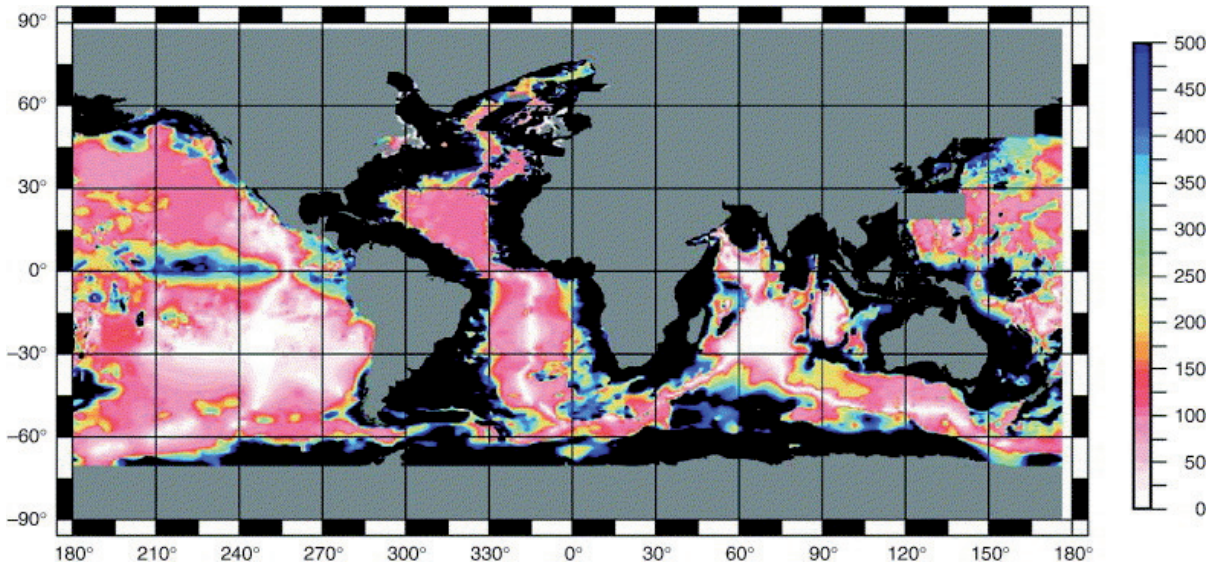

Figure 2: A digital map of global sediment thickness distribution for the oceans and marginal seas of the world as complied by the National Geophysical Data Center (http://www.ngds.noaa.gov/mgg/sedthick/sedthick.html). Areas with sediment thickness >500m appear black while continents and areas with no data appear gray (Edwards et al., 2005).

The deep-sea seabed lies in water depths where the light intensity is too low to sustain photosynthetic production and where water temperatures range between -1°C and 4°C (Svedrup et al., 1942). Benthic productivity and biomass is therefore controlled by the availability of organic matter from the euphotic zone or of chemical energy generated by geological processes. Depending on the productivity of the overlying water, organic matter which arrives to the ocean depths is not evenly distributed in time and space. As well as the sedimentation of organic material from above, plate tectonics and other geological processes transport chemical energy to the seafloor from below, which provides a significant fraction of the deep-sea energy flux.

1.1 Permanently cold systems

The deep-sea seabed which is not influenced by geological process represents permanently cold systems. Almost all deep-sea floors has temperatures <5°C, so that most deep-sea floors are covered by permanently cold sediments. Exceptions are the Mediterranean Sea which is 13°C between 0.6 and 4 km depth and the Red Sea where the bottom temperature can be 21.5°C at 2 km depth. The lowest temperatures found are -1.9°C in the deep waters of the Antarctic (Schulz and Zabel, 2006).

The sedimentation rate decreases with increasing distance from a sediment source, which could either be a continent or an area of high biogenic productivity. The primary production

in the phototrophic layer is controlled by local upwellings of nutrients into the euphotic zone, induced mostly by wind stress and ocean circulation patterns (vertical and horizontal) (Palter et al., 2005). The most oligotrophic seafloor environments are found in the central ocean gyres, particularly in the South Pacific (Figure 3) (Claustre and Maritorena, 2003). The middle of the gyre is furthest from the continents and productive ocean regions than any other site on Earth. The central South Pacific Gyre has been described as Earth's largest oceanic desert as its area of low-chlorophyll concentration is more as twice the area of the North

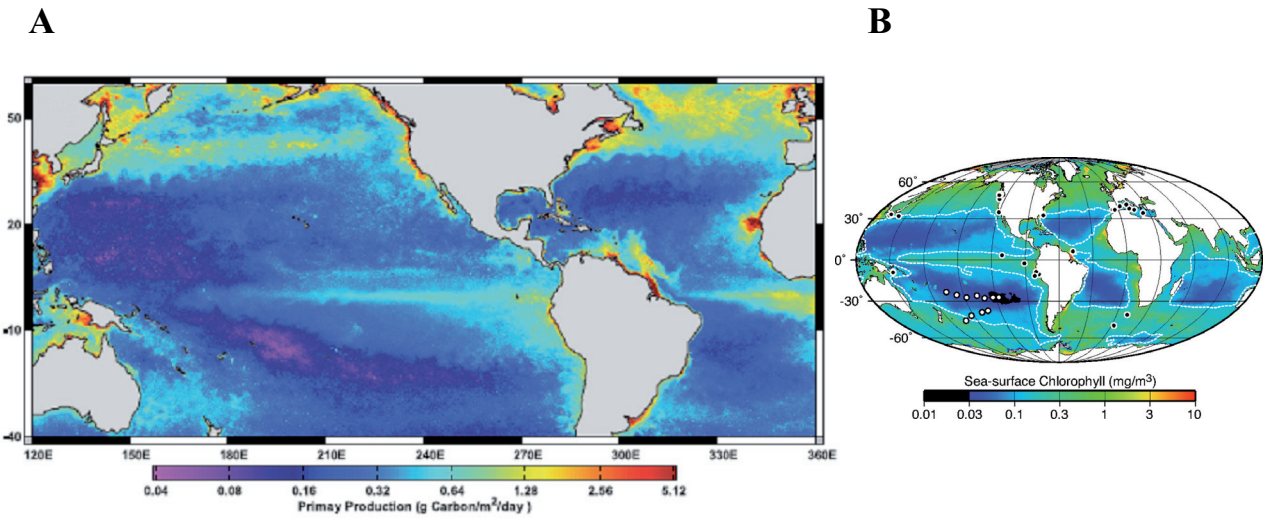

Figure 3: (A) Patterns of primary production in the Pacifc and Atlantic Ocean, July 2006 (Kolber, 2007) and (B) a map of time-averaged sea-surface chlorophyll-a (chl-a) concentrations (Global SeaWiFS Chlorophyll) mean of September to December 1997. Dashed white lines delimit the area in each gyre where the sea-surface chl-a concentration is 0.14 mg/m^3.

America (Dandonneau et al., 2003). In contrast to these oligotrophic seafloors, deep-sea floors near coastal areas have higher primary productivities (Douglas et al., 2007) resulting in very high sedimentation rates (1 to 2 mm/year), which are due to pelagic and terrigenous input (Curray et al., 1979; Weber and Jørgensen, 2002; Teske et al., 2002). The largest part of the ocean seafloor receives on average 1 g of organic carbon per m^2 per year from sedimentation (Jørgensen and Boetius, 2007).

Apart from the pelagic clays, the various clay minerals in the sediments are a function of their original source and the route of transportation (Petschick et al., 1996). Their transport into the area of deposition occurs either by eolian or volcanic transport, or by means of water and ice. According to their composition or origin, deep-sea sediments constituents are described in a three-component system as (i) biogenic carbonate, (ii) biogenic opal, and (iii) non-biogenic mineral (terrigenous, volcanogenic, and authigenic). The biogenic oozes mainly consist of shells and skeletal material from the planktonic organisms (Figure 4).

The benthic oxygen availability and therefore the oxygen penetration depth ranges from millimeters in organic-rich and highly reactive sediment, to the full penetration of the sediment cover, which is known from oligotrophic sediments of the South Pacific gyres. Transport processes, such as advection and mixing, are normally limited to a few centimetres per year by sediment porosity and compaction in the sedimentary seafloor.

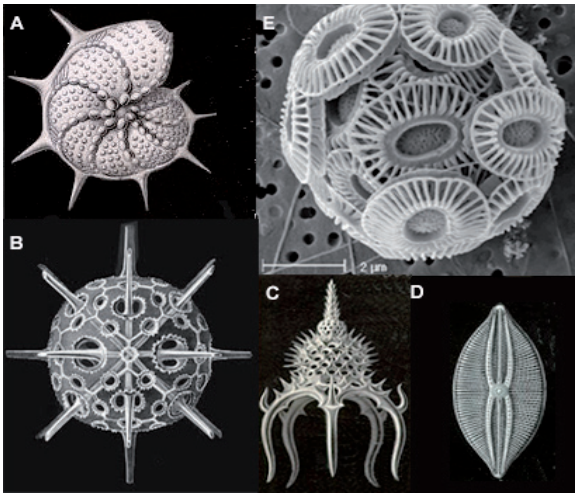

Figure 4: (A) Calcareous foraminifers, (B and C) opal/siliceous radiolarian, (D) siliceous diatom (Haeckel E 2006), and (E) calcareous coccolithophorid (Schulz and Zabel, 2006; REM, Lab University of Basel).

1.1.1 The eastern South Atlantic Ocean

The geophysical settings of the eastern South Atlantic Ocean were investigated in several studies, as this ocean represent an unusually productive ocean. Such a high productivity is only known from two other areas, which are the eastern tropical Pacific and the northern Indian Ocean (Berger and Wefer, 1996). High biological productivity in the eastern South Atlantic is induced by strong trade winds in austral winter at the equatorial upwelling zone (equatorial divergence) and at the centers of coastal upwelling of Namibia (17° to 30°C) (Figure 5B) (Peterson and Stramma, 1991). In addition, seasonal shoaling of nutrient-rich subsurface waters in the Angola cycling gyre area (oceanic upwelling) leads to enhanced oceanic productivity (Schneider et al., 1996).

The Cape, Angola and Guinea basins comprise the abyssal plains in the eastern South Atlantic Ocean (Figure 5A). The circulation in the deep eastern South Atlantic is dominated by the interaction between the North Atlantic Deep Water (NADW) flowing towards the south and Circumpolar Deep Water (CPDW) flowing to the north (Figure 5C). The NADW, which is warmer and has a higher salinity (higher density), divides the CDW into two layers, above and below the NADW (Reid, 1996). The Walvis Ridge separates the Guinea and the Angola basins from the Cape Basin and forms a barrier to the northward and southward flow of water below a depth of about 3,000 km. Therefore, the deepest parts of the Angola and Guinea basins are filled almost by NADW, while the Cape Basin is dominated by LCPDW below 4,000 km (Figure 5C) (Diekmann et al., 1996).

Clay minerals found in the deep-sea basins of the South Atlantic Ocean are mainly of terrigenous origin and are strongly influenced by African sources. They are transported to the deep sea as a result from a complex interaction of river and wind input. Their distribution is

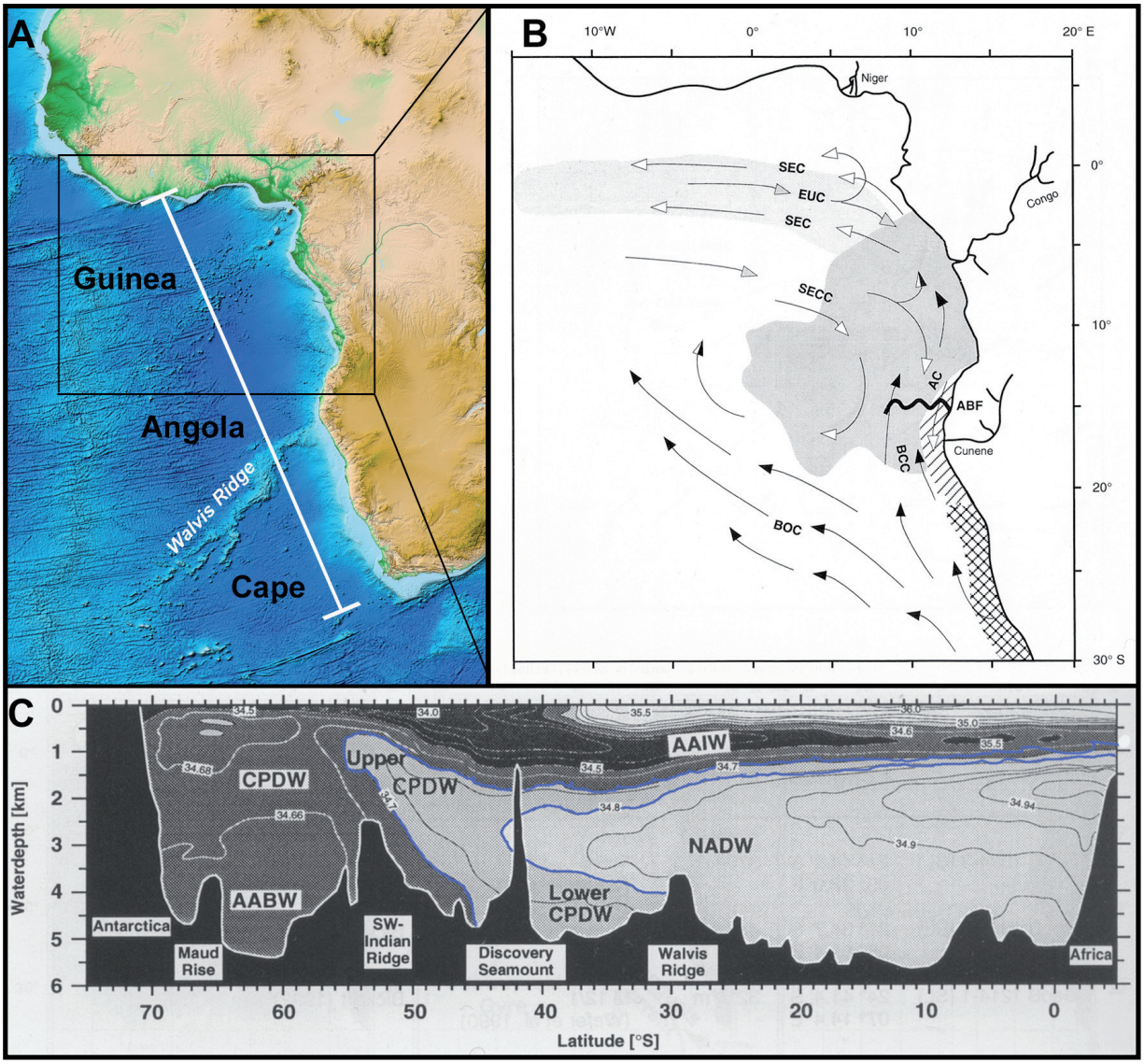

Figure 5: (A) The South Atlantic Ocean showing the Walvis Ridge that separates the Cape Basin from the Angola and Guinea basins. A part of the transect, presented in (C) (0° to 40°S) is indicated as a white line. (B) Surface currents (black arrows – cold; white arrows – warm; grey arrows – subsurface currents), and areas with high primary productivity (light gray – equatorial divergence; grey shaded area - oceanic upwelling with thermocline shoaling and frontal mixing; hatched area – seasonal coastal upwelling; and cross hatched area – permanent upwelling) in the eastern South Atlantic Ocean (Schneider et al., 1996). (C) Deep-sea water masses circulation showing the mixing of the northern flow NADW and southern flow CPDW.

controlled by ocean currents, while water advections are most effectively (Petschik et al., 1996). Primary clay mineral input is driven by the large African river systems (Pastouret et al., 1978; Bremner and Willis 1993), NE- and SE-trade winds from African desert (eolian transport) (Bremner and Willis, 1993), and glaciogenic supply from the continental margin of Antarctica (Ehrmann et al., 1992; Petschik et al., 1996). Guinea and Angola basins sediments originate from the Central African soil transported by e.g. the Congo (Zaire) River and from wind-borne dust transported by NE-trades from the Sahel-Zone (Gingele and Schulz, 1993). Both are kaolinite-rich zones, but the Central Angola Basin is as well characterized by high content of poorly crystallized smectite (>30%), supplied by Southwest African rivers like the

Kunene and carried to the north by the Benguela Coastal Current (Table 1). The Cape Basin comprises an illite-rich zone, originating from Namibian and South African deserts as well as from South African soils, which are rich in illite (Diekmann et al., 1996).

Table 1: Mineralogy of main lithogeneous sediment components.

Clay mineral	Composition (idealized)
Kaolinite	$Al_2Si_2O_5(OH_4)$
Mica group, e.g. Illite	$K_{0.8\text{-}0.9}(Al,Fe,Mg)_2(Si,Al)_4O_{10}(OH)_8$
Smectite group, e.g. Montmorillonite	$Na_{0.33}(Al_{1.67}Mg_{0.33})Si_4O_{10}(OH_2 \cdot nH_2O$

1.2 Hydrothermal systems

Besides sedimentary deep-sea floors, young bare seafloor is formed near ridge axes, which represent unsedimented rock. The predominant rock substrate of the young seafloor is basaltic in composition, which is exposed to the seawater and undergoes seawater-rock alteration reactions. At these sites, active hydrothermal vents were discovered associated with continental fracture zones at the Galapagos Rift west of Ecuador in 1977 (Corliss and Ballard, 1977; Lonsdale P, 1977; Francheteau et al., 1979). This hydrothermal vents represented rich areas of very high productivity with dense communities of unusual animals. Today, 30 years later, more than 100 sites of high-temperature hydrothermal venting deposits are known on the modern seafloor (Kesler and Hannington, 2005; Tivey 2007) (Figure 6).

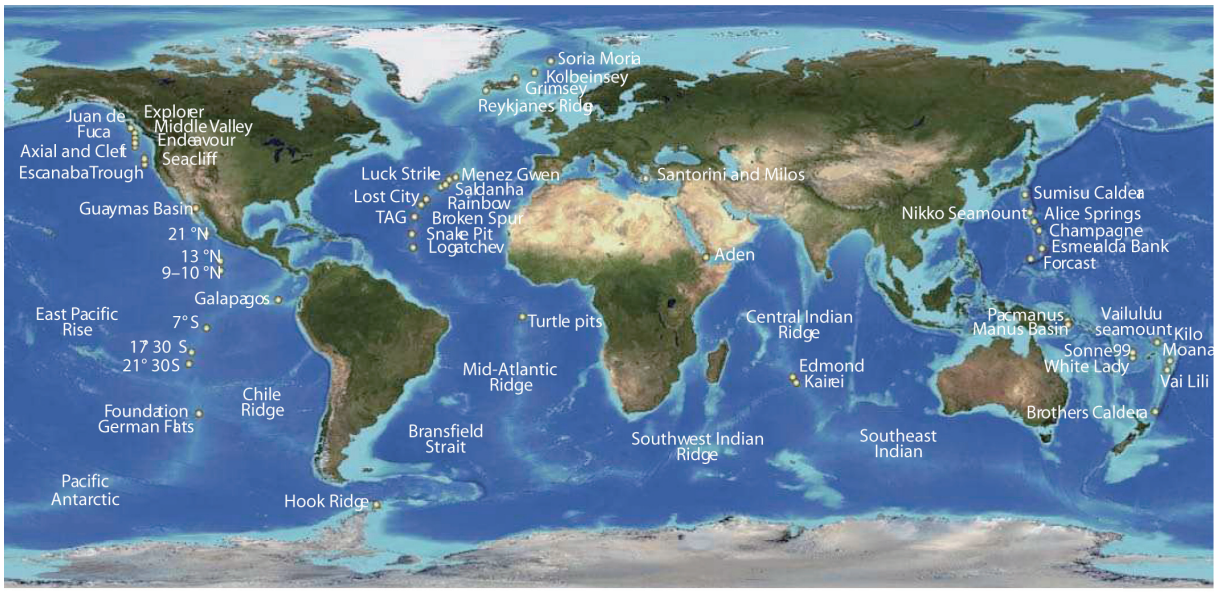

Figure 6: Global distribution of hydrothermal vents. Temperature and chemical anomalies hint that many more sites exist throughout the world's ocean (Martin et al., 2008).

There are strong similarities among all seafloor vent fields in terms of the processes of heat and mass transfer that result in venting of hydrothermal fluids to the oceans, formation of mineral deposits, and creation of chemical and thermal environments conducive to biological activity. These processes have a major impact on the chemistry of the oceans (Edmond et al., 1982) and have been responsible for extensive alteration of oceanic crust (Alt, 1995). It has been estimated that 25-30% of the earth's total heat flux is transferred from the lithosphere to the hydrosphere by the circulation of seawater through oceanic spreading centers (Lowell, 1991; Stein and Stein, 1994). Besides high-temperature venting, off-axis diffuse fluid flow plays an important role in transferring mass and energy from the crust and mantle to the oceans.

Mid-ocean ridges are located at boundaries between tectonic plates where the ridge plates separate and deeper mantle rises to fill the crack created by spreading. Molten rock from deep in the earth fills the void between the plates and creates new seafloor and a volcanically active ridge. The vast majority of all Earth's mid-ocean ridges form a single, continuous, globe-encircling volcanic chain that is roughly 60,000 km in length and lies at around 2000 to 5000 m depth. According to their spreading rate, mid-ocean ridges are classified in superfast-spreading to ultralow-spreading ridges (Table 2). Spreading rates, besides melt supply rate and the effectiveness of hydrothermal cooling, are important in shaping ridge morphology, which leads to differences in their across-axis morphology (segmentation) (MacDonald et al., 1991; Kelley et al., 2002). Fast-spreading ridges have an axial rise with a very narrow summit rift that is the locus for most volcanic and tectonic activity, while slow-spreading ridges have rugged rift mountains enclosing a broad axial valley (Figure 7) (MacDonald et al., 1991).

Table 2: Mid-ocean ridges and their classification according to their spreading rates.

Mid-ocean riges	**Spreading rate [mm yr^{-1}]**	**Classification**
East Pacific Rise (27 to 32°S)	130-170	superfast-spreading
East Pacific Rise (8 to 13°N)	90-130	fast-spreading
Central Indian Ridge	50-90	intermediate-spreading
Mid-Atlantic Ridge	20-50	slow-spreading
Southwest Indian and Arctic Gakkel Ridge	< 20	ultraslow-spreading

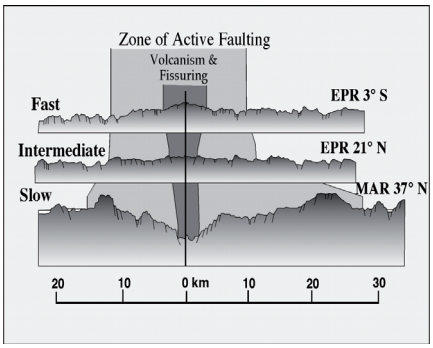

Figure 7: Ridge morphologies of fast, intermediate und slow spreading ridges (Kelley et al., 2002).

At fast-spreading ridges, where magma supply is large, an axial shallow (1300 to 1600 m) magma chamber is present, receiving enough magma to build a magmatic crust that is 6 km thick on average (Chen et al., 1992). Seismic data indicate a zone of high-velocity material that generally occurs over a depth interval of 1-4 km below the seafloor, with faster-spreading systems in general having shallower magma chambers (Kelley et al., 2002). Therefore, fast-spreading ridges tend to be dominated by volcanism, surpassing the effects of tectonics. In contrast, the morphology of slow-spreading ridges is more dominated by tectonic processes, so that the ridge axis may be offset by >30 km (MacDonald, 1998). However, the presence of an axial magma chamber and deep penetrating faults suggests that there is a continuous interplay between magmatic and tectonic processes (Singh et al., 2006a). In comparison to fast-spreading ridges, crustal thickness varies at slow-spreading ridges from 3 to 8 km.

Besides mid-ocean ridges, volcanic systems are found at spreading centers in back-arc basins close to subduction zones. At convergent boundaries, oceanic lithosphere is consumed at deep-sea trenches, leading to the eruption of chains of andesitic arc volcanoes near the edge of the overriding plate (Martinez et al., 2007). Back-arc basins are formed behind subduction zones by rifting volcanic arcs and accreting new volcanic seafloor. The magmatic chamber may be initially close to the arc volcanic front driven by hydrous flux melting, but as they separate from the arc with time, magma production evolves towards mainly pressure-release melting. Therefore, the morphology changes from a deep, flat axis to a peaked, shallow axial high. As spreading rates are decreasing by over a factor of two back-arc basins show a wide variety of spreading styles and lithospheric compositions (Martinez, 2007).

1.2.1 Basalt- and peridotite-hosted hydrothermal systems

At hydrothermal vent field, the composition of the rocks and the shape of the heat source (temperature) influence the composition of the emitted hot fluids. Hydrothermal systems are hosted by basalt or peridotites, which are sections of the upper mantle. During hydrothermal circulation, dense, cold seawater percolates downward through fractured oceanic crust, becomes heated and modified due to a heat source (magma chamber or newly solidified hot rock), and exit again at the vent field (Ramirez-Llodra et al., 2007). Chemical reactions between fluid and rock change the composition of both the aging ocean crust and the seawater.

Most mid-ocean ridges are basalt-hosted where hydrothermal fluids deliver slightly acidic, anoxic, alkali-rich, Mg-poor, and transition metal-enriched (e.g. iron, zinc, copper, manganese) fluids (Figure 8, Table 3). In addition, close to the magma chamber when

temperature and pressure exceed those of the boiling curve for seawater, volatiles (e.g. sulfide, hydrogen, methane, carbon dioxide) are accumulated in high concentrations. The mixing of these metal- and sulfide-rich, high-temperature, acidic fluids with the surrounding cold, alkaline seawater causes the metal sulfide to precipitate and results in the formation of sulfide deposits and supports unique ecosystems.

Vent-fluid compositions can also be affected greatly by reaction with sediment. Sediment-hosted basaltic hydrothermal vent are located near the coast. The presence of carbonate and organic matter buffers the pH in the vent fluid (Tivey, 2007) so that all sedimented systems are similar in exhibiting a higher pH (5.1-5.9 at 25°C) and lower metal contents than fluids formed in unsedimented settings (Table 3). Sediment-hosted ridge systems fall into two general classes depending on the relative importance of biogenous (Guaymas Basin, Gulf of California) and terrigenous deposits (Middle Valley, northern Juan de Fuca Ridge; Escanaba Trough, Gorda Ridge).

Table 3: Fluid compositions retrieved from different venting settings (Tivey, 2007).

	Mid-Ocean Ridge	Back-Arc	Rainbow	Lost City	sediment-hosted	seawater
T (°C)	405	278-334	365	91	100-315	2
pH (25°C)	2.8-4.5	< 1-5	2.8	10-11	5.1-5.9	8
H_2S, mmol/kg	0-19.5	1.3-13.1	1	< 0.064	1.1-5.98	-
H_2, mmol/kg	0.0005-38	0.035-0.5	13	< 1-15	-	-
CH_4, mmol/kg	0.007-2.58	0.005-0.06	0.13-2.2	1-2	-	-
CO_2, mmol/kg	3.56-39.9	14.4-200	-	-	-	2.36
SO_4, mmol/kg	0	0	0	1-4	0	53
Fe, µmol/kg	7-18700	13-2500	24000	-	0-180	-
Mn, µmol/kg	59-3300	12-7100	2250	-	10-236	-

Mantle-derived peridotite rocks occur on the seafloor at slow- and ultraslow spreading axes. Although the total length of ridge axis along which peridotites are exposed are unknown, estimation revealed that about one third of the 55,000 km global ridge system comprises ultraslow ridges (Dick et al., 2003) and that these are expected to be hosted in peridotites. In peridotite-hosted systems (=ultramafic-hosted), active carbonate chimneys are found besides sulfide deposits (Figure 8). The Lost City Field was the first discovered off-axis peridotite-hosted active vent field at the Mid-Atlantic Ridge (MAR) (Atlantis Fracture Zone 30°N; Kelley et al., 2001). It is located tens of kilometers off-axis so that they rarely contain volcanic rocks and are formed by sustained fault activity that has lasted for millions of years (Kelley et al., 2005). The peridotites are usually intimately associated with gabbroic intrusions (Cannat, 1996; Escartin and Cannat, 1999). The seawater mantle peridotite interaction leads to exothermic serpentinization reactions, producing Mg-poor, methane- and hydrogen-rich, and

very high pH (10-11) fluids (Table 3). The ultramafic underpinnings of the Lost City system have a similar chemical composition to lavas that erupted into the primordial ocean on early Earth (Kelley et al., 2005). Consequently this hydrothermal vent field provides insights into past mantle geochemistry and therefore in the physiological characteristics that resemble the earliest microbial ecosystems on Earth. The Lost City hydrothermal vent field is therefore a very interesting study concerning the origin of life. Examples of active sulfide-forming hydrothermal sites situated on peridotite mantle rocks at the MAR include the Rainbow field (German et al., 1996; Douville et al., 2002), Logatchev field (Gablina et al., 2000), Ashadze field (Beltenev et al., 2003), and the newly discovered Nibelungen field (Melchert et al., 2008).

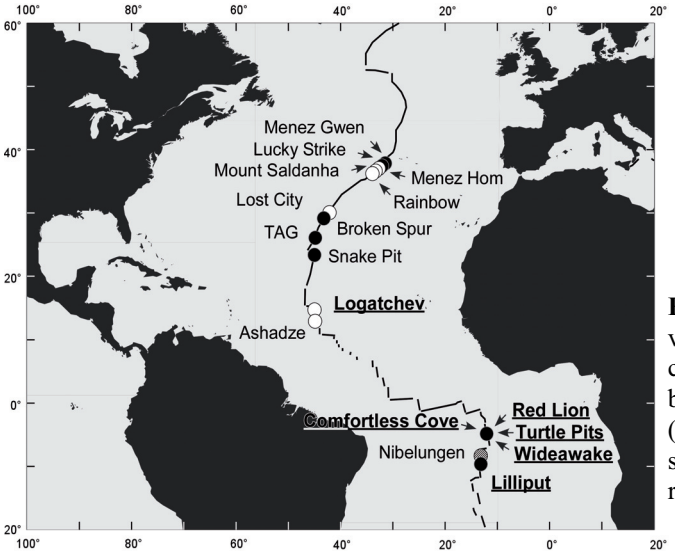

Figure 8: Currently known active hydrothermal vent fields along the Mid-Atlantic Ridge. White circles denote ultramafic-hosted and black circles basalt-hosted vent field. Fluids of the Nibelungen (gray circle) field support an ultramafic-hosted setting, while only basaltic rocks were so far recovered (Koschinsky et al., 2006).

1.2.2 The peridotite-hosted Logatchev hydrothermal vent field

The Logatchev hydrothermal vent field was discovered by Russian scientists during the cruise of RV 'Professor Logatchev' in 1993-1994 (Gebruk et al., 2000). It is positioned at the Mid-Atlantic Ridge at 14°45'N and is therefore as well known as '14º45' or as '15ºN'. The hydrothermal field is located at the lower, eastern ridge-flank at about 3000 m water depth, close to the axial valley. The field consists of several hydrothermal structures which are aligned along roughly 520 m distance in NW-SE direction between the north-western smoking crater "Quest" and the south-eastern chimney structure of "site A" (Figure 9). Besides smoking craters and chimney complexes, Logatchev is covered by thick sediment layers (Nercessian et al., 2005; Petersen et al., 2009). They likely result from a combination of tectonic activity due to rifting processes (Gao, 2006), low-temperature water/rock interaction (Daczko et al., 2005), and high-temperature hydrothermal alteration.

The Logatchev hydrothermal vent field at the MAR is an active peridotite-hosted hydrothermal site. The fluids at the Logatchev field reach temperatures up to 350°C and are enriched in hydrogen and methane, which are produced during serpentinization of ultramafic rocks at high temperatures (Schmidt et al, 2007). As exothermic serpentinization cannot contribute significantly to the required heat, gabbroic intrusions and the lithospheric mantle could be other possible heat sources (German and Li, 2004). The fluid geochemistry is very similar at all vent sites. This suggests a common source in the reaction zone, feeding the different vents (Figure 10) (Schmidt et al., 2007). The fluids exposed a temporal stability over nine years, which indicates a stable system with continuous serpentinization in the sub-seafloor without significant changes in alteration patterns and heat supply.

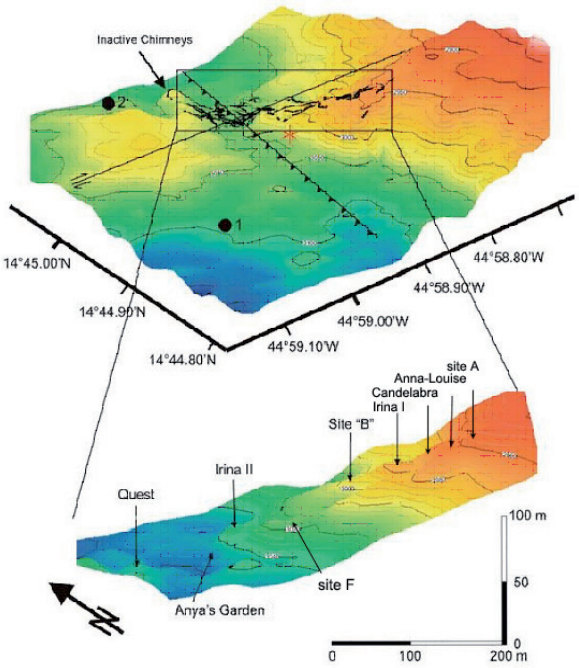

Figure 9: The locations of the hydrothermal structures at the Logatchev hydrothermal vent field.

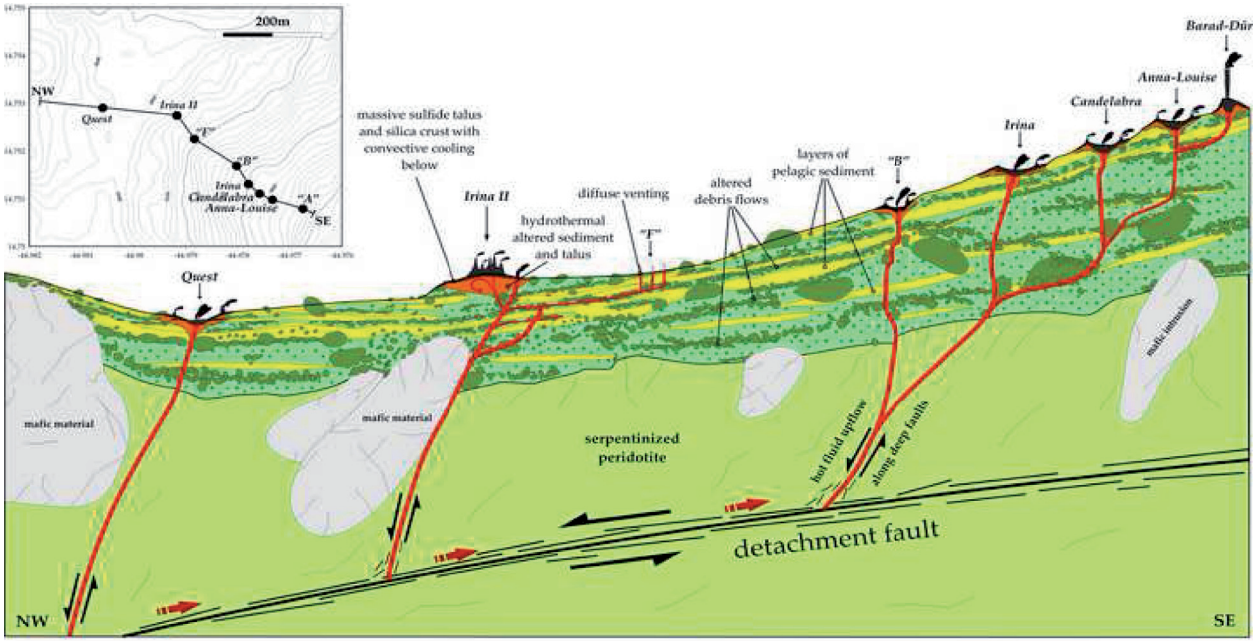

Figure 10: Schematic profile through the Logatchev hydrothermal field showing the proposed fluid flow from an underlying detachment fault through a sequence of gravitational landslides and intercalated sedimentary material (Petersen et al., 2009).

2 Microbial diversity

The microbial biomass in deep-sea surface sediments ranges from 1.6×10^7 to 1.5×10^9 cells per g sediment which is comparable to coastal sediments (Deming and Colwell, 1982; Harvey et al., 1984; Guezennec and Fiala-Medioni, 1996; Wang et al., 2004). The benthic habitat contains 10-10,000-fold more cells per unit volume than the pelagic habitat of productive ocean-surface water (Glud, 2008). Where chemical energy is transported from the subsurface up to the seafloor, rich and diverse microbial communities can proliferate. The highest biomass with up to 10^{12} cells per cm^3 was found in methane-fuelled microbial communities in anoxic sediments above gas hydrates and gas vents (Michaelis et al., 2002; Treude et al., 2007). In the beginning, these seafloor microbial communities were identified only by using traits that require culturing in the laboratory. Culture techniques, however, reveal only a small fraction of the diversity of microbial life (Table 4) (Whitman et al., 1998). The development of molecular methods, such as the analysis of 16S rRNA genes (Amann et al., 1995) and fingerprint techniques (DGGE, T-RFLP, ARISA) enable a more comprehensive view of microbial diversity. These genetic based methods enabled the detection of a vast amount of yet uncultured microbial diversity (Head et al., 1998).

Table 4: Cultivation efficiency of different habitats.

Habitat	Cultivation efficiency [%]	Reference
Marine Water	0.01 to 1	Ferguson et al., 1984
Lake water	0.1 to 1	Staley and Konopka 1985
Estuary	0.1 to 3	Ferguson et al., 1984
Sediment	0.2 to 23	Köpke et al., 2005
Soil	0.3	Torsvik et al., 1990

2.1 Microbial diversity in permanently cold systems

Microorganisms in cold deep-sea sediments mineralize 97% of the organic carbon that reaches the deep-sea floor from the photic zone so that only 3% is buried in the sediment. The oxic zone in deep-sea sediments can reach depths of many cm or dm (Reimers, 1987; Wenzhöfer and Glud, 2002), so that organic carbon can be mineralized completely to CO_2 through aerobic respiration. The general diversity and distribution of *Bacteria* and *Archaea* at the vast ocean seafloor are largely unknown.

In 1957, Zobel and Morita (Zobel and Morita, 1957) were among the first researchers who attempted to isolate microorganism that were specifically adapted to high pressures. They called them barophilic bacteria (=piezophile) (Yayanos 1995). Since then, barophilic isolates

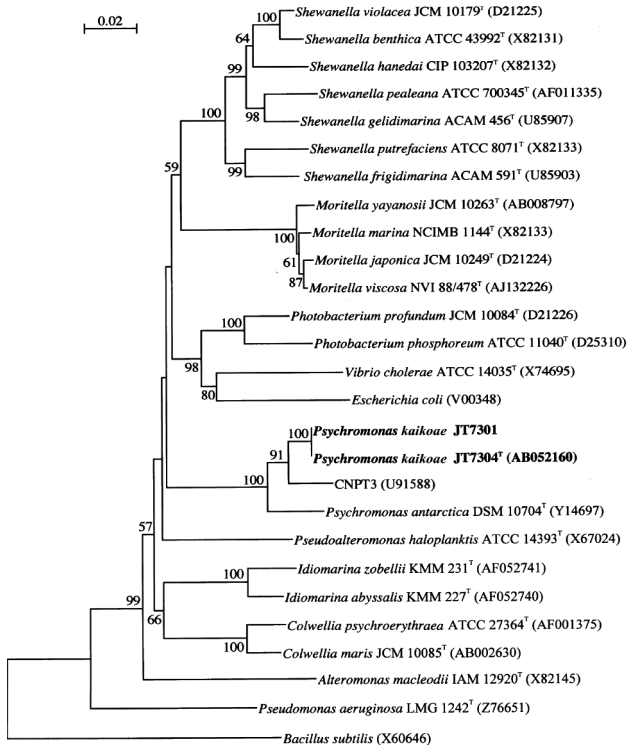

Figure 11: Phylogenetic tree showing isolates within the Gammaproteobacteria. The tree was calculated by the neighbour-joining method and bootstrap values were calculated from 1000 trees (Nogi et al., 2002).

have been obtained from a variety of different deep-sea habitats by a number of different laboratories (DeLong et al., 1997). All of the barophilic bacteria isolated belong to the *Gammaproteobacteria*. Most isolates are related to the five genera *Shewanella*, *Photobacterium*, *Colwellia*, *Moritella*, and *Psychromonas* (Figure 11) (Margesin and Nogi, 2004). *Shewanella benthica* is the most commonly isolated barophilic species, recovered from a variety of abyssal and hadal environments in Pacific, Atlantic, and Antarctic Oceans (DeLong et al., 1997). Notable exceptions are two sulfate-reducing isolates of the genus *Desulfovibrio* (Bale et al. 1997) and one Gram-positive member of genus *Carnobacterium* (Lauro et al. 2007).

In contrast to cultivation studies, all culture-independent studies of microbial communities in deep-sea surface sediment of the Pacific, North Atlantic, Arctic and Antarctic Oceans and the Mediterranean Sea showed a high microbial diversity. This supports the hypothesis that in deep-sea sediments, competition is assumed to be weak and symmetrical, contributing to a high diversity (Grant, 2000). The microbial community of the detritus-fuelled oxic seafloor is dominated by *Bacteria* (Santelli et al., 2008). *Archaea* seem to comprise a small portion of the oxic seafloor microbial community, in contrast to the anoxic subsurface, where *Archaea* might dominate (Biddle et al., 2006). The highest richness of *Bacteria* was found in deep-sea basaltic seafloors, which were shown to be colonized by variably abundant microbial communities that may be as diverse as farm soils (Figure 12) (Santelli et al., 2008). The bacterial community identified by 16S rRNA gene sequencing, is comprised of at least 16

different taxonomic groups, including all subdivisions of the *Proteobacteria*. However, current results of Templeton et al. (2009) indicate that this basalt floors are influenced by external hydrothermal sources so that the seafloor biomass does not subsist only on energy largely derived from basalt alteration reactions. Therefore, further investigations are clearly necessary to determine the maximum richness in permanently cold deep-sea floors.

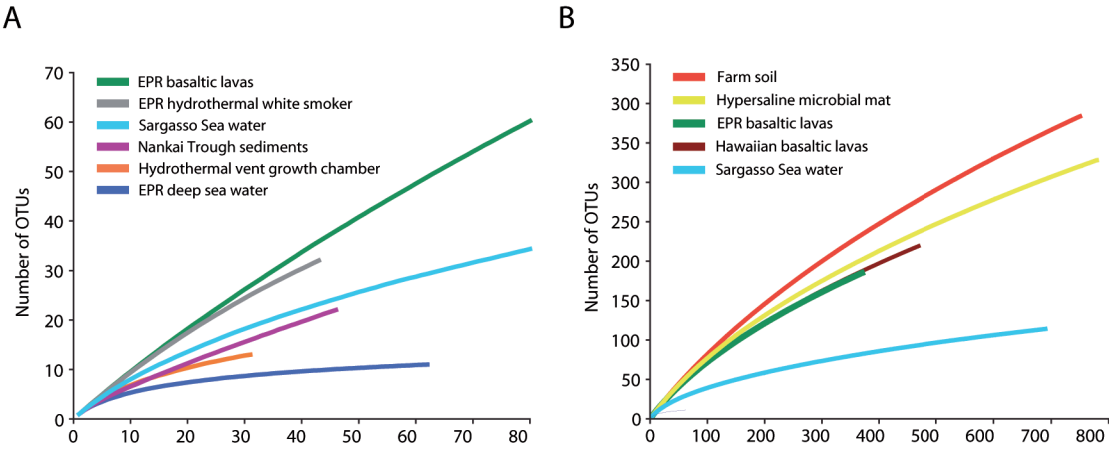

Figure 12: Relative bacterial richness from several environmental studies shown through rarefaction analyses. (A) Species richness of Bacteria inhabiting EPR seafloor lavas is compared with that of other ocean environments, such as the Sargasso Seawater, a MAR hydrothermal vent in situ growth chamber, an EPR hydrothermal white smoker spire, Nankai Trough deep-sea sediments, EPR deep sea water and a basalt-hosted community from Hawaii. (B) The bacterial richness of the EPR basalts is also compared and other known high-richness environments such as a farm soil, a hypersaline microbial mat from the Guerrero Negro (Santelli et al., 2008).

In all investigated deep-sea floors, sequences affiliated with *Gammaproteobacteria* represent the dominant part of the clone libraries (Ravenschlag et al., 1999; Bowman and McCuaig, 2003; Polymenakou et al., 2005; Hongxiang et al., 2008; Santelli et al., 2008). Many sequences clustered with cultivated members of the family *Alteromonadaceae* (*Shewanella*, *Moritella*, *Idiomarina*, and *Pseudoalteromonas*) that have been isolated from different deep-sea sediment and water column (DeLong 1997; Kato et al., 1997; Yanagibayashi et al., 1999; Ivanova et al., 2000). Sequences related to environmental groups lacking cultivated members are also found in a high frequency in clone library studies of seafloors from different oceans (JTB255/BD3-6; Polymenakou et al, 2005; Yanagibayashi et al, 1999; Li et al, 1999; Urakawa et al, 1999; Zhao and Zeng, 2005; Xu et al, 2005; Bowman and McCuaig, 2003; Ravenschlag et al, 1999). Other groups of so far uncultured members are BD7-8/MERTZ and JTB23/Sva0091, which form distinct lineages among the sulfur-oxidizing members of the orders *Thiotricales* and *Chromatiales*. The comparison of different clone library data obtained from deep-sea sediments suggests that many prokaryotic groups, taxons equivalent to the species to family level, were ubiquitously distributed.

2.2 Microbial diversity at hydrothermal systems

Instead of the uniform conditions present in surface sediments in cold systems, hydrothermal systems consist of different habitats exhibiting steep temperature and chemical gradients (Jørgensen and Boetius, 2007). Free-living microorganisms can establish themselves at hydrothermal habitats, where the hot, electron donor-rich vent fluids meet cold, electron acceptor-rich ocean waters (Figure 13A). The huge range of inorganic and organic compounds produced abiotically by magmatic degassing and subsurface rock-water reactions at high pressure and temperatures can be use by free-living microorganisms (Figure 13B). Hydrothermal fluids can either mix with cold seawater and emanate at low speed and mild temperatures (diffuse fluids) into the overlying seawater, or be ejected directly into the cold seawater. In the latter case, iron and manganese salts precipitate as black particle clouds reminiscent of smoke, which is known as the plume. These precipitations of dissolved materials can form energy-rich solid surfaces such as chimneys structures and sediments that can also be exploited by microorganism. Animal surfaces can also provide a substrate for colonization by free-living microorganisms.

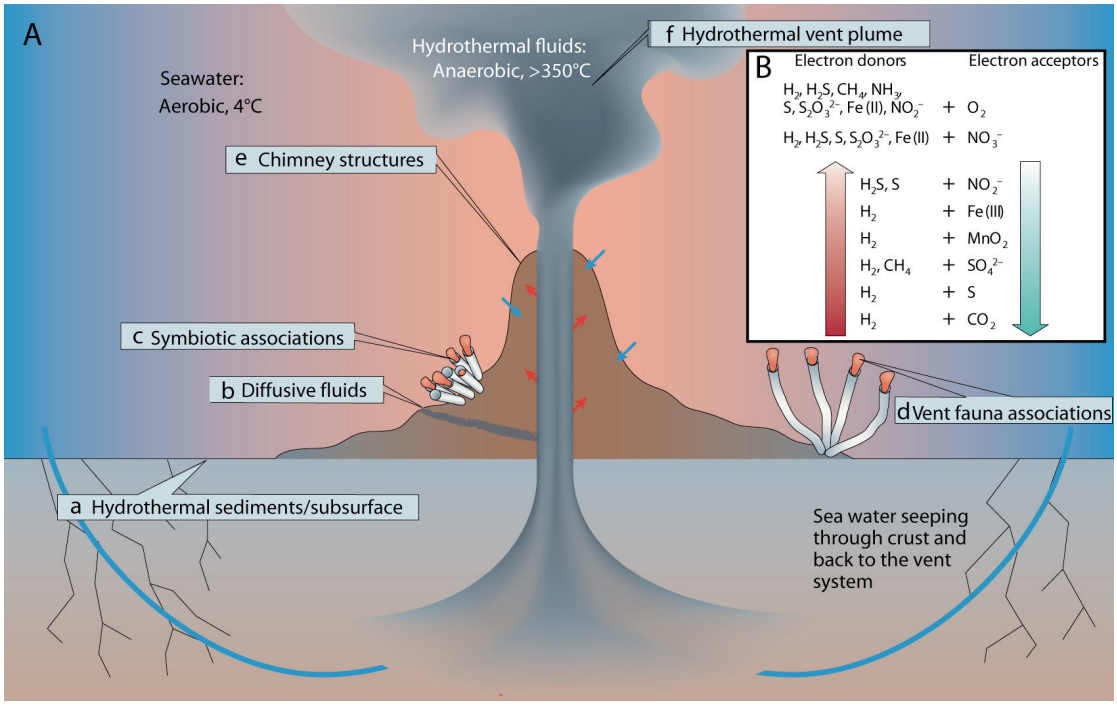

Figure 13: (A) Different vent-habitats exhibiting gradients of chemistry and temperature. Coloured arrows indicate the flow of either hot hydrothermal fluids (red) or cold seawater (blue) (Campbell et al., 2006). (B) Energy sources available at hydrothermal vents showing the redox couples of electron donors (in vent fluids) and electron acceptors (in bottom water) (Jørgensen and Boetius, 2007).

2.2.1 Culture-dependent studies of free-living microorganisms

Hydrothermal fluids and the interior of chimney structures represent high-temperature habitats and therefore extreme environments. Cultivation approaches showed that this kind of habitats allow life and survival only to a few groups of specialists. Isolated microorganisms that grow at temperatures higher than 75°C (optima 80°C) are define as hyperthermophiles. The isolation of microorganism at increasing temperatures has raised the question of whether there is an upper limit to life and, if so, what it is. Since life needs liquid water, hyperthermophiles growing above 100°C are found preferentially in high-pressure environments where the boiling point of water is higher. As there are physicochemical

Table 5: Representatives of metabolic groups of mostly hyperthermophiles found at marine vents.

Isolates	T_{max} [°C]	Metabolism	Habitat	Domain	Reference
Strain 121	121	Fe(II)-reduction heterotroph (formate)	Endeavor segment of the Juan de Fuca Ridge, water sample from black smoker	Archaea	Kashefi and Lovely, 2003
Pyrolobus fumarii	113	hydrogen-oxidation	TAG at the Mid-Atlantic Ridge black smoker wall	Archaea	Blöchl et al., 1997
Methanopyrus kandleri	110	methanogen	Guaymas Basin, sediment Mid-Atlantic Ridge of Kolbeinsey, north of Iceland, sediment and water	Archaea	Kurr et al., 1991
Pyrodictium occultum	110	hydrogen-, sulfur-oxidation	Submarine solfatanic field of Vulcano, Italy, geothermal heated water and sulfurous deposits	Archaea	Stetter et al., 1983
Archaeoglobus fulgidus	95	sulfate-reduction	Vulcano, Italy and at Stufe di Nerone, Naples, Italy, geothermally heated seafloor	Archaea	Stetter, 1988
Aquifex pyrophilus	95	hydrogen-thiosulfate- and elemental sulfur-oxiditation	Kolbeinsey Ridge, Iceland, hot sediments	Bacteria	Huber et al., 1992
Methanococcus igneus	91	methanogen	Kolbeinsey Ridge, Iceland blackish sandy sediment and venting water	Archaea	Burggraf et al., 1990
Methanococcus jannaschii	86	methanogen	East Pacific Rise base of white smoker chimney	Archaea	Jones et al., 1983
Thermus aquaticus	75	heterotroph (sugar and organic acids)	Yellowstone National Park, thermal springs water California, thermal spring water	Bacteria	Brock and Freeze, 1969
Sulfurimonas autotrophica	40	elemental sulfur-, sulfide- and thiosulfate-oxidation	Hatoma Knoll in the Mid-Okinawa Trough, sediment	Bacteria	Inagaki et al., 2003
Sulfurovum lithotrophicum	40	elemental sulfur- or thiosulfate-oxidation	Iheya North hydrothermal system in the mid-Okinawa Trough, Japan, gas-bubbling sediment	Bacteria	Inagaki et al., 2004
Thiomicrospira sp. (strain L-12)	25	sulfur-oxidation	Galapagos Rift Rose-garden vent, from mussel	Bacteria	Ruby and Jannasch, 1982

limitations to biochemistry at high temperatures (e.g. for ATP and DNA), the upper limit of life is assumed to be between 121 and 130°C (Lengeler et al., 1999). The archaeon *Pyrolobus fumarii* and Strain 121 are two isolates holding the global record of growth at high temperatures (113 and 121°C, respectively) (Table 5) (Blöchl et al., 1997; Kashefi and Lovely, 2003). Most hyperthermophilic microorganisms known today are *Archaea*, and only a few hyperthermophiles belong to the domain *Bacteria* (*Thermotoga*, *Aquifex*). Environments with temperatures beyond 95°C appear to contain only *Archaea* as living inhabitant. Besides hyperthermophiles, different microorganisms of moderate habitats were isolated (Karl, 1995). To date, most enrichment culture studies of deep-sea vents were designed to isolate specific chemolithoautotrophic microorganisms rather than to define the total community composition.

2.2.2 Culture-independent approaches for studying free-living microorganisms

Most of the initial research on hydrothermal microbial life concentrated on the microbial diversity that is associated with vent plumes at mid-ocean ridges (Takai et al., 2006). The discovery of microbial corrosion structures and mineral alteration in ocean basalts, submarine lava, hydrothermal precipitates and vented rocks has shifted the focus from vent plumes to the huge undiscovered microbial realm in the seabed (Thorseth et al., 2001; Bach and Edwards, 2003; Santelli et al., 2008). Massive biomass accumulations were observed at seawater-substrate interfaces (Karl, 1995) forming biofilms and microbial mats which control the rates of redox reactions, modifying their environment. The cell numbers in these microbial mats reach up to 6.9×10^7 to 5.3×10^8 cells per ml (Emerson and Moyer, 2002) in comparison to cell numbers of 5×10^2 cells per ml in fluids (Takai et al., 2009) and 1.0×10^5 to 1.1×10^5 cells per ml in plumes (Sunamura et al., 2004).

2.2.3 Seabed microbial diversity in basalt-hosted hydrothermal vent fields

The seabed at hydrothermal vent fields is characterized by chimney structures. Active hydrothermal chimneys are habitat with steep chemical and thermal gradients (Kristall et al., 2006) as they are locations where hot-temperature fluids are emitted to the cold surrounding seawater (Figure 14A). The exterior to the interior of the structure represent diverse environments, which provides a wide range of microhabitats for microorganisms. In the interior layers, representing a high-temperature environment, *Archaea* were found to be important and major components of the diversity (Schrenk et al., 2003). The uncultured group

named "Deep-sea hydrothermal Vent Euryarcheaota group" (DHVEG) is distributed throughout the global deep-sea vent system (Takai et al., 2006). Archaeal biofilms in the sulfide chimney Finn (Juan de Fuca Ridge) were found to be composed of members of the *Thermococcales* (*Crenarchaeota*) and *Methanococcales* (*Euryarchaeota*) (Schrenk et al., 2003).

The greatest abundance of microorganism was detected in the middle and exterior zones (interior=5.5 × 10^4 cells/g, exterior=6 × 10^7 cells/g; Takai et al., 2009), which reflect habitats with moderate temperatures (Schrenk et al., 2003). These zones are colonized by multispecies biofilms composed of *Bacteria* and *Archaea* (Figure 14A, and B). The detected bacterial community represents phylogenetically diverse bacterial populations, including diverse phyla (Takai et al., 2006; Takai et al., 2009). *Epsilonproteobacteria* were found to dominate microbial mats which were associated with a chimney (Southern EPR, 17°S; Longnecker and Reysenbach, 2001) and were highly abundant in sulfur chimney structures (TOTO caldera in the Mariana Volcanic Arc, Nakagawa et al., 2006).

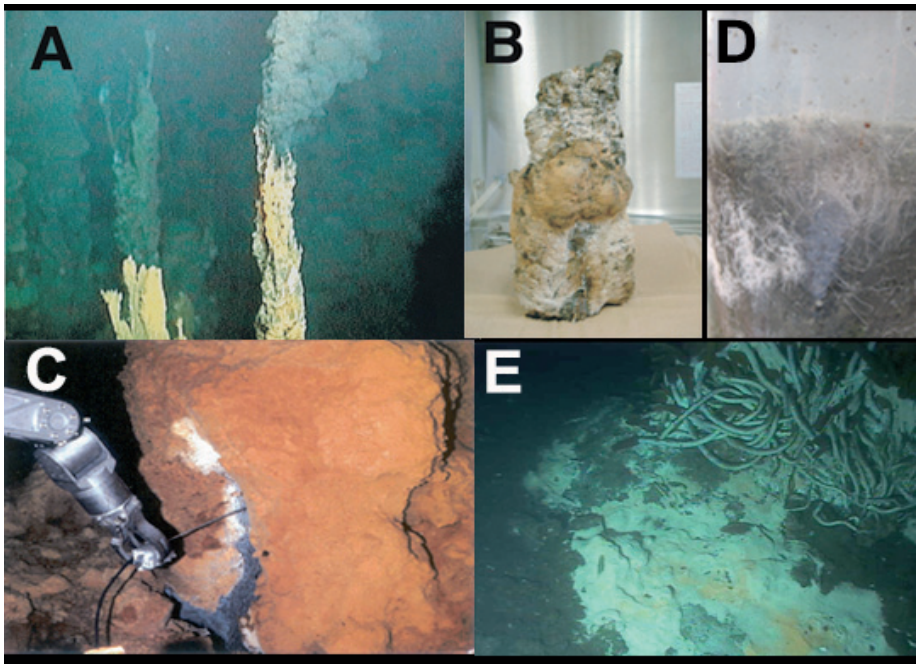

Figure 14: (A) Photographs of black smoker vents of the PACMANUS field and (B) of a successfully recovered chimney structure. (C) Microbial mat presents at the hydrothermal vent system Loihi Seamount, Hawaii. (D) Filamentous *Beggiatoa* spp. in a recovered sediment core and (E) a corresponding photograph of the microbial mats at the Guaymas Basin.

The active vents at the Loihi Seamount are characterized by high concentration of Mn and Fe(II) (up to 268 µM) in the fluids (Emerson and Moyer., 2002). Instead of sulfur chimney structures, here microbial mats are attached to the seafloor and are heavily encrusted with rust-colored Fe oxides (Figure 14C). These mats are dominated by *Leptothrix ochracea* and

Gallionella spp. (*Betaproteobacteria*) and *Mariprofundus ferrooxydans,* which represent the first isolates of the new candidatus class of the *Proteobacteria*, the *Zetaproteobacteria* (Emerson et al., 2007). These strains were found to be most common in a variety of microbial mats collected at different times and locations at Loihi. Furthermore microbial mats at Loihi Seamount include members related to *Archaea* and *Gammaproteoabacteria*, *Deltaproteobacteria*, and *Epsilonprotoebacteria*. Further Fe(hydr)oxide mats were found at the hydrothermal environment of the Vailulu'u Seamount which is the eastern-most island of American Samoa (Sudek et al., 2009). In contrast to the Loihi Seamount communities, most sequences were related to *Epsilonproteobacteria*. Sequences affiliated with *Alphaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria*, *Bacteroidetes* and *Planctomycetes* were present in a lower frequency.

Besides chimney and seafloor structures, sediments represent a habitat where dense communities are developed. The Guaymas Basin represents one of the best-investigated sediment-hosted hydrothermal systems to date. This basin has a very high sedimentation rate (1 to 2 mm/year) due to high biological productivity in surface waters and a large terrigenous input. The thick layer of organic-rich, diatomaceous sediments (100 m, and up to 500 m) undergoes pyrolysis and thermal alteration to a wide variety of petroleum hydrocarbons, steranes and diasteranes, and aromatic hydrocarbons (Teske et al., 2002). This leads to a reduced metal content and an increased methane content (12 to 16 mM) in the vent fluids. On top of these sediments, massive bacterial mats of filamentous *Beggiatoa* spp. are found (Figure 14D, and E) (Nelson et al., 1989; Gundersen et al., 1992). Further common bacterial phylotypes include members of the *Epsilonproteobacteria*, *Deltaproteobacteria*, the *Chloroflexi*, and the Candidate Division OP11. In addition, sequences affiliated with anaerobic methanotrophic (ANME) archaea of the ANME-1 and ANME-2 group were for the first time detected in hydrothermal systems (Teske et al., 2002).

Although all above mentioned investigated hydrothermal vent field belong to basalt-hosted systems, different seabed structures represent different habitats for microbial communities. Therefore, microbial diversity differed between hydrothermal vent fields. Cultured-independent methods confirmed that extreme hot environemts are predominantly inhabited by *Archaea*. Besides classes of minor importance, *Epsilonproteobacteria* were dominant at sulfur structures, iron encrusted microbial mats represented favorable habitats for *Betaproteobacteria*, and microbial mats at a sedimented field consisted of *Gammaproteobacteria*.

2.2.4 Seabed microbial diversity in peridotite-hosted hydrothermal vent fields

Hydrothermal systems associated with peridotite rocks were discovered around 20 years after basalt-hosted hydrothermal vent fields. Therefore, the microbial diversity of this system is less documented in comparison to microbial communities at hydrothermal systems associated with basaltic lava. At the peridotite-hosted field Lost City, the mixing of hot fluids and cold seawater leads to the formation of carbonate chimneys that rise up to 60 meters above the ultramafic seafloor (Figure 15A) (Kelley et al., 2001). Dense biofilms of *Methanosarcinales* were found at carbonate chimneys which emit fluids >80°C. They make up ~100% of the archaeal community (Brazelton et al., 2006). In chimneys that have little or no active venting, the Lost City *Methanosarcinales* (LCMS) group is replaced by a single phylotype, the clade ANME-1. In contrast, a diverse bacterial assemblage populates the chimney exteriors. Therefore, *Archaea* were present at high-temperature environments and *Bacteria* were present in moderate habitats, which is similar to the microbial compositions in chimneys found at basalt-hosted fields.

Another ultramafic-hosted hydrothermal vent field which in contrast to Lost City emits high-temperature fluids (365°C, pH 2.8) is the Rainbow hydrothermal vent field on MAR (36°N). The active chimneys are surrounded by heterogeneous sediments contributed by a mixture of pelagic sediments, iron oxides and oxidized fragments of dead chimneys

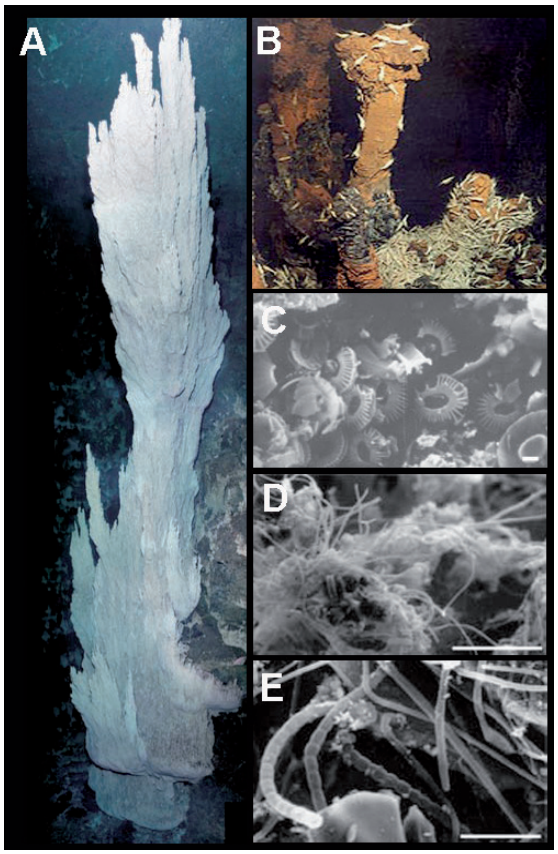

Figure 15: (A) The Lost City 10-m-tall actively venting carbonate chimney (Graceful) growing directly out of a serpentinite cliff on the eastern side of the field (Kelley et al., 2005). (B) Photographs of chimneys at the Rainbow hydrothermal vent field. (C) Scanning electron microscopy photographs of Rainbow sediment showing coccoliths and foraminifer shells (scale bars=1 µm) and (D and E) of prokaryotic cell types observed in different colonization after 15 days exposure to a fluid source (scale bars=10 mm (López-Garcia et al., 2003a, and b).

(Figure 15B) (Nercessian et al., 2005). These sediments are primarily composed of carbonates, mostly by haptophyte coccoliths and foraminifer shells (Figure 15C) (López-Garcia et al., 2003a). However, the sediments showed no apparent sign of hydrothermal activity. The bacterial composition revealed by 16S rRNA gene clone libraries was highly diverse. Most of the bacterial clones were affiliate with the *Gammaproteobacteria* or *Acidobacteria*. (Nercessian et al., 2005; López-Garcia et al., 2003a). The majority of archaeal sequences (84%) was related to the euryarchaeon *Methanocaldococcus infernus*. This methanogen was recently identified in the walls of black smokers suggesting a possible thermophilic lifestyle. Microcolonizers exposed to hot fluids (~365°C) showed in contrast to the sediment samples a highly specialized community. After 15 days of incubation, dense bacterial mats were detected (Figure 15D, and E). Most of the obtained sequences were related to *Epsilonproteobacteria* and clustered with the *Sulfurimonas*, *Sulfurovum* and *Arcobacter* group (López-Garcia et al., 2003a).

2.2.5 Driving forces of microbial diversity at hydrothermal systems

Most of the understanding of microbial diversity of deep-sea hydrothermal vents stems from research focused on mid-ocean ridges. However, recent research in the microbial diversity of mid-ocean ridges, back-arc basins, and volcanic arcs revealed a greater heterogeneity of microbial communities between vent systems than previously thought (Takai et al., 2006). All studies support the idea that microbial community composition will be strongly modulated by the geochemical structure and dynamics of local environments. The heterogeneity in microbial composition between different vent fields can be directly correlated to differences in the physical and geochemical conditions of the vent habitats. It was shown that the concentration and composition of the gas components in seafloor hydrothermal vent fluids could be one of the potential driving forces behind intra-field variability in active microbial communities (Takai et al., 2006).

2.2.6 Symbioses at hydrothermal systems

Deep-sea hydrothermal vents were the first habitats in which chemosynthetic primary production was shown to fuel large animal communities that are considered to be among the most productive on earth (Van Dover, 2000). Chemosynthetic symbionts can be epibionts, which are found to be attached to a specific part of an animal and endobionts, which can be extracellular (e.g. below the cuticle) or intracellular (e.g. gill, trophosome). Their role is to

transform the chemical energy from vent fluids into food for their host. The analyses of 16S rRNA gene sequences revealed *Gammaproteobacteria* as a common symbiont in vent animals (vent mussels *Calyptogena magnifica*, *Bathymodiolus thermophilus*, vent snail *Alviniconcha* sp., seep mussel *Maorithyas hadalis*). Phylogenetic analyses of these symbionts revealed at least nine phylogenetically distinct clades, most of which were interspersed with sequences from free-living bacteria (Dubilier et al., 2008). Symbionts detected from the seep mussel *Bathymodiolus childressi* clustered to a single clade within the *Gammaproteobacteria*, with the free-living methane oxidizers of the genera *Methylobacter* and *Methylomicrobium* (Distel and Cavanaugh, 1994). Some symbionts clustered with *Epsilonproteobacteria* (vent shrimp *Rimicaris exoculata*, Pompeii worm *Alvinella*). Besides the association between one animal and one symbiont, multiple co-occuring symbionts have been found. Until now, up to six co-occuring bacterial symbionts were shown to exist in cold-seep mussels (*Idas* sp.) (Duperron et al., 2008).

3 Microbial activity

Microbial diversities determined by comparative 16S rRNA analysis and fluorescence *in situ* hybridization (FISH) were often used to hypothesize metabolic processes in a given habitat. Thereby it is assumed that the metabolic capabilities of detected taxa are similar to those of the closest cultivated relatives. Phylogeny may however not be reliable for predicting physiology. Even closely related bacteria might have very different metabolic repertoires, e.g. resulting from lateral gene transfer events, enabling them to occupy differing ecological niches (Suen et al., 2007). Therefore, attempting to identify a niche by phylogenetics alone is proving to be difficult as distant species sometimes share the same niche, and phylogenetically close species sometimes occupy very different niches. To examine what microorganisms are doing in their habitats, how they interact with each other and with their environment, genomic, *in situ* and *ex situ* approaches can be used. Until recently, most of what we knew about microbial activity was derived from cultured microorganisms and *ex situ* laboratory experimental investigations.

3.1 Genomics and metagenomics

Genomic and metagenomic studies increased our understanding of the genetic mechanisms underlying responses of organisms to their environments. Moreover, functional elements beyond just the protein coding regions were analyzed, providing insights into gene regulation

and genome evolution (Kahvejian et al., 2008). Sequencing of whole prokaryotic and eukaryotic genomes was facilitated by the continuous introduction of new methods based on DNA sequencing. This techniques follow a cyclic pattern in which new technologies are introduced, rapidly take up, and then undergo a slow decline in use as newer techniques are developed that supersede them (Figure 16).

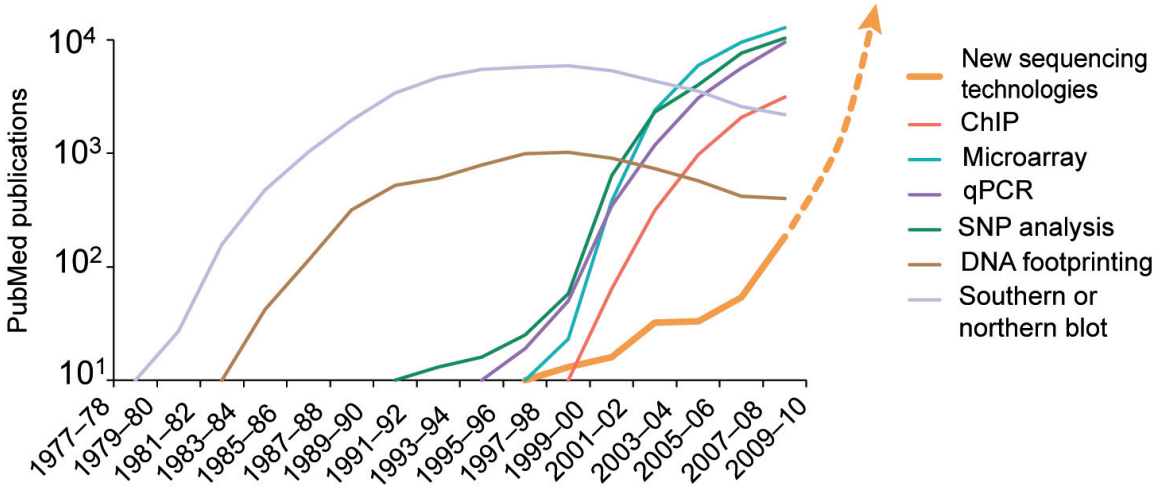

Figure 16: The number of publications with keywords for nucleic acid detection and sequencing technologies. PubMed (http://www.ncbi.nlm.nih.gov/sites/entrez) was searched in two-year increments for key words and the number of hits plotted over time. For 2007–2008, results from January 1–March 31, 2008 were multiplied by four and added to those for 2007. Key words used were those listed in the legend except for new sequencing technologies ('next-generation sequencing' or 'high-throughput sequencing'), ChIP ('chromatin immunoprecipitation' or 'ChIP-Chip' or 'ChIP-PCR' or 'ChIP-Seq'), qPCR (TaqMan or qPCR or 'real-time PCR') and SNP analysis (SNPs or 'single-nucleotide polymorphisms' and not nitroprusside (nitroprusside is excluded because sodium nitroprusside is sometimes abbreviated as 'SNP' but is generally unrelated to genetics)) (Kahvejian et al., 2008).

Genomics has classically focused on pure, easy-to-obtain samples, such as microbes that grow readily in culture or large animals and plants. Therefore, the first obtained complete bacterial genome sequence was of *Haemophilus influenzae*. On October 21th2009, a landmark of genome sequencing was reached with the completion of the thousandth whole genome sequence, that of *Methanocaldococcus vulcanius* M7 (Figure 17A). This microorganism is a hyperthermophilic methanogen archaeon isolated from a deep-sea hydrothermal chimney on the East Pacific Rise at a depth of 2600 m. The inventory of bacterial and archaeal isolates with complete or draft sequences is approaching the two thousand mark (Liolios et al., 2008). The complete genomes include those of many important model and reference organism, and multiple sequences are available for many of the key human and animal pathogens. Consequently, the phylogenetic distribution of the completed sequences is biased towards organism of specific medical or economic interest. Thus, more than 80% of the available sequences represent just three major lineages: *Proteobacteria*, *Firmicutes* and *Actinobacteria*

(Figure 17B). Therefore, a phylogenetic-driven pilot project called 'Genomic Encyclopedia of Bacteria and Archaea (GEBA) was started in order to derive maximum knowledge from existing and new microbial genomes data. The analysis of the first 56 of those demonstrated pronounced benefits in diverse areas including the reconstruction of phylogenetic history, the discovery of new gene families, and the prediction of function for known genes from other organisms (Wu et al., 2009).

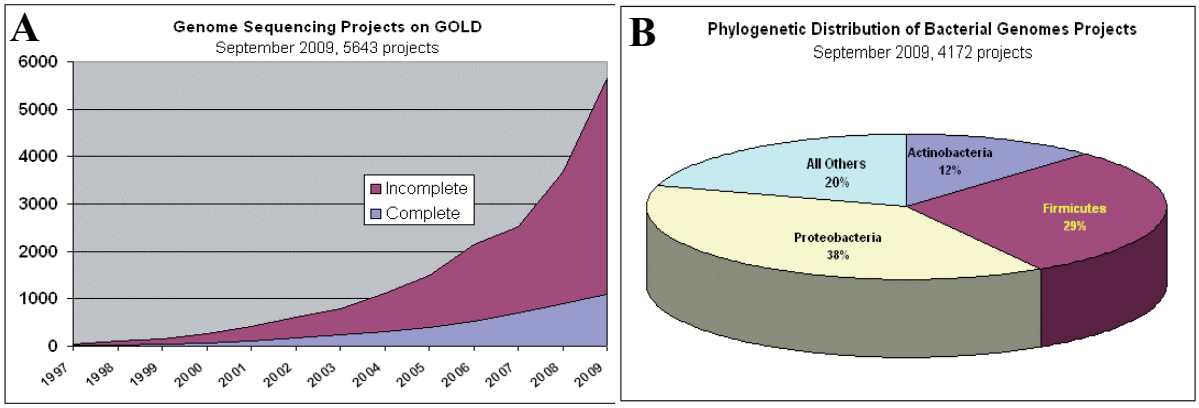

Figure 17: (A) Number of sequences available in the Genbank as of September, 2009. The biological data explosion in mid 90's kick-started the 'omics' revolution which can be easily seen with the exponential growth from 1995. (B) Phylogenic distribution of investigated bacterial gemome, showeing than 80% of the available sequences represent just three major lineages: *Proteobacteria*, *Firmicutes* and *Actinobacteria* (http://genomesonline.org/gold_statistics.htm)

However, the great majority of recognized bacterial and archaeal diversity in not represented by pure cultures (Table 4) (Whitman et al., 1998). As DNA can be isolated directly from living and dead cells in various contexts, instead of sequencing genomes of isolates, DNA sequences directly from the environment can be analyzed. Therefore, in principle, any microorganism that cannot be isolated or is difficult to grow in lab can be studied. This has led to the emergence of a new field, which is referred to as metagenomics. Improvements in cloning vectors including cosmids or fosmids (35-40 kb insert size), bacterial artificial chromosomes (BAC, 80-120 kb inserts), and yeast artificial chromosomes (YAC, 200-800 kb inserts) allowed to investigate a wide range of organisms that are otherwise difficult to study (Tringe and Rubin, 2005).

Meanwhile, the progressive reduction of the time and costs of high-throughput sequencing by technologies such as 454 FLX Titaniium pyrosequencing (Solexa GA platform) made it feasible to sequence libraries that are constructed from mixtures of organisms, even those that are contaminated with genomes other than that of the targeted organism (Rothberg and Leamon, 2008). The constantly increasing read length obtained by the new high-throughput sequencing generation allows direct sequencing of DNA without the traditional initial cloning

step excluding cloning bias. This has opened the door to sequence-based studies of organism and environments that were previously thought to be inaccessible, including obligate pathogens and symbionts, environmental microbes (which cannot be grown in pure cultures), and ancient organisms.

DNA-based studies do not differentiate between active and inactive microorganisms. As it is known that a large proportion of the cells in a given environment is inactive at any time (Bakken, 1997), this will affect the interpretation of diversity studies. Therefore, the application of new sequencing techniques will not be enough to understand underlying responses of living organisms to their natural environments. A deeper understanding will necessarily require a multidisciplinary approach, combining organismal analyses with molecular genetics and genomics, laboratory experiments with field studies all within an ecologically relevant framework (Figure 18) (Ungerer et al., 2008; Prosser et al., 2007). The combination of ecological and genomic approaches has led to a new interdisciplinary field that is called ecological genomics.

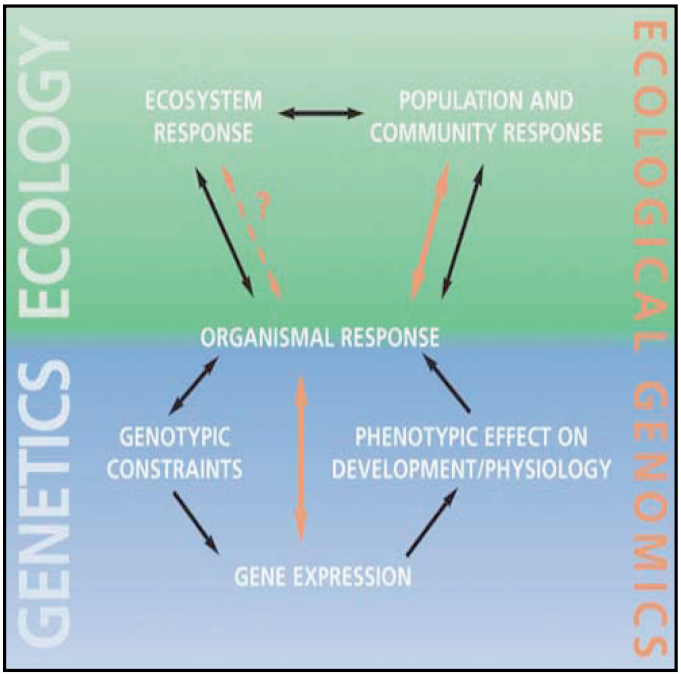

Figure 18: Conceptual framework for Ecological Genomics. In the upper part, the black arrows indicate ecological interactions between the organism, the population and community levels and the ecosystem. The properties of organisms affect the functions of the other levels and vice versa. In the lower part, the black arrows also indicate the interactions between the levels, with organismal responses affecting and being affected by its genotype, which in turn affects what genes are expressed and at what levels, which in turn has effects on the phenotype of the organism, ultimately leading to its overall response. Ecological genomic studies seek to integrate these disciplines (orange arrows) through the use of functional genomics approaches (Ungerer et al., 2008).

3.2 *In situ* and rate measurments

Besides genomic and metagenomic studies, which examine the genetic mechanisms underlying responses of microorganisms to their environment, the development of new *in situ* techniques enables the detection of microbial activity in their environment. Environmental parameters such as high-resolution chemical gradients in sediments can be measure by a microprofiler (Figure 19A). These microprofiles allow the quantitative evaluation and therefore the calculation of different consumption rates in environments revealing steady state gradient concentrations. Dissolved gases and volatile organic compounds can be detected by *in situ* mass spectrometry (Figure 19B). These new *in situ* technology achievements were a step forward to detect even extremely steep, temporally and spatially variable biochemical and physicochemical gradients, which are essential in zones of elevated microbial activity such as hot-spot environments (e.g. cold seeps and hydrothermal vents).

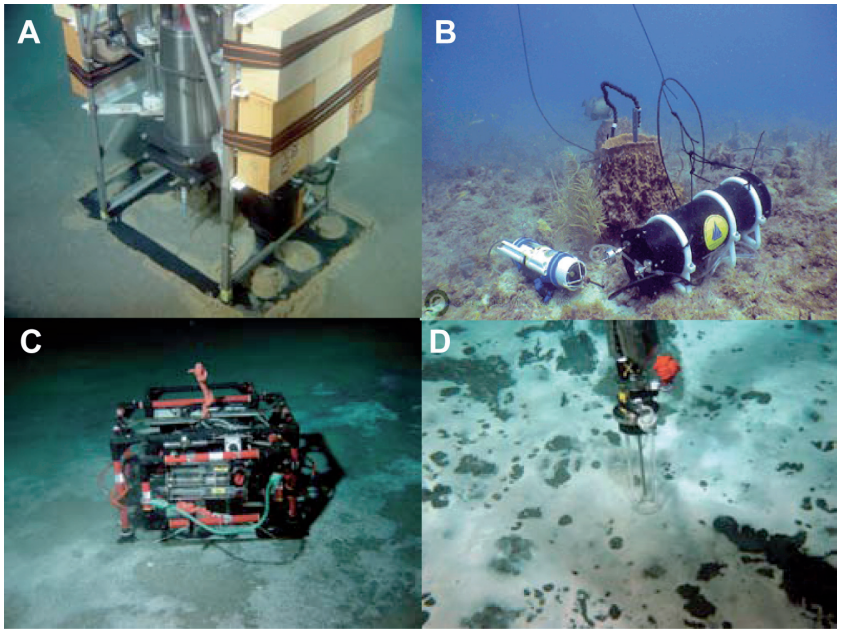

Figure 19: Different *in situ* measurement instrument: (A) microprofiler, (B) *in situ* mass spectrometry, (C) benthic chamber , and (D) INSINC module (Boetius and Wenzhöfer et al., 2009)

Furthermore, *in situ* respiration rates including oxygen, methane and sulfide consumption rates, can be determined by benthic chamber measurements (Figure 19C), providing more information about the exchange rates and total fluxes (Boetius and Wenzhöfer et al., 2009). To quantify the *in situ* sulfate reduction rates, an INSINC module was developed (Figure 19D). For this measurement, intact seafloor sediments are *in situ* injected with tracer substances ($^{35}SO_4^{2-}$). Besides *in situ* rate measurement, ex situ rates detection and incubation experiments provide information about microbial activities in their environment.

3.3 Activity of seafloor microbial communities in cold systems

Microorganisms comprise a large fraction of total benthic biomass and dominate the turnover of organic matter in deep-sea sediments (Deming and Baross, 1993). Variation in biomass and activity of microbial communities in the deep sea can largely be explained by the regional and temporal variation *in* particulate organic carbon (POC) flux (Boetius et al., 2000). Very little is known about the adaptation allowing for growth and survival of microbes in the deep-sea. Deep-sea bacteria have been shown to possess piezo-specific adaptations in terms of membrane phospholipid fatty acid unsaturation (Delong and Yayanos 1985; Allen et al. 1999; Allen and Bartlett 2000) and cell division (Jannasch 1987; Yayanos and DeLong 1987; Bidle and Bartlett 1999; Ishii et al. 2004). Moreover, hydrostatic pressure has been shown *in vitro* to affect many protein-protein (Silva and Weber 1993) and protein-DNA interactions (Chilukuri et al. 1997; Tang et al. 1998), and *in vivo* to hinder essential cellular processes such as replication and translation (Welch et al. 1993; Ishii et al. 2005). To cope with long periods of starvation, studies conducted with the moderate piezophile *Psychromonas* sp. CNPT3 under low-nutrient conditions have shown that cells decreased their biovolume and membrane unsaturated fatty acid content and increased their ability to attach to a glass substratum, particularly during incubations at high pressure. Furthermore one long-term experiment, a batch culture of *Psychromonas* HS11 (Delong and Yayanos 1986) has revealed, that piezophiles can survive with little or no food at high pressure for over 20 years (Lauro and Bartlett, 2008).

Genomic studies of first genomes of psychropiezophilic (cold and pressure-loving) bacterial isolates revealed a high ratio of rRNA operon copies/genome size (Lauro and Bartlett, 2008). Highest numbers of operons are present in bacteria that respond most rapidly to environmental changes (Klappenbach et al. 2000). These observations suggest that most of the deep bathytypes in culture have an opportunistic (r-strategy) lifestyle with a high degree of gene regulation. Motility is arguably the most pressure-sensitive cellular process in surface-water prokaryotes (Meganathan and Marquis 1973; Bartlett 2002). Gene clusters for motility and chemotaxis are among the most divergent between different deep-sea and shallow environments isolates (Campanaro et al. 2005). In fact, microarray-based genome comparison between three strains of *Photobacterium profundum* has shown that the deep barophilic isolates (SS9 and DSJ4; Nogi et al. 1998) have an additional gene cluster that is lacking in the shallow type (3TCK) which resembles genes for the production of lateral flagella (Campanaro et al. 2005).

Cultivation experiments revealed that most microorganisms of the detected highly diverse microbial communities are heterotrophic. Because of the abundance of sulfate in the seawater, its bacterial reduction to sulfide represents the main terminal pathway during the anaerobic mineralization of organic matter in the seabed (Jørgensen, 1982). Maximal *ex situ* sulfate reduction rates in the deep sea ranged between 3.3 and 4.9 nmol cm^{-3} d^{-1} (Ferdelman et al., 1999). In the surface sediments (0-5 cm) of the deep Arabian Sea, carbon remineralization by sulfate reduction was detected to comprise 40% of the total carbon input (Boetius et al., 2000).

3.4 Activity of seafloor microbial communities at hydrothermal vent fields

Many microorganisms at hydrothermal vent fields obtain their energy from the oxidation of inorganic substrates and are known as chemolithotrophs. If the carbon source is also inorganic, the microorganisms are called chemolithoautotrophs or simply chemoautotrophs. This process was observed more than 100 years ago (Winogradsky 1887 in Van Dover, 2000) and is known as chemosynthesis or lithotrophy. Due to their modest but specific nutritional requirements, chemolithotrophic bacteria were the first physiological type of bacteria to be isolated by using selective culture methods, widely known as enrichment culture techniques (Lengeler et al., 1999).

Chemosynthesis is a process that is well known but was considered to play no significant, quantitative role in the carbon cycle of the photosynthetically dominated Earth's surface. As seafloor and subseafloor microbial processes occur in the dark, chemosynthetic microbial processes predominate in these environments. Given the variety of physical and chemical conditions at deep-sea vents, it is not surprising that chemoautotrophs inhabiting these environments exhibit considerable physiological diversity (Nakagawa and Takai, 2008). However, studies concerning the activity of microorganisms from deep-sea hydrothermal vent fields are rare. High-throughput sequencing methods lead to an increase of genomic and metagenomic approaches (Figure 20). Further interdisciplinary studies are necessary to understand the microbial activity and their interaction with each other and with their environment at hydrothermal vent sites.

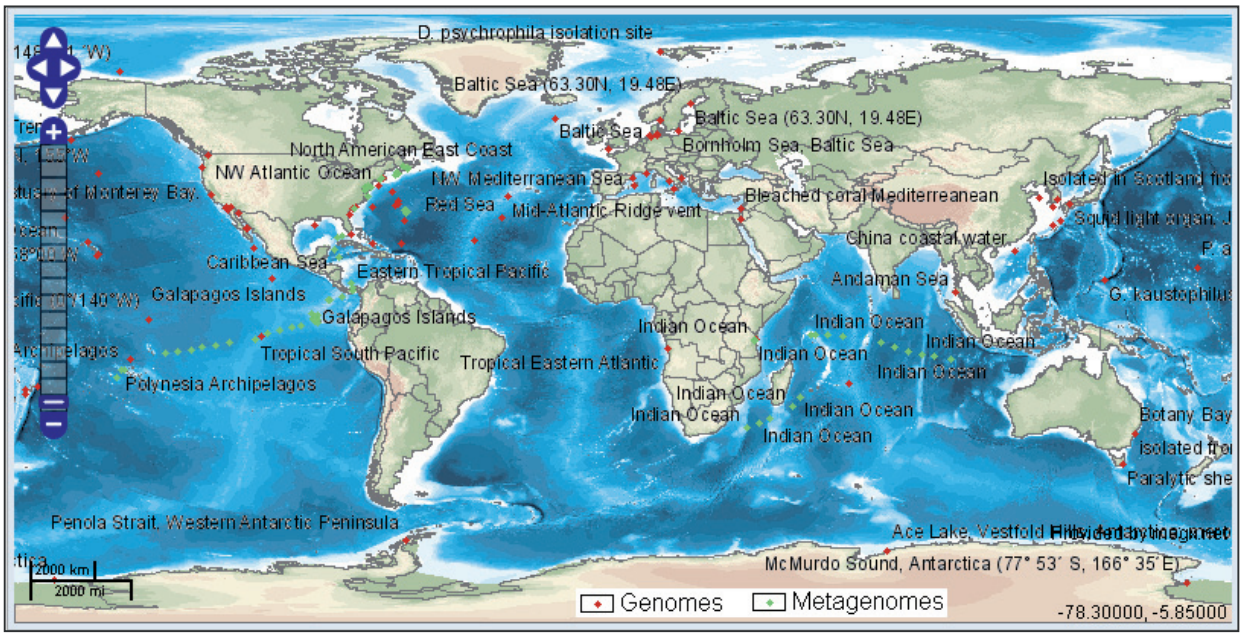

Figure 20: Distribution of metagenomic and genomic studies available with the megx marine ecological genomics (http://www.megx.net) map server. From hydrothermal vent sites 27 prokaryotic genomes are present. In the GOLD database (http://www.genomesonline.org) only 4 metagenomic approaches concerning hydrothermal seafloor are listed.

3.4.1 Basalt- and peridotite-hosted hydrothermal vent fields

In basalt-hosted systems the precipitations of massive mineral deposits primarily consist of metal sulfides (Tivey et al., 1995). The amount of energy available to support microbial growth based on oxidation of minerals (principally pyrite) exceeds what would be available based on the oxidation of aqueous chemicals (principally H_2S) by nearly an order of magnitude per kilogram of vent fluid (McCollom, 2000). First isolates from the Galapagos Rift ocean floor were obtained from microbial mats covering surfaces in the immediate vicinity of the vents (Ruby et al., 1981). Sulfur-oxidizing bacteria of the chemolithotrophic genus *Thiomicrospira* (*Gammaproteobacteria*) supported the hypothesis that sulfur-oxidizing bacteria are the primary producers at vents with sulfide-containing hydrothermal fluids. Since then, sulfur-oxidizing bacteria have been obtained from a variety of different seabed structures from hydrothermal vent fields (Longnecker and Reysenbach, 2001; Taylor et al., 1999; Inagaki et al., 2003; Inagaki et al., 2004).

The sediment-hosted Guaymas Basin supports also massive bacterial mats of autotrophic, sulfur-oxidizing, filamentous *Beggiatoa* spp. (Nelson et al., 1989; Gundersen et al., 1992). High sulfate reduction rates in the surface sediments at pressures representing the *in situ* conditions showed that sulfate reduction represents an important metabolic pathway in these sediments (Elsgaard et al., 1994; Weber and Jørgensen, 2002). Anaerobic oxidation of

methane (AOM) was also detected, but the detected rates were so low that only 1 to 5% of the high SR rates can be fuelled by AOM (Kallmeyer and Boetius, 2004). Therefore, high rates of methane-dependent SR have been found so far only in cold environments (cold seeps, gas hydrates, and mud volcanoes), at *in situ* temperatures of -1.5 to +12°C (Wegener et al., 2008).

The isolation of chemolithoautotrophic Fe-oxidizing bacteria revealed that Fe is also an important energy source at hydrothermal vents (Karl et al., 1989; Edwards et al., 2003; Emerson et al., 2007). Thermodynamic and bioenergetic calculations support Fe-oxidation, Fe-reduction, and sulfide-oxidation as potential energy sources for rock-hosted chemoautotrophs at basalt-hosted fields (Bach and Edwards, 2003). In anoxic environments, such as hot vents, hydrogen oxidation and methanogenesis may also be favored metabolisms as revealed by cultivation studies (Table 5). However, genomic analyses of different epsilonproteobacterial isolates from oxic habitats revealed different sets of genes of hydrogenases. Therefore, hydrogen oxidation could occur in oxic mesophilic habitats (Nakagawa et al., 2007). In particular, peridotite-hosted fields, where hydrogen is the most dominant energy source in vent fluids, could represent a habitat for hydrogen-oxidizing microorganisms. As most studies to date have focused on basalt-hosted systems, little is known about the microbial activity of ultramafic-hosted fields. So far only culture-independent studies revealed the microbial diversity of these systems. To date, cultivation experiments and rate measurements have been crucial in our understanding of the physiological diversity at basalt-hosted deep-sea vents.

3.4.2 Potential metabolic pathways

Metagenomic and genomic studies provided additional information about potential metabolic capabilities of microorganism at hydrothermal vent fields. Genome analyses of recently isolated mesophilic strains of *Epsilonproteobacteria* have led to the detailed characterization of their sulfur-oxidation pathway (Takai et al., 2005; Nagakawa et al., 2007). This strains use the Sox-dependent sulfur-oxidizing pathway consisting of sulfite:cytochrome c oxidoreductase (Sor), Sqr and Sox multienzyme complex (Figure 21). Most of the predicted sox genes formed two spatially separated gene clusters (Nakagawa et al., 2007; Sievert et al., 2008) in comparison to the single sox gene cluster identified for *Paracoccus pantotrophus* (Friedrich et al., 2001). In addition, recent genomic analyses of the gammaproteobacterial endosymbionts of tubeworms, mussels and clams have identified the genes for adenylyl-sulfate reductase (AprAB) and ATP sulfurylase (Sat), indicating that they oxidize sulfite to

sulfate via adenylylphosphosulfate (Nelson and Fisher, 1995). Furthermore additional enzymes are involved in the sulfur oxidation pathway, including the reversible dissimilatory sulfite reductase (Dsr), sulfide:quinone oxidoreductase (Sqr) and sulfur oxidation (Sox) multienzyme complex lacking soxCD (Figure 21; Kuwahara et al., 2007; Markert et al., 2007; Newton et al., 2007). Metagenomic analysis of the sulfide-oxidizing gammaproteobacterial endosymbiont of *Riftia pachyptila* revealed the presence of genes involved in the reductive tricarboxylic acid cycle (rTCA) and in the Calvin cycle (Markert et al., 2007). This indicates that two pathways can be used for CO_2 fixation. Analyses of the metagenome from an epsilonproteobacterial *A. pompejana* episymbiont, whose next relative is the vent-associated, free-living bacterium, *Sulfurovum* sp. NBC37–1 (Grzymski et al., 2008), revealed genes encoding the complete rTCA cycle for carbon fixation, sulfur oxidation, and denitrification. Based on these genomic analyses, it has been hypothesize that the success of *Epsilonproteobacteria* in hydrothermal vent ecosystems is a product of their adaptive capabilities, broad metabolic capacity, and strain variance (Nakagawa et al., 2007; Grzymski et al., 2008).

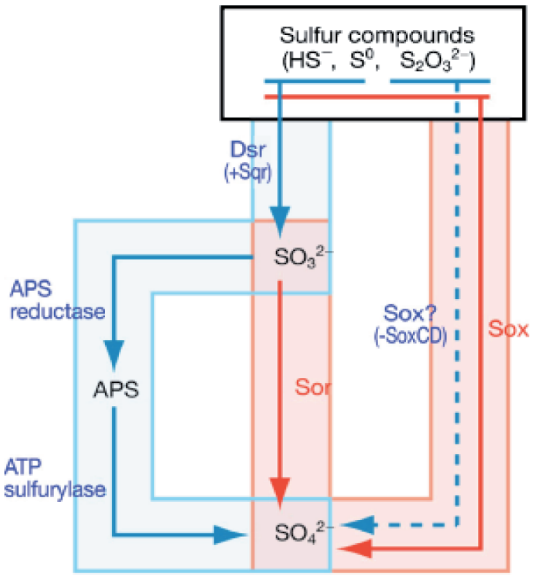

Figure 21: Sox-dependent (shown in red) and Sox-independent (shown in blue) sulfur-oxidation pathways of deep-sea vent chemoautotrophs (Nakagawa and Takai, 2008).

4 Microbial biogeography

Microbial biogeography can be defined as the science that documents the spatial distribution of prokaryotic taxa in the environment at local, regional, and continental scales. In a broader sense, this discipline examines variation of microbial features (e.g., genetic, phenotypic, physiological) at different spatial scales, between distantly located sampling sites or along large environmental gradients (Ramette and Tiedje, 2007). Its scope also encompasses the understanding of the processes generating and maintaining those distribution patterns. The ultimate goals are to propose and evaluate theories regarding the creation and evolution of such diversity patterns in the environment. The current theory of prokaryotic biogeography and diversification proposes two factors to explain those variations (Martiny et al., 2006;

O'Malley, 2008). Contemporary environmental heterogeneity (EH) has long been considered a very powerful factor acting on microbial populations. On the basis of the cosmopolitan hypothesis, 'everything is everywhere, but the environment selects' (Baas-Becking, 1934), environmental conditions have long been considered to have a strong influence on microbial biogeography. Thus, niche-based explanations for the environmental variation in abundance and diversity of microbes have long been the paradigm among microbial ecologists. The effects of historical events on microbial populations have recently been proposed as another factor that structures microbial life (Papke et al., 2003; Whitaker et al., 2003; Ramette and Tiedje, 2007). Spatial distance (SD) may thus be seen as a proxy that represents differential community dynamics, which are themselves related to past historical events and disturbances (e.g. physical barrier, anthropogenic activity, dispersal history, and past heterogeneity) whose legacies have been maintained because of spatial isolation between populations (Borcard and Legendre, 1994).

Although it is now well accepted that both EH and SD may help create and maintain microbial diversity in terrestrial ecosystems, little is known about their relative contribution and interactions on the intraspecific abundance and diversity of microbes (Martiny et al., 2006). To answer this question, information on the current abiotic and biotic conditions and the spatial arrangements of the sample assemblages are necessary. Although the statistical partitioning of the ecological variation has sometimes been undertaken in previous microbial biogeography studies (Whitaker et al., 2003; Horner-Devine, 2004), it is still not clear how much of the microbial variation can be explained when both SD and EH are considered, especially at different levels of taxonomic resolution and at different spatial scales.

4.1 Statistical analyses in biogeographic studies

There are technical and conceptual reasons for the lack of understanding of the scaling of microbial diversity. Technically, it has been challenging to quantify microbial diversity. The development of molecular approaches has enabled a more comprehensive view of microbial diversity. However, a commonly accepted theoretical species concept for microbes is still lacking. This is possibly because all of the current concepts include methodological consideration. Microbes are currently assigned to a common species if their reciprocal, pairwise DNA re-association values are ≥70% in DNA-DNA hybridization experiments. This hybridization is chosen because it yields species consistent with the phenotypic (pragmatic) taxonomy. In addition, microbes with 16S ribosomal RNAs (rRNAs) that are ≤98.7% identical are most ofthen members of different species, because such strong differences in

rRNA correlate with <70% DNA–DNA similarity (Stackebrandt and Ebers, 2006). However, the opposite is not necessarily true, and distinct species have been occasionally described with 16S rRNAs that are >98.7% identical. Most uncultured microbes cannot be assigned to a classical species because we do not know their phenotype. Therefore, other methods, such as multilocus sequencing analysis (MLSA) (Hanage et al., 2006), average nucleotide identity (ANI) of all orthologous genes in complete genome sequences (Konstantinidis and Tietje, 2005; Richter and Rossello-Mora, 2009), and concepts, such as metapopulation lineages (de Queiroz, 2005), genomic-phylogenetic species concept (Staley, 2006) and ecotype-based population-structure (Cohan and Perry, 2007), for defining microbial species have been suggested.

To answer questions concerning whether microbes are cosmopolitan or endemic, in practice, many studies of microbial diversity abandon species definitions entirely. They define instead operational taxonomic units (OTUs) based on the ribosomal gene sequences that do not require cultures. The first step to analyse biogeographic patterns in a microbial community is to estimate the microbial diversity and to detect how well a sample reflects a community's "true" diversity. Measures of microbial diversity include richness (number of species/OTUs) and evenness (relative abundance of OTUs).

Different statistical tools are available to estimate richness of a community from a sample. These estimators fall into three main classes: extrapolation from accumulation curves, parametric estimators, and nonparametric estimators (Hughes et al., 2001). Nonparametric estimators are the most promising for microbial studies. The computer program DOTUR was developed by Schloss et al. (2005), which assigns sequences to OTUs based on the genetic distance between sequences and estimates richness and diversity. This tool provides a community membership and structure comparison based on OTUs. Furthermore, the calculation of rarefaction curves provides the comparison of observed richness among sites, treatments or habitats that have been unequally sampled. A rarefied curve results from averaging randomizations of the observed accumulation curve (Heck et al., 1975). Further statistical tools for comparing the structure of microbial communities describing the abundance of each member include ∫-LIBSHUFF (Schloss et al., 2004), TreeClimber (Schloss and Handelsman, 2006b), Unifrac (Lozupone and Knight, 2005), AMOVA (Martin, 2002) and SONS (Schloss and Handelsman, 2006a).

The detection of differences and similarities of microbial communities between different habitats leads to the understanding of the effect of special scaling and environmental heterogeneity in biodiversity on local and global scale. One approach to test how community

composition changes with geographic distance is the distance-decay relationship (Figure 22A) (Nekola and White, 1999; Green and Bohannan, 2006). When coupled with environmental data, the distance-decay relationship offers a means to assess the relative importance of both the environmental heterogeneity and dispersal history in controlling the spatial scaling of biodiversity. The taxa-area relationship detects the relationship between species richness and sampled area (Figure 22B). Based on biogeographic studies of macroorganisms, it has been suggested that cosmopolitical microorganisms should be characterized by relatively taxa-area curves, with z values (slopes) lower than those reported for macroorganism (0.1 to 0.3 within contiguous habitats, $0.25 < z < 0.35$ for discrete islands) (Rosenzweig, 1995).

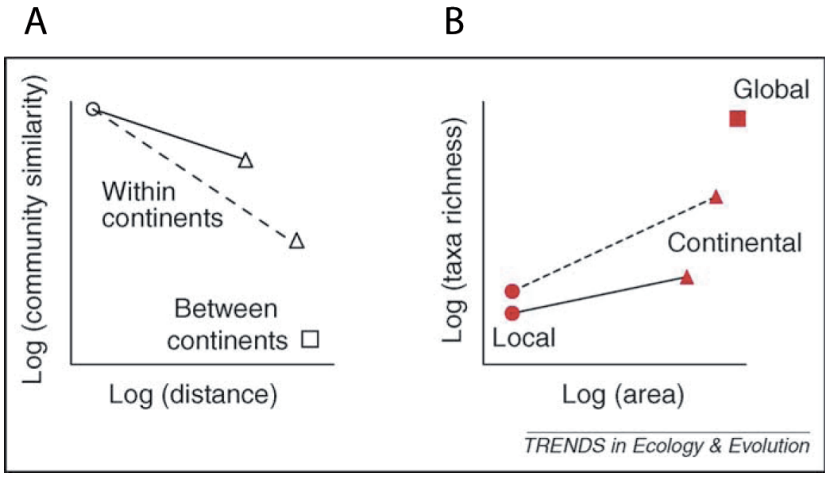

Figure 22: Hypothetical spatial patterns of microbial diversity. (A) The distance-decay relationship within two different continents (solid and dashed lines) and the similarity in community composition between those continents (open square). Community similarity is equal for each continent at local scales (open circles) in the limit where replicate samples are completely censused from the same location. (B) The taxa-area relationships for two continents. A greater rate in community composition turnover results in a steeper taxa-area relationship slope (dashed line). The local:global richness ratio on a given continent is equal to the taxa richness estimated at the local scale (solid circle) divided by the taxa richness estimated at the global scale (solid square) (Green and Bohannan, 2006).

To visualize and compare the different datasets (matrices), different clustering algorithms are used (e.g. non-metric multidimensional scaling (nMDS), principal component analysis (PCA)). The results of this analysis can be displayed as a dendrogram (Kuske et al., 2002) or along dimensionless axes with multidimensional scaling (Yannerell and Triplett, 2005). The significance of the calculated correlation of different matrices is determined by spatial statistics such as the Mantel test and the spatial Mantel test.

4.2 Biogeographic pattern of microbial communities

Studies of biogeographic patterns of free-living deep-sea microbes are so far not available. However, numerous studies in the 1990s began to address the question of whether microbes have endemic or cosmopolitan distribution. To date, only a few studies have so far investigated the relationship between EH and SD. At extreme environments, such as hot spring, endemicity has been uncovered several times amongst hyperthermophilic bacteria and archaea (e.g. Hudson et al., 1989; Petursdottir et al., 2000; Sompong et al., 2005). Martiny and colleagues found only two studies that examined distribution distance in relation to environmental factors (Papke et al., 2003; Whitaker et al., 2003). Both of those papers, as well as a study of high-mountain lake bacteria (Reche et al., 2005), concluded that the organismal distributions they investigated could not be adequately explained by EH, for example hot spring chemistry, and that the most important influence was that of SD. Furthermore, Martiny and colleagues observe that large distances (>3000 km) between populations overwhelm environmental correlations, while at very close distances (<10 km), SD has almost no influence when compared to environmental factors. At in-between spatial scales (10-3000 km), both factors appear to be important (Table 6).

Table 6: Studies of the effects of spatial distance (SD) and environmental heterogeneity (EH) on microbial composition

Organisms	Approximate scale (km)	Habitat	OTU	Effect of		Reference
				SP	EH	
Synechococcus	20,000	Hot springs	16S/ITS sequence	Yes	No	Papke et al., 2003
Sulfolobus	12,000	Hot springs	MLS of isolates	Yes*	No*	Whitaker et al., 2003
Bacteria	3,000	Coral	16S sequence	No	Yes*	Rohwer et al., 2002
Bacteria	500	Lakes	ARISA	Yes*	Yes*	Yannerell and Triplett, 2005
3-CBD bacteria	500	Soil	ARDRA	No	Yes*	Mantel, 1967
Ascomycetes	100	Soil	ARISA	Yes*	Yes*	Green et al., 2004
Bacteria	100	Aquatic	ARISA	No	Yes	Hewson and Fuhrmann, 2004
Bacteria	10	Lakes	DGGE of 16S	Yes*	No*	Reche et al., 2005
Bacteria	0.3	Marsh sediment	16S sequence	No*	Yes*	Horner-Devine et al., 2004
Bacteria	0.1	Soil	TRFLP	No	Yes*	Kuske et al., 2002

The studies are ordered by the geographical scale over which the samples were taken, reported as the approximate furthest distance between sampling points. *The effect was tested for statistical significance. 3-CBD, 3-chlorobenzoate-degrading; ARDRA, amplified ribosomal DNA restriction analysis; ARISA, automated ribosomal intergenic spacer analysis; DGGE, denaturing gradient gel electrophoresis; ITS, intergenic transcribed space; MLS, multilocus sequencing; OTU, operational taxonomic unit used in the study; TRFLP, terminal RFLP.

5 Aims of this study

Within this thesis, the diversity, function and biogeographic patterns of microbial communities from different deep-sea sediment were investigated. This includes sediments from the peridotite-hosted hydrothermal vent field on the Mid-Atlantic Ridge (Logatchev) and from three permanently cold deep-sea basins in the South Atlantic Ocean.

Most investigations of microbial communities of hydrothermal systems focused on basalt-hosted vent fields, as peridotite-hosted systems were discovered around 20 years later. Nevertheless, microbial diversity of hydrothermally influenced sediments was so far only known from the coastal near basalt-hosted hydrothermal field of the Guaymas Basin (Gulf of California section of the EPR) (Teske et al., 2002). Studies of the microbial diversity in sediments from a peridotite-hosted system were so far limited to a single study of the Rainbow hydrothermal vent field (Nercessian et al., 2005). However, these sediments do not represent hydrothermally influenced habitats. Within this thesis, in the framework of the German Research Foundation's priority program on hydrothermal vents (SPP 1144 'From Mantle to Ocean: Energy-, Material-, and Life-cycles at Spreading Axes'), different sediments within a peridotite-hosted hydrothermal vent field were investigated.

The first goal of this thesis was to reveal the diversity and abundance of deep-sea microbial communities in hydrothermally influenced sediments by 16S rRNA gene analyses and fluorescence *in situ* hybridization techniques. The detected microbial diversity should be compared with the microbial diversity of non-hydrothermally influenced deep-sea surface sediments to identify vent specific microbial groups. Furthermore the comparison with detected microbial diversity of basalt-hosted systems should reveal differences in the microbial composition due to different fluid compositions.

Another objective of this thesis was to link phylogenetic information with biogeochemical processes, and the function of certain microbial groups. The potential and present activity of free-living microorganisms at an ultramafic-hosted hydrothermal system should be determined, focusing on reduced sulfur compounds, methane and hydrogen, as the hypothesized driving energy sources for chemosynthetic life in this vent system. *In situ* techniques should be applied to determine environmental parameters. Moreover, *ex situ* microbial turnover rate measurements should be conducted to evaluate the importance of hydrogen in comparison to sulfide in a peridotite-hosted field. The diversity of key genes of relevant chemosynthetic pathways should be determined. Metagenome analysis should be applied to determine the genetic context of these key genes, and to reveal more information on the genetic capabilities of the key microbial groups.

The third project within this thesis was the investigation of biogeographic pattern of deep-sea floor microbial communities. The effect of environmental heterogeneity and spatial distance should be examined by testing the correlation between microbial diversity, environmental conditions and spatial distance. For small scale, the detected microbial diversity of different sediment of the Logatchev hydrothermal vent field should be used. For large distances, the microbial diversity of three deep-sea sediment separated by at most ~3500 km were investigated. As spatial distribution and the factors affecting it are unknown for deep-sea microbial communities, the investigation of different deep-sea habitats as well as at different scales will help to answer the question which factors create and maintain microbial diversity.

II List of Publications

Contributions to the publications and manuscripts presented in this thesis:

1. **Regina Schauer, Hans Røy, Nico Augustin, Hans-Hermann Gennerich, Marc Peters, Frank Wenzhöfer, Rudolf Amann, and Anke Meyerdierks.** The bacterial sulfur cycle in surface sediments of an ultramafic hydrothermal vent field.
   Manuscript is under revision for publication in *Environmental Microbiology*.
   R.S.: developed the concept, did the 16S rRNA sequencing and analyses, performed FISH and CARD-FISH experiments, determined the sulfate-reduction and AOM rates, performed sampling during the cruise MSM04-3, conceived and wrote the manuscript H.R.: developed the concept, provided in situ microprofiler data, calculated oxygen consumption rates N.A.: determined the mineral composition of the surface layer H-H.G.: provided temperature data M.P.: did isotopic measurements R.A.: developed the concept, conceived and edited the manuscript A.M.: developed the concept, performed sampling during the cruise M64-2, conceived and edited the manuscript

2. **Regina Schauer, Hanno Teeling, Sixing Huang, Chai-I Huang, Sven Klages, Richard Reinhardt, Frank-Oliver Glöckner, Rudolf Amann, and Anke Meyerdierks.** Metagenomic and functional analysis of the importance of sulfide as an energy source for primary production in an ultramafic hydrothermal vent field.
   Manuscript in preparation.
   R.S.: developed the concept, amplified and sequenced all functional genes, did the phylogeny of aprA- and soxB-genes, did fosmid library construction, screening and sequencing, extracted DNA for pyrosequencing, analysis of the metagenomic dataset, conceived and wrote the manuscript H.T.: developed the concept, helped with the analysis of the metagenomic dataset S.H.: developed and applied the tool PAOLA C-I.H.: sampling during the cruise MSM10-3, incubation experiments S.K.; R.R.: did the pyrosequencing F.O.G.: analyzed 16S and 23S rRNA gene sequences of the pyrosequencing dataset R.A.: developed the concept, conceived and edited the manuscript A.M.: developed the concept, conceived and edited the manuscript

3. **Regina Schauer, Christina Bienhold, Alban Ramette, and Jens Harder.** (2010) Bacterial diversity and biogeography in deep-sea sediments of the South Atlantic Ocean.

   The ISME Journal **4:**159-70.

   R.S.: developed the concept, performed sampling during the cruise M63-2, did the 16S rRNA sequencing and its statistical analysis, conceived and wrote the manuscript C.B.: performed the T-RFLP experiments and its statistical analysis A.R.: helped with the statistical analysis J.H.: developed the concept, conceived and edited the manuscript

1 The bacterial sulfur cycle in surface sediments of an ultramafic hydrothermal vent field

The bacterial sulfur cycle in surface sediments of an ultramafic hydrothermal vent field

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Running title: bacterial sulfur cycle in ultramafic sediments
Keywords: bacterial diversity, deep sea, diffusive transport, Logatchev, sulfur isotopes, microprofiles

Summary

Surface sediments at two sites of the ultramafic Logatchev hydrothermal vent field were found to be covered with white mats reminiscent of those found on sulfidic sediments at basalt-hosted fields. To study the importance of sulfide as an energy source fueling microbial communities in ultramafic vent fields, we conducted an interdisciplinary study. Geochemical analyses detected metal-sulfides in the sediments, elemental sulfur in mats and an intensive sulfide flux from below. Temperature profiling of the two sulfur-mat sites revealed conductive heating of sediments by underlying hydrothermal fluids that differed from known advective heating. High oxygen consumption rates were found, indicating high biomass production coupled to high sulfate reduction rates. Comparative 16S rRNA gene sequence analyses identified various bacteria related to those found in basaltic systems. However, fluorescence *in situ* hybridization revealed that the overlying sulfur-mats were dominated by filamentous *Epsilonproteobacteria* or a vibrioid *Arcobacter* type instead of mat-forming *Beggiatoa* which dominate similar mats at basaltic systems. The analyses of the surface sediment confirmed high abundances of *Epsilonproteobacteria* (7-21%), *Deltaproteobacteria* (20-21%), and *Bacteroidetes* (19-20%). We propose that sulfur cycling is one of the driving forces for primary production and biomass formation in sediments of ultramafic hydrothermal vent fields.

Introduction

Deep-sea hydrothermal vents are highly productive ecosystems with symbiotic and free-living chemolithoautotrophic microorganisms as primary producers (Jannasch and Wirsen, 1979). The hydrothermal systems discovered to date are either hosted by ultramafic components or basalt. These geological differences lead to different fluid compositions (Tivey, 2007). In basalt-hosted systems, sulfide oxidation as well as iron oxidation and reduction are the principal energy sources for microbial ecosystems (Bach and Edwards, 2003). In ultramafic systems, peridotite-seawater reactions result in fluids which are in addition to sulfide and reduced metals also enriched in methane and hydrogen (Wetzel and Shock, 2000; Schmidt et al., 2007). Accordingly, biofilms of methane-metabolizing *Archaea* were identified on high-temperature carbonate chimneys (60-100°C) in the ultramafic rock-hosted Lost City hydrothermal vent field at the Mid Atlantic Ridge (Kelley et al., 2005; Brazelton et al., 2006). Further known ultramafic vent systems on the Mid-Atlantic Ridge are Logatchev (15°N) and Rainbow (36°N). Both systems are characterized by high temperature fluids of >300°C and a low pH (Schmidt et al., 2007). Besides smoking craters and chimney complexes, these two

sites are of special interest as thick sediment layers were found on top of the ultramafic rocks (Nercessian et al., 2005; Petersen et al., 2009). These layers likely result from a combination of tectonic activity due to rifting processes (Gao 2006), low-temperature water/rock interaction (Daczko et al., 2005), and high-temperature hydrothermal alteration.

Large patches of the sediments at Logatchev were found to be covered by white mats (Lackschewitz and Shipboard Scientific Party 2005) similar to those reported from basaltic hydrothermal systems and cold seeps (Omoregie et al., 2008; Teske at al., 2002). Previously reported white mats consist of sulfide-oxidizing bacteria, which store or excrete elemental sulfur or of amorphous silica precipitates (Wenzhöfer et al., 2000). Whitish microbial mats at Galapagos (Jannasch and Wirsen, 1981) and Guaymas Basin (Gundersen et al., 1992) contained *Beggiatoa*-like and *Thiothrix*-like filaments. Furthermore, *Epsilonproteobacteria* represented a major group at the southern East Pacific Rise (Longnecker and Reysenbach, 2001), Loihi Seamount (Moyer et al., 1995) and the Guaymas Basin (Teske at al., 2002). The epsilonproteobacterial genera *Sulfurimonas* (Group B) and *Sulfurovum* (Group F) were recently identified in *in situ* colonization experiments at the East Pacific Rise (EPR 13°N, Alain et al., 2002) and at the Mid-Atlantic Ridge (MAR 23°22' N, Reysenbach et al., 2000; MAR 23°22' N, Corre et al., 2001; MAR 37°17' N, Lopez-Garcia et al., 2003). Microbial mats are therefore well characterized in basaltic systems. Microbial communities in such white mats at ultramafic-hosted systems have not yet been investigated, despite of the radical difference in fluid chemistry.

To investigate microbial communities in white mats at the ultramafic-hosted Logatchev hydrothermal vent field, four sites were sampled during two research cruises (Figure 1). Two of these sites, “F” and Anya’s Garden, exhibited white mats on top of the sediments. We determined geochemical parameters, microbial turnover rates, and investigated the microbial communities in all four sediment surface layers (0-1 cm) as well as in the overlying white mats when present. Biogeochemical and molecular analyses revealed that sulfide has the most important influence on microbial communities in the surface sediments although ultramafic fluids are typically enriched in methane and hydrogen.

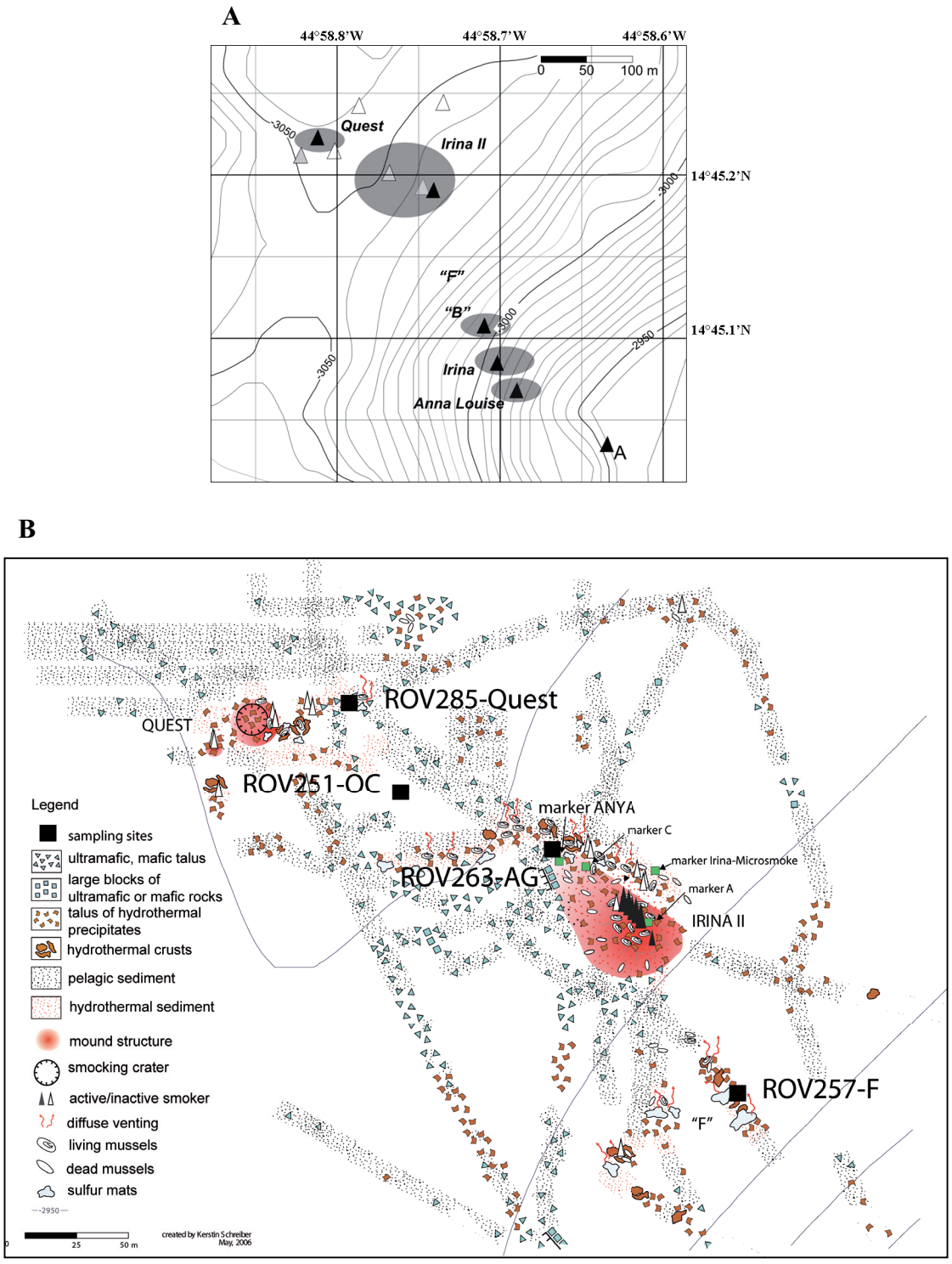

Figure 1: (A) Active black smoker and diffuse venting sites at the Logatchev hydrothermal vent field and (B) a more detailed map of the sampling sites in this area (Lackschewitz and Shipboard Scientific Party 2005).

Results

Geochemistry

Sediments from four sites within the Logatchev area were analyzed (Table 1, Figure 2). Two mat-covered sediments were taken at site F and Anya's Garden (AG), respectively. These were compared with one mat-free sediment sample from site Quest within the vent field. The fourth sediment was sampled 25 m outside of the hydrothermally influenced area (oceanic sediments) for a comparison with the hydrothermal sediments.

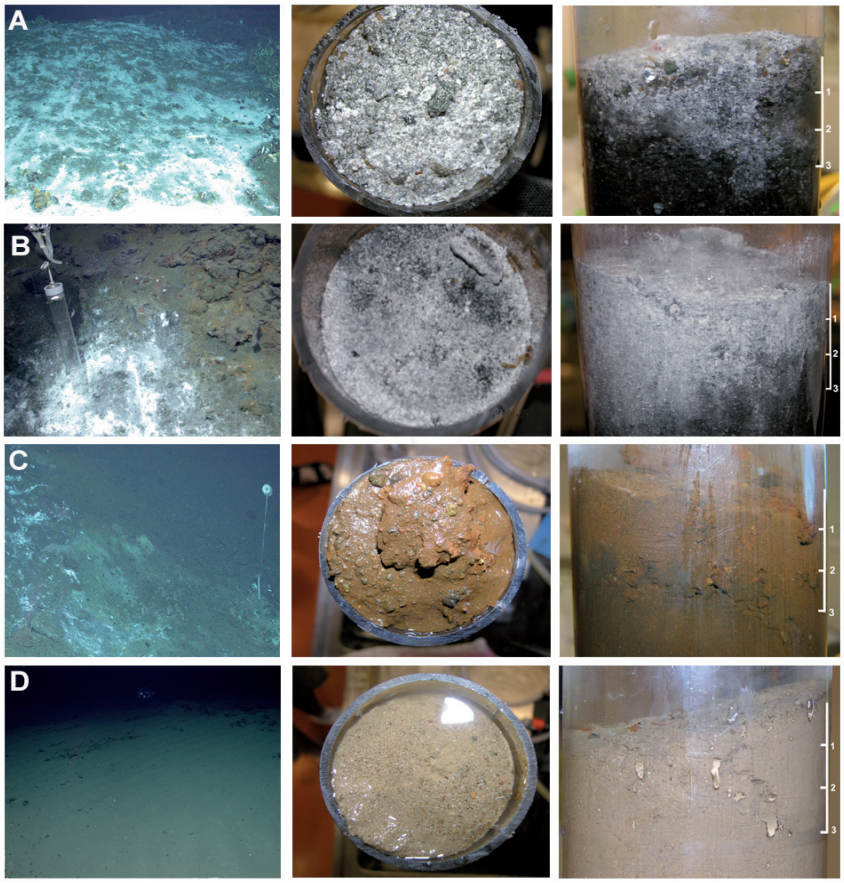

Figure 2: Different sediments from the Logatchev hydrothermal vent field at the Mid-Atlantic Ridge. Photographs of the sampling site (left), surfaces of recovered sediment cores (diameter = 6.35 cm) (middle), and side view of the first 0-3 cm of the sediment cores (left). (A) Sediments from site F, (B) Anya's Garden, (C) site Quest, and (D) oceanic sediment.

Mineralogical analyses of the surface layers (0-1 cm) of sediment cores taken at site F, AG and Quest revealed a relatively high diversity in primary and secondary silicate minerals as well as abundant oxides and sulfides (Table 1). The mineralogy of the white-mat sediments at site F and AG was similar. Both contained hydrothermally generated iron- and copper-sulfides and either plagioclase or chlorite, suggesting mainly altered mafic material (gabbro) in these samples. The presence of elemental sulfur on top of the AG sediment was remarkable

Table 1: Location of the sampling sites and geochemical data of the sediments.

Site	Latitude [N/S]	Longitude [E/W]	Water depth [m]		Sediment mineralogy	Temp. [cm]	Temp. [°C]	S-Fract. [µmol/g]	δ^{34}S-Isotopes [‰]
F	14°45.134 N	44°58.731 W	3000	xxx :	plagioclase*	0	2.3	1015	-15.7
				xx :	pyrite^{-}	36	100		(± 0.011)
				x :	marcasite^{-}, hematite^{+}, corrensite*, smectite group*, quartz*, hornblende*				
Anya's Garden	14°45.174 N	44°58.768 W	3038	xx :	sulfur, sphalerite^{-}, pyrite^{-}, chlorite group*, calcite$^{\#}$	0	2.4	1060	-7.2
				x :	chalcopyrite^{-}, marcasite^{-}, wurtzite^{-}, talc*, chrysotile*	24	64.8		(± 0.092)
Quest	14°45.179 N	44°58.833 W	3224	xxx :	talc*	0	2.4	125.7	-0.6
				xx :	wurztzite^{-}	22	7.5		(± 0.160)
				x :	hematite^{+} , chrysotile*, lizardite*				
oceanic sediment	14°45.181 N	44°58.767 W	3034	xxx :	calcite$^{\#}$	0	2.4	7.6	+4.1
				x :	quarz*	24	4.8		(± 0.081)

xxx = abundant, xx = common, x = less
mineral types: silicate*, oxide^{+}, sulfide^{-}, carbonate$^{\#}$

as the Logatchev hydrothermal vent (LHV) field is metal-saturated (Augustin et al., 2008). The white surface material at site F could not be sufficiently separated from the rest of the sediment for mineralogical analyses. In contrast to these two surface sediments, the mineralogy of the Quest sediments reflected altered ultramafic material. Talc, a typical alteration product of serpentine minerals (lizardite, chrysotile) was abundant as well as hematite. The oceanic sediment contained carbonate and quartz minerals, indicating a pelagic origin of this sediment. Temperature measurements revealed a steep temperature gradient of 270-277°C m^{-1} in the sulfur-mat sediments at site F and AG (Figure 3A, and B). This gradient was tenfold steeper than at Quest and in the oceanic sediment (20-34°C m^{-1}). The temperatures increased linearly with depth, indicating conductive heat transfer rather than venting of hot fluids. Radial temperature profiles from the center to the outside of the white mats at site F and AG revealed that the mats coincided with hotspots below.

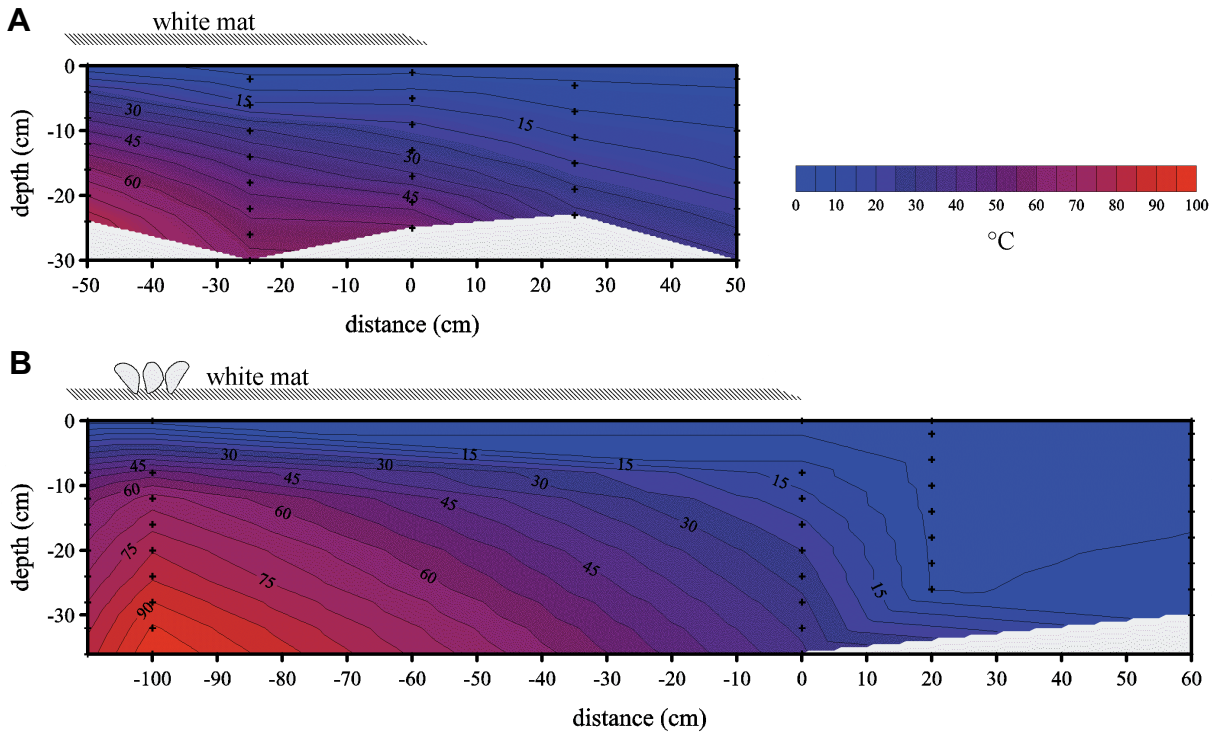

Figure 3: (A) Temperature profiles (°C) in sediments covered by sulfur-mats at Anya's Garden and (B) site F, showing a temperature decrease from the center of the sulfur-mats to their outside.

The sulfur isotope analysis of the surface layer at site F revealed a $\delta^{34}S$ value of -15.7‰ (Table 1). This large sulfur isotope fractionation of dissolved seawater sulfate (+20‰; ±0.12‰; Longinelli 1989) showed that the sulfide had a biogenic origin due to intense microbially-mediated sulfate reduction. The heavier sulfur isotopic composition for sulfide

from AG and Quest (-0.6 to -7.2‰) revealed a lower amount of biogenic sulfide in the sediments but indicated as well a microbial influence. In contrast, the sulfur isotope composition of the oceanic sediment indicates an inorganic sulfur source. The isotopic composition reflects the isotopic composition of dissolved sulfide from hot vent fluids at the Logatchev field (+0.2 and +8.8‰) reflecting the mixing process between leached mantle sulfur and thermochemically reduced seawater sulfate (Peters et al., 2009).

Sulfide-profiles of AG showed that sulfide was diffusing from deeper sediment layers (Figure 4B). Sulfide was depleted approximately 2 cm below the sediment-water interface. The concentration of reduced inorganic sulfur compounds (pyrite, elemental sulfur, acidic volatile monosulfides) in the surface layer was comparable for site F (1,015 µmol/g) and AG (1,060 µmol/g) (Table 1). Corresponding values were much lower for the Quest (125.7 µmol/g) and oceanic sediments (7.6 µmol/g).

Fluxes and rates

Oxygen fluxes measured with microsensors at site F and AG were 30 mmol O_2 m^{-2} day^{-1}, similar to those in eutrophic coastal, estuarine marine (Jørgensen, 2001) and cold seeps sediments (Niemann et al., 2006) (Figure 3). Oxygen consumption rates in the oceanic sediment were below the detection limit in benthic chamber experiments (Røy et al., unpublished).

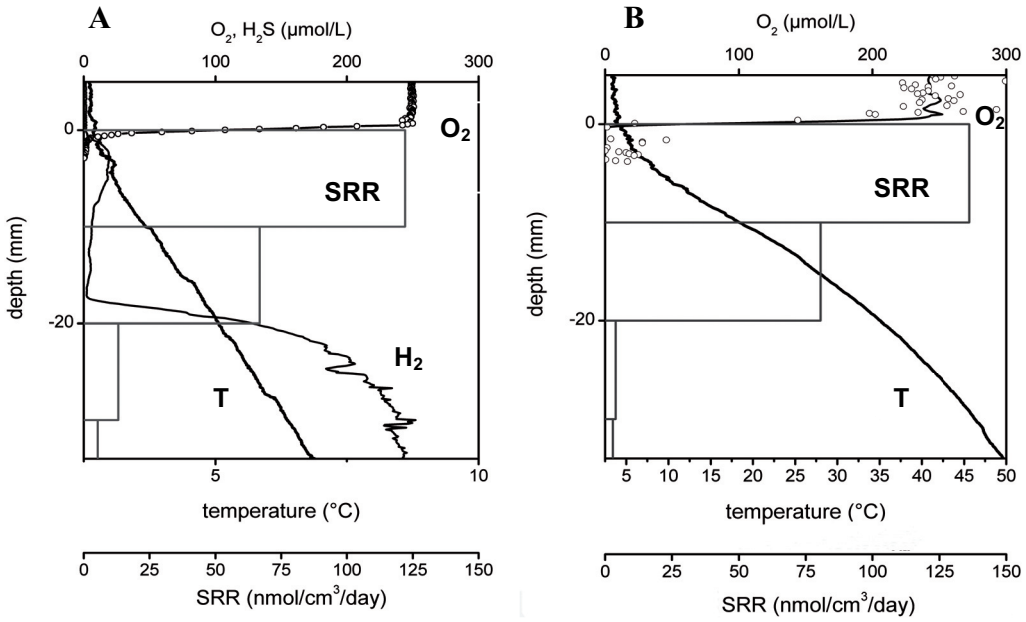

Figure 4: (A) Biogeochemical microprofiles for sulfur-mat sediments from Anya's Garden and (B) site F, showing temperature (T), oxygen (circles), and sulfide profiles (only for B) as well as sulfate reduction rates measured by ^{35}S-technique in the top three centimeters.

Maximum sulfate reduction rates (SRRs) were determined for the surface layer (0-1 cm) (AG = 122 nmol cm^{-3} day^{-1}, site F = 136 nmol cm^{-3} day^{-1}) and decreased to less than 1 nmol cm^{-3} day^{-1} at a depth of 5 cm (Figure 3). In contrast, SRRs were not detectable at Quest. Anaerobic oxidations of methane (AOM) rates were low throughout the whole sediment cores, with a maximum at the surface layer at site AG (11 nmol cm^{-3} day^{-1}) and in a sediment depth of 0-3 cm at Quest (13-11 nmol cm^{-3} day^{-1}). These rates are comparable to those measured in the sulfate methane transition zones in coastal and margin sediments (Knittel and Boetius, 2009). SRRs were tenfold higher than AOM rates in site F and AG surface sediments.

Microbial diversity

Comparative analysis of samples taken in 2005 and 2007 by denaturing gradient gel electrophoresis (DGGE) showed that the hydrothermally influenced sediments from AG, site F, and Quest were stable over a period of two years (Figure 5A). At site F, different patterns were observed for the 0-2 cm layer and deeper layers, showing a shift in the microbial community (Figure 5B).

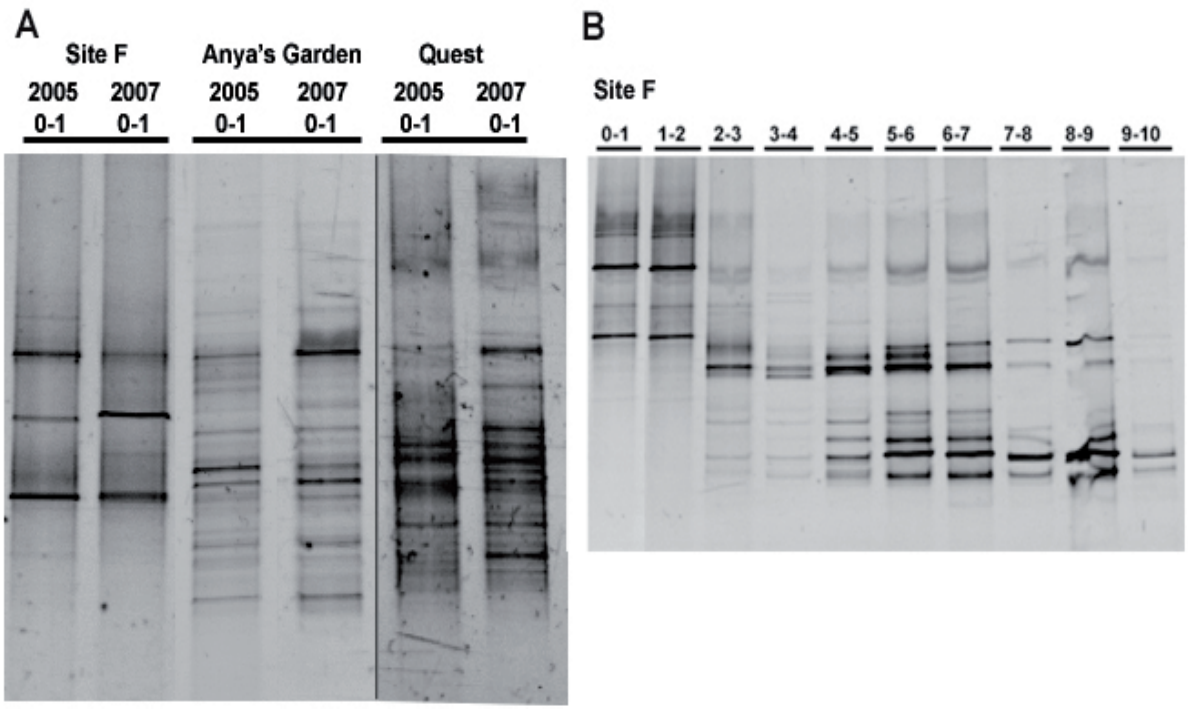

Figure 5: (A) DGGE fingerprints of PCR-amplified bacterial 16S rRNA sequences from the surface sediments (0-1 cm) of site F, Anya's Garden and Quest in 2005 and 2007. (B) Depth profiles of site F sediment to a depth of 10 cm from 2007.

Table 2: Cell and CARD-FISH counts, number of 16S rRNA gene sequences, and estimated Chao1 richness for all four sediments.

Site	Cell counts [cell/g]	CARD-FISH [%]		No. of 16S rRNA gene sequences[b]		Richness estimator Chao1[c]	
		EUB338 I-III[a]	ARCH 915	bacterial	archaeal	Bacteria	Archaea
F	4.2×10^9	75	6	111 74 F/37 P	52 46 F/6 P	173 (115, 301)	64 (35, 165)
Anya's Garden	2.9×10^9	78	2	137 84 F/53 P	13 13 F/0 P	107 (88, 147)	10 (8, 20)
Quest	3.6×10^8	69	4	93 62 F/31 P	84 56 F/28 P	102 (74, 170)	13 (11,27)
Oceanic sediment	6.8×10^7	70	8	154 78 F/76 P	81 55 F/26 P	139 (117, 181)	14 (13, 21)

[a] equimolar mixture of probes EUB338, EUB338-II, and EUB338–III covering about 90% of all members of *Bacteria* (Amann and Fuchs, 2008)
[b] total numbers of sequences as well as number of full-length (F) and partial (P) sequences
[c] Chao1 richness with lower and upper bound of 95% confidence interval

In this study, the microbial diversity in the surface layers was further examined by comparative 16S rRNA sequence analysis. All four sites revealed an even bacterial richness (Table 2). *Proteobacteria* represented the largest group of sequences in all bacterial libraries (59% to 71%) (Figure 8). Sequences related to *Epsilonproteobacteria* were exclusively found in the surface sediments from AG and site F (21% and 8% of all sequences, respectively). Most epsilonproteobacterial sequences from AG (15%) affiliated with the genus *Sulfurovum* (Group F) (Inagaki et al., 2004) (Figure 6). There was one particular cluster of 19 sequences that was most closely related to environmental sequences retrieved from hydrothermal sediment from the Guaymas Basin (Teske et al., 2002). Most of the epsilonproteobacterial sequenes from Site F were related to symbionts affiliated to the genera *Sulfurimonas* (Group B) and *Sulfurovum* (Group F). Sequences related to the ectosymbiont of *Rimicaris exoculata* from South MAR (Petersen et al., 2009) were retrieved exclusively from site F, while sequences from site F and AG were related to the endosymbiont of *Alviniconcha* sp. Type 2 (Suzuki et al., 2006). These symbionts-affiliated sequences suggest that sulfur-mat sediments could provide a niche for the free-living forms of symbiotic bacteria. Other sequences recovered from both sites were affiliated with sequences retrieved from a sulfur spring (Milk Lake, Tawain) or to filamentous bacteria on a *in situ* colonizer (Rainbow, Lopez-Garcia et al., 2003).

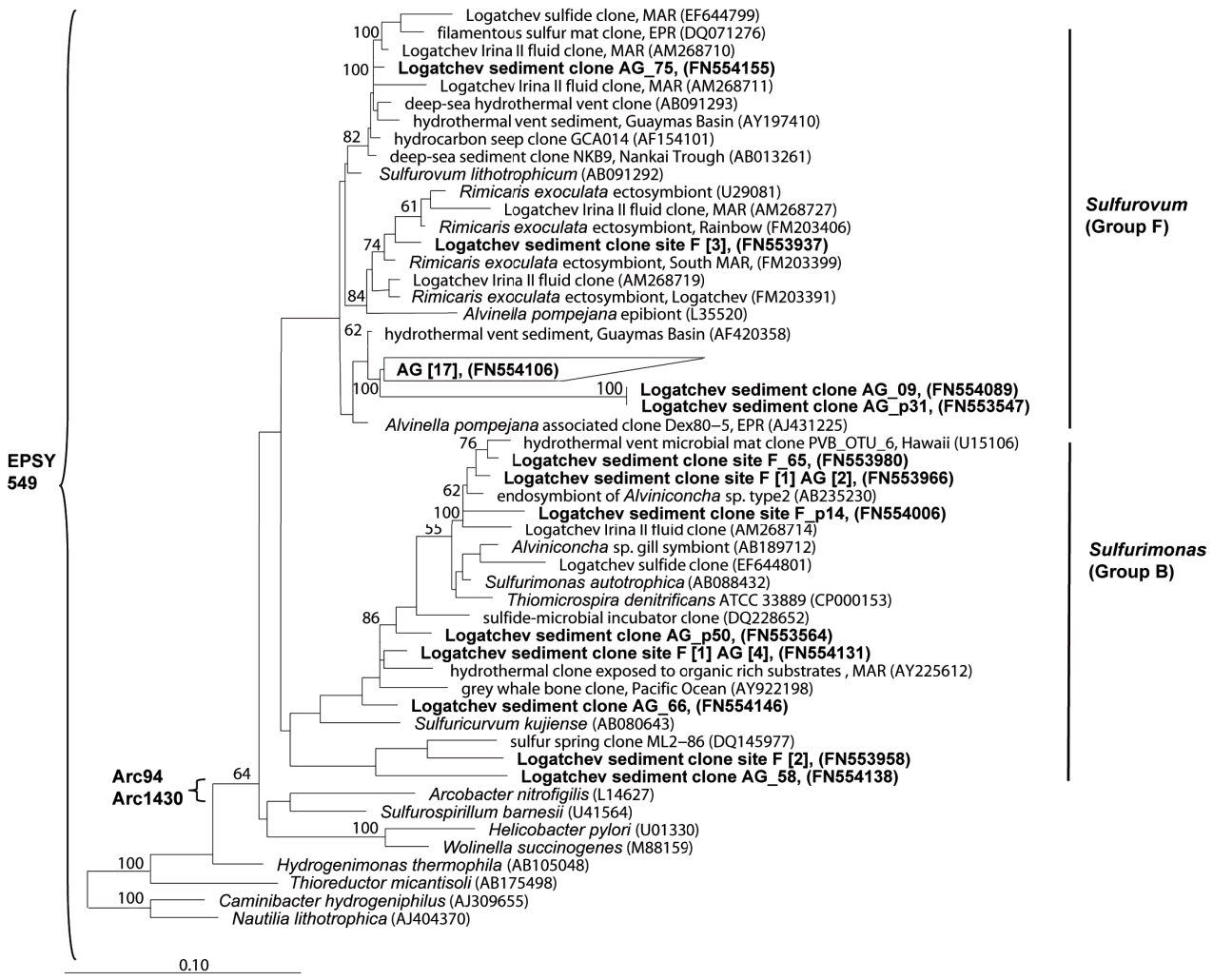

Figure 6: Phylogenetic tree showing the affiliation of 16S rRNA gene sequences from *Epsilonproteobacteria*. The tree was calculated by maximum-likelihood analyses with bootstrap values at the nodes showing percentages based on 100 replicates. Sequences obtained in this study are indicated by bold type, and the number of sequences with 97% identity is shown in squared brackets. The bar represents 10% estimated sequence divergence. Sequences that are targeted by EPSY549 or Arc94, Arc1430 probes are indicated.

A second major group of sequences from site F and AG libraries was related to *Deltaproteobacteria* (16% and 19% of all sequences, respectively). All of the AG sequences and most of the sequences from site F were related to the sulfate-reducing families *Desulfobulbaceae* and *Desulfobacteraceae* (Figure 7). Similar sequences were obtained from a mat-covered mud volcano (Omoregie et al., 2008), gas hydrate sediment (Mills et al., 2005) and hydrothermal sediment from the Guaymas Basin (Teske et al., 2002). Further sequences from site F were related to *Desulfuromonadaceae*. Members of this family are capable of sulfur and Fe(III) reduction (Pfennig and Biebl, 1976; Roden and Lovley, 1993). In contrast, sequences from the Quest and oceanic sediment clone libraries were mainly affiliated with *Nitrospinaceae* and *Bdellovibrionaceae* as well as with 16S rRNA sequences that originated from other deep-sea or marine sediments.

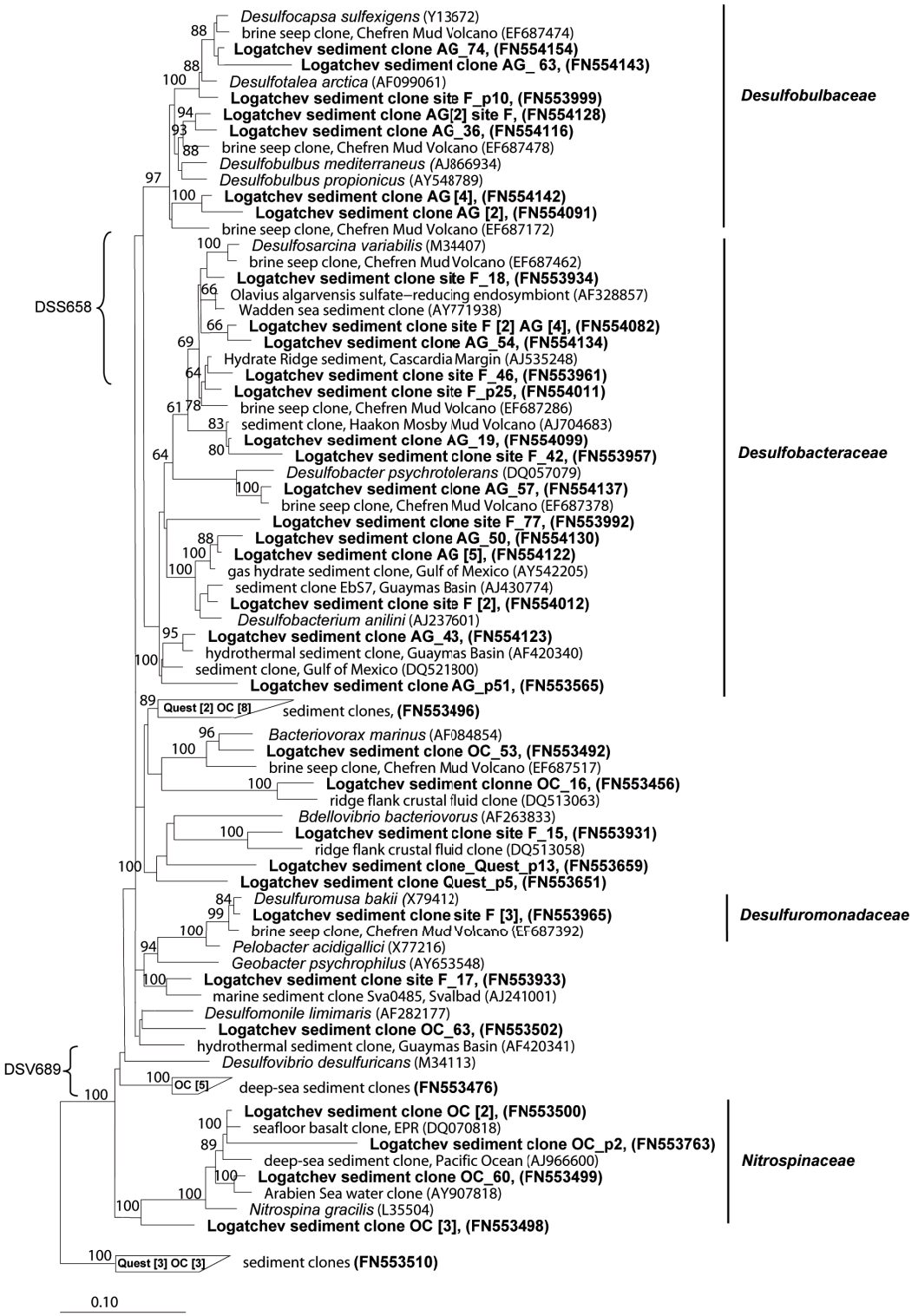

Figure 7: Phylogenetic tree showing the affiliation of 16S rRNA gene sequences from *Deltaproteobacteria.* The tree was calculated by maximum-likelihood analysis with bootstrap values at the nodes showing percentages based on 100 replicates. Sequences obtained in this study are indicated by bold type, their number of sequences with 97% identity is shown in squared brackets. The bar represents 10% estimated sequence divergence. Sequences that are targeted by the DSS658 or the DSV689 probes are indicated.

Most of the sequences from the oceanic sediment site (40%), Quest (60%) and site F (37%) libraries and a major group of sequences from the AG library (19%) belonged to the *Gammaproteobacteria*. The largest number of sequences of the Quest (49%) and oceanic sediment (35%) libraries clustered with members of the group JTB255/BD3-6, which includes sequences of different deep-sea sediments (Schauer et al., 2009), and permanently cold marine sediments (Ravenschlag et al., 1999). In contrast, most of the *Gammaproteobacteria*-related sequences from site F and AG clustered with lineages among the sulfur-oxidizing members of the orders *Chromatiales, Thiotrichales*, to methanotrophic gill symbionts (Fujiwara and Uematsu, 2002), and with the aerobic, anoxygenic, phototrophic NOR5/OM60 clade (Fuchs et al., 2007).

Bacteroidetes-related sequences from site F (17%) and AG (13%) clustered with sequences associated with hydrothermal vent animals (Alain et al., 2002; Goffredi et al., 2004), Guaymas Basin hydrothermal sediments (Phelps et al., 1998), and cold-seep sediments (Li et al., 1999). These sequences of *Bacteroidetes* were often found in environments with high abundance of sulfide-oxidizing or sulfate-reducing bacteria (Macalady et al., 2006).

The archaeal sequences from site F and AG differ as well from those retrieved from Quest and oceanic sediments (Figure 4). Most of the site F and AG sequences were related to *Euryarchaeota* (51.9% and 92.3 %, respectively), while 89 to 100% of all sequences from Quest and oceanic sediment were related to *Crenarchaeota*. Site F and AG contained mainly sequences affiliated with anaerobic methane oxidizers (ANME-1; 21.2%, 30.7%, respectively), and with the Marine Benthic Group D, which is frequently detected in habitats where AOM occurs (Knittel et al., 2005). Almost all sequences derived from Quest and oceanic sediment affiliated with the crenarchaeotal Marine Group I, the most abundant and widely distributed group in the ocean (Takai et al., 2004).

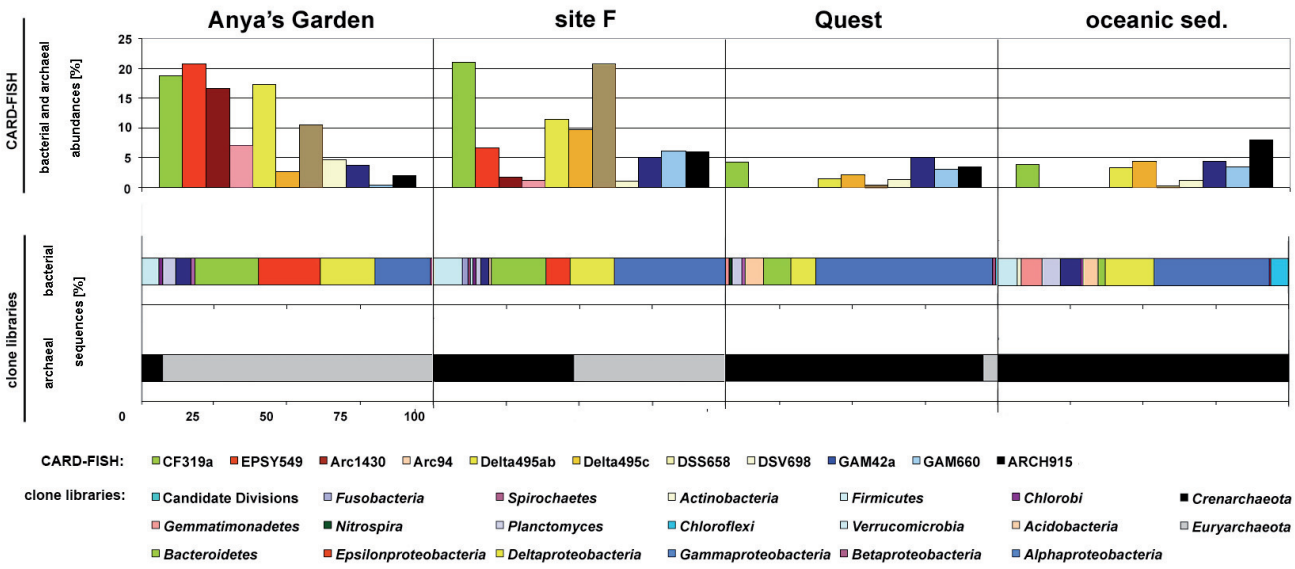

Figure 8: Bacterial and archaeal abundances (CARD-FISH) and 16S rRNA gene based diversities (clone libraries) in the four different sediments of the Logatchev hydrothermal vent field.

Microbial community structure

The highest total cell numbers were detected in the white-mat sediments (0-1 cm) (site F=4.2×10^9 cells/g, AG=2.9×10^9 cells/g), and the lowest in the oceanic sediment (6.8×10^7 cells/g) (Table 2). Catalyzed reporter deposition-fluorescence *in situ* hybridization (CARD-FISH) revealed that all four sites were dominated by *Bacteria* (69 to 76% of total cells), while *Archaea* accounted for less than 8% of total cells (Table 2). *Epsilonproteobacteria* were only detected at site F and AG where they constituted 7% and 21% of the microbial communities, respectively (Figure 8). The probes Arc1430- and Arc94, which are specific for the genus *Arcobacter*, targeted 2% and 1% at site F and 16% and 7% at AG, respectively (Table 3). *Deltaproteobacteria* were more abundant in site F (21%) and AG (20%) surface sediments compared to Quest (4%) and oceanic sediments (8%) (combined counts of probe Delta495_ab and Delta495_c). Cells targeted by probe DSS658 made up 21% at site F and 11% at AG, indicating that sulfate-reducing bacteria of the *Desulfosarcina/Desulfococcus* clade were highly abundant at these sites. The relative abundance of GAM42a-hybridized cells ranged from 4-5% at all sites. Potentially sulfide-oxidizing *Gammaproteobacteria* targeted by probe GAM660 were most abundant at site F (6%). *Bacteroidetes* detected by probe CF319a, accounted for 21% at site F and 19% at AG of the microbial community. They are ubiquitous degraders of polymers in the marine environment. *Bacteroidetes* were highly abundant in white-mat sediments and less abundant at Quest (4%) and in the oceanic sediment (4%). In summary, microbial communities from mat-covered sediments were dominated by *Deltaproteobacteria*, *Epsilonproteobacteria* and *Bacteroidetes* (site F: 48%, AG: 60%), while *Gammaproteobacteria* and *Deltaproteobacteria* were most abundant at Quest (9%) and in the oceanic sediment communities (12%). As all probes used detected only 16-24% of the total community at site Quest and in the oceanic sediment, the dominance of other groups cannot be excluded. Microscopic examination of the overlying white-mat at site F and AG showed that filamentous bacteria were present on top of site F but not of AG sediments (Figure 9A). The white material on top of both sediments was further investigated by FISH without prior sonication. The filamentous bacteria at site F were targeted by probes specific for *Epsilonproteobacteria* (EPSY549, Figure 9B and EP404). As different filamentous morphologies were targeted by EPSY549 and the EP404 probe, different filamentous *Epsilonproteobacteria* were most likely present. These filaments were not detected in the sonicated surface sediment sample from site F. Therefore, these filaments are sensitive to sonication, so that CARD-FISH counts of *Epsilonproteobacteria* did not include these filaments. Thus, *Epsilonproteobacteria* are underrepresented in the surface sediment counts of site F. In both white mats, Arc1430- (Figure 9C, G), CF319a- and DSS658-hybridized cells were detected.

GAM42a-targeted cells were large (2-4 µm diameter, Figure 9D, H) and formed aggregates. *Archaea* targeted by the ARCH915 probe were not found in the white layer.

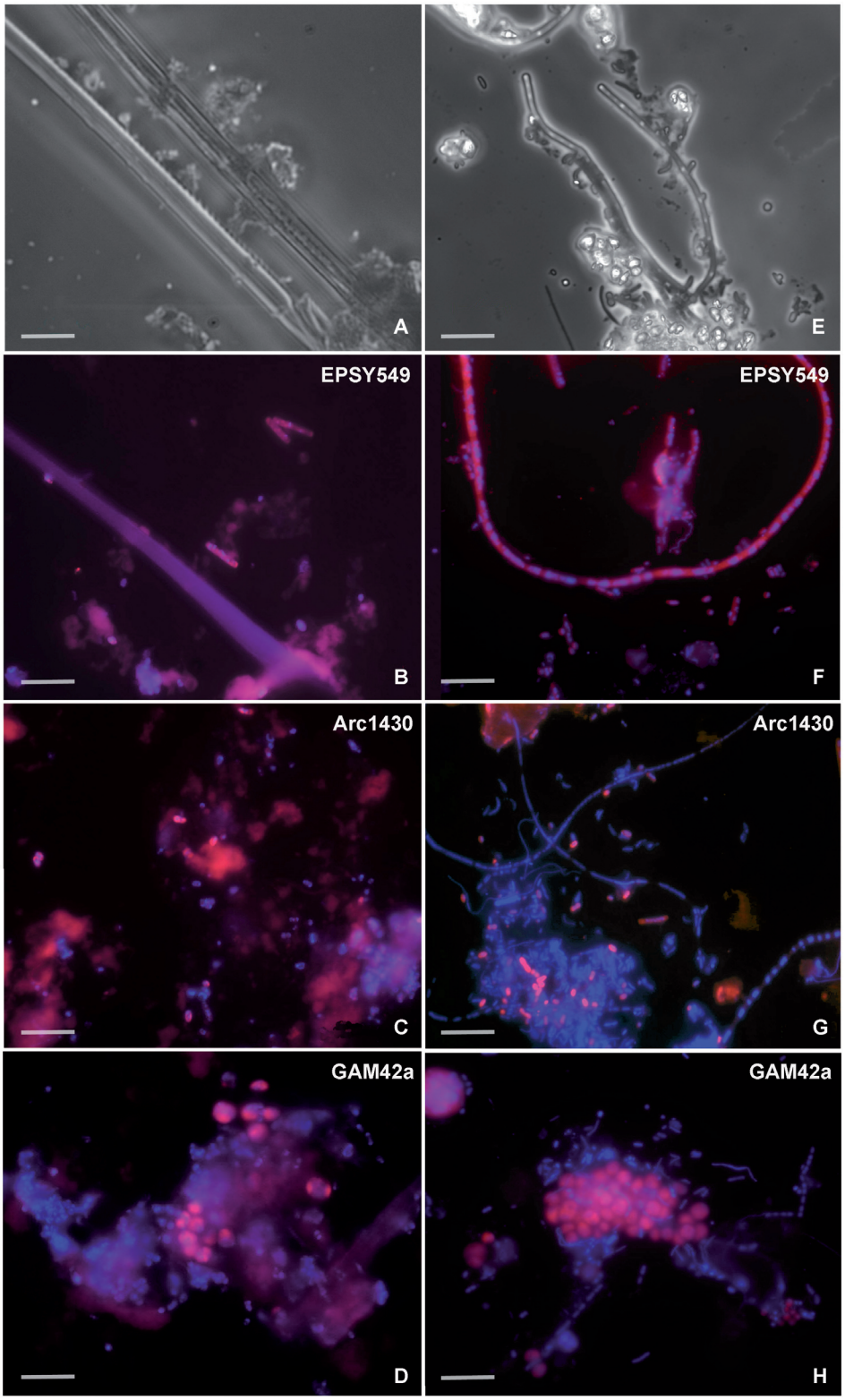

Figure 9: Photomicrographs of overlying mat from Anya's Garden (A-D) and site F (E-F). Cells of different morphologies are shown targeted by the EPSY549- (B+F), the Arc1430- (C+G), and the GAM42a-probe (D+H). Scale bars: 10 µm.

Table 3: Oligonucleotide probes and hybridization conditions used in this study.

Target group	Probe	Sequence (5' to 3')	Label	FA [%][a]	Hybridization Temp (°C)	Reference
Most Archaea	ARCH915	GTGCTCCCCCGCCAATTCCT	HRP, Cy3	35	46	Stahl and Amann, 1991
Most Bacteria	EUB338	GCTGCCTCCCGTAGGAGT	HRP, Cy3	35	46	Amann et al., 1990
	EUB338-II	GCAGCCACCCGTAGGTGT	HRP, Cy3	35	46	Daims et al., 1999
	EUB338-III	GCTGCCACCCGTAGGTGT	HRP, Cy3	35	46	Daims et al., 1999
control probe complementary to EUB338	NON338	ACTCCTACGGGAGGCAGC	HRP, Cy3	35	46	Wallner et al., 1993
Epsilonproteobacteria	EPSY549	CAGTGATTCCGAGTAACG	HRP, Cy3	35	46	Lin et al., 2006
Epsilonproteobacteria	EP404	AAAKGYGTCATCCTCCAA	Cy3	30	46	Macalady et al., 2006
Arcobacter sp.	Arc1430	TGCGCCACTTAGCTGACA	HRP, Cy3	20	46	Snaidr et al., 1997
Arcobacter sp.	Arc94	TGCGCCACTTAGCTGACA	HPR	20	46	Snaidr et al., 1997
Most *Deltaproteobacteria* and *Gemmatimonadetes*	Delta495a[b]	AGTTAGCCGGTGCTTCCT	HRP	35	46	Loy et al., 2002
Some *Deltaproteobacteria*	Delta495b[b]	AGTTAGCCGGCGCTTCCT	HRP	35	46	Loy et al., 2002
Some *Deltaproteobacteria*	Delta495c[b]	AATTAGCCGGTGCTTCCT	HRP	35	46	Loy et al., 2002
Desulfosarcina-related bacteria	DSS658	TCCACTTCCCTCTCCCAT	HRP, Cy3	60	46	Manz et al., 1998
Most *Desulfovibrio spp.*	DSV689	GTTCCTCCAGATATCTACGG	HRP	40	46	Manz et al., 1998
Methanotrophic, RuMP-metabolism, Gammaproteobacteria	MOGAM	GGTCCGAAGATCCCCCGCTT	HRP	20	46	Tsien et al., 1990
Gammaproteobacteria	GAM42a[b]	GCCTTCCCACATCGTTT	HRP, Cy3	35	46	Manz et al., 1992
Potential sulfur-oxidizing Gammaproteobacteria	GAM660	TCCACTTCCCTCTAC	HRP	35	46	Ravenschlag et al., 2001
most *Flavobacteria*, some *Bacteroidetes*, some *Sphingobacteria*	CF319a	TGGTCCGTGTCTCAGTAC	HRP, Cy3	35	46	Manz et al., 1996

[a] Formamide (FA) concentration in hybridization buffer.
[b] Competitor probes are required.

Discussion

Diffusive transport of fluid components

Sulfur-mats were found on top of heated sediment at AG and site F. The linear temperature profiles showed that the heat flux to the surface was conductive and not carried by fluid emission. The impression of shimmering water at AG and site F was therefore caused by heating of ambient seawater and subsequent rising due to decreased density. The effect can be observed at the bottom of a pot of water on a stove. As fluid emissions were not detected, our analyses do not support the description of these sites as "diffuse venting sites" (Gebruk et al., 2000), although these sites may have exhibited diffuse venting in the past. Low concentrations of methane, sulfide and hydrogen measured in the shimmering water above these sediments (Borowski and Shipboard Scientific Party 2007) confirmed that the surface sediment layers were not exposed to direct vent emission. Nevertheless, the heat sources below the white patches are most likely conduits carrying hydrothermal fluids. The highest detected fluid temperature at Logatchev is around 350°C (Douville et al., 2002). Extrapolation of the temperature gradient of 270°C m^{-1} to 350°C shows that the heat source is within 1.3 m or even closer if the temperature of the source water is below 350°C. The hot fluids at LHV contain high concentrations of reduced substances (dissolved hydrogen 19 mM, methane 3.5 mM, sulfide, 2.5 mM) (Schmidt et al., 2007). These electron donors diffuse towards the sediment-water interface along with the heat and fuel the chemolithoautotrophic community in the sulfur-mats. This is in agreement with a decrease of temperature and electron donor supply from the center of the mats to the outside. The upper temperature limit for microbial life of around 121°C (Kashefi and Lovley, 2003) is reached in only 45 cm depth. Thus, microbial layers controlled by the gradients of electron donors and acceptors (e.g. methane sulfate transition zone) must be concentrated near the surface where the temperature is compatible with life. Our analyses showed that regions of diffuse flow at hydrothermal vent fields lead as well to massive biomass accumulations at the seawater-sediment interface, besides previously reported regions exposed to vent fluid flow (Karl 1995).

The sulfur cycle in sediments of an ultramafic vent field

Biogeochemical profiles for sediments at AG revealed an intense sulfide flux from below. This sulfide flux was either generated by microbial dissimilatory sulfate reduction from a maximum of 45 cm depth or/and supplied by upwards diffusing hydrothermal fluids. Sulfide is depleted about two centimeters below the sediment surface. The slightly increasing sulfide concentration in the first centimeter could be due to changes in the pH or to dissimilatory

sulfate reduction. As corresponding pH profiles are missing, this remains unclear. Oxygen was already consumed after a few millimeters sediment depth. The separation of sulfide and oxygen prevents the direct sulfide oxidation by oxygen, resulting in a suboxic zone (upper 4-18 mm) where neither oxygen nor sulfide is present. Consequently, sulfide cannot be directly oxidized with oxygen. Sulfide oxidation by oxygen requires bioirrigation by burrowing animals that pump oxic water down in the sulfidic sediment. High temperatures in the sediment probably prevent massive biorrigation by animals, so that chemical oxidation of sulfide to metal sulfides is more likely. The presence of various iron- and copper-sulfides in these sediments suggests that metal oxides constitute an efficient sulfide barrier by oxidizing and binding sulfide (Thamdrup et al., 1994) (Figure 10). Therefore, most sulfides precipitate as metal sulfides and elemental sulfur. Oxygen becomes available in the first few millimeters (mm), as revealed by oxygen profiles. Near the sediment-surface interface, the reduced metal-sulfides likely react with oxidants such as oxygen or manganese oxide, and intermediates of pyrite oxidation such as thiosulfate and polythionates are produced (Figure 10) (Jørgensen and Kasten, 2006). These intermediate sulfur compounds can serve as energy sources for chemolithoautotrophic bacteria. High oxygen consumption rates in the first millimeters, and highest cell numbers in site F and AG sediments confirmed the presence of highly active microbial communities.

Mat-forming *Beggiaotoa* and *Thiothrix* as well as vibrioid *Arcobacter* typically occur in sediments with high sulfide fluxes (Jannasch and Wirsen, 1981; Taylor and Wirsen, 1997). *Arcobacter*-related species were identified as one of the dominant groups at AG, which are

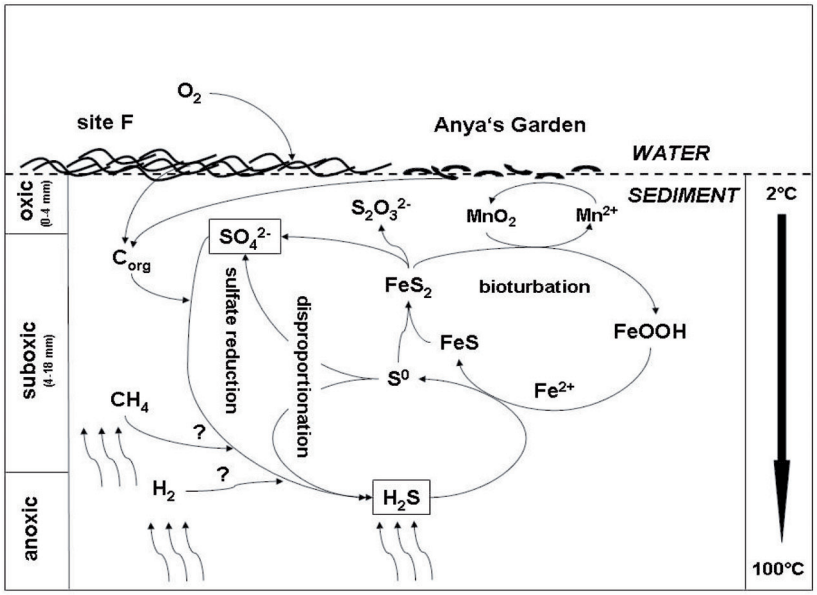

Figure 10: A model of the bacterial sulfur cycle in the sulfur-mat sediments from the Logatchev hydrothermal vent field. Upwards diffusing components of the underlying hydrothermal fluids, particularly sulfide, drive the sulfur cycle.

known to be autotrophic sulfide-oxidizing bacteria that excrete filamentous sulfur (Wirsen et al., 2002; Sievert et al., 2007). Therefore, the production of filamentous sulfur by sulfide-oxidizing *Arcobacter* could account for the detected sulfur-mat on top of AG sediments.

Mat-forming *Epsilonproteobacteria* were identified by FISH on top of the sediment at site F. These *Epsilonproteobacteria*-mats probably provide the sediments with the whitish appearance, instead of mat-forming *Beggiaotoa* and *Thiothrix,* which were found in hydrothermal vent systems at Galapagos and Guaymas Baisn. Phylogenetic analysis of the total surface sediment layer (0-1 cm) indicates that these filaments are likely related to the genus *Sulfurimonas* (Group B). Indeed, one member of this genus, *Sulfurimonas autotrophica*, was isolated from deep-sea hydrothermal sediments (Inagaki et al., 2003). Cultivated members of this genus are chemoautotrophic, mesophilic, elemental sulfur-, sulfide- and thiosulfate-oxidizers. *Gammaproteobacteria* affiliated with *Chromatiales* and *Thiotrichales,* which we detected by 16S rRNA gene analyses could also contribute to sulfide-oxidation in these sediments.

The presence of sequences closely related to those of sulfur-disproportionating *Desulfocapsa sulfoexigens* (Finster et al., 1998), and sulfur-reducing bacteria belonging to the *Desulfuromonadacea*, are in accordance with the presence of elemental sulfur (Figure 6). These bacteria are important in sulfur transformation processes in aquatic sediments (Jørgensen, 1990) (Figure 8). The biomass produced by autotrophic bacteria thriving on reduced sulfur compounds probably fuels sulfate-reducing bacteria, enabling high SRRs (Figure 3a, b). Highly abundant *Deltaproteobacteria* were found in the sulfur-mat sediments, which grouped with sulfate-reducers of the genera *Desulfosarcina*, *Desulfobacter*, and *Desulfobulbus*. These genera were detected at cold seeps (Omoregie et al., 2008) and in hydrocarbon-rich sediments of the Guaymas Basin (Teske et al., 2002). The SRRs in the surface layers of site F and AG were 100-times higher compared to those in deep-sea sediments (0.6-1.97 mmol m^{-2} d^{-1}) (Weber et al., 2001) which was reflected by the sulfur isotope fractionation. However, detected SRRs were much lower in comparison to the highest SRR found at hot-spots of methane flux from cold seeps or gas hydrates (803 mmol m^{-2} d^{-1}) (Arvidson et al., 2004). At these sites, SR is likely fueled by the oxidation of hydrocarbons and oil, rather than by the anaerobic oxidation of methane (AOM) (Joye et al., 2004). We detected sequences from AG and site F affiliated with anaerobic methane oxidizers (ANME-1; 21.2%, 30.7%, respectively), and with the Marine Benthic Group D, which is frequently detected in habitats where AOM occurs (Knittel et al., 2005). Low anaerobic methane oxidation rates and the low abundance of *Archaea* in the surface sediment layer (0-1 cm)

indicate that sulfate reduction (SR) fuelled by methane is only of minor importance. This suggests that SR is coupled to organic material as known from other habitats with high SRRs (Kallmeyer and Boetius, 2004a; Lloyd et al., 2006; Omoregie et al., 2008; Robador et al., 2009). The primary degradation of high molecular weight organic compounds by highly abundant heterotrophic *Bacteroidetes* (Kirchman 2002), likely provides sulfate-reducing bacteria with low molecular weight compounds.

Biogeochemical and molecular analyses propose that the cycling of sulfur-compounds is one of the dominant processes in sediments from site F and AG (Figure 8). In non-hydrothermally influenced marine sediments, microbial communities are typically fueled by deposited organic material that is oxidized by e.g. sulfate-reducing bacteria. In contrast, the microbial communities at site F and AG are fueled by additional electron donors supplied by the underlying hot fluids. Therefore, we hypothesize that chemolithoautothrophic bacteria produce enough biomass to support secondary sulfate reducers. Quest and the oceanic sediments did not reveal the electron donors supplied from below and therefore showed clear differences in their bacterial and archaeal diversity. These sediments revealed undetectable oxygen consumption, sulfate reduction rates and an inorganic origin of sulfide, and therefore appeared unaffected by the intense chemolithoautotrophic turnover, taking place 25 m away.

Microbial community of basalt and ultramafic-hosted hydrothermal systems

Hydrothermal vent fields hosted by ultramafic rocks emit fluids enriched in methane and hydrogen compared with basalt-hosted systems. As fluids are generally emitted from chimney structures or diffuse vent sites at high flow rates, the surrounding environments are directly exposed to the fluid flow (Karl 1995). In contrast to these sites, we showed that in sediments of the ultramafic Logatchev vent field fluids slowly pass through the sediment in a diffusive process until they reach the surface layer. We propose that typical ultramafic fluid components such as methane, hydrogen and sulfide are consumed by microorganisms or are oxidized to metal sulfides in deeper layers indicated by the presence of various iron- and copper-sulfides. Thus, we detected little influence of the hydrogen- and methane-enriched fluids on the composition of the microbial communities in the surface sediments. This is in contrast to studies of microbial communities in hot fluids at Logatchev (Perner et al., 2007) or at high-temperature carbonate chimneys (60-100°C) at Lost City (Brazelton et al., 2006).

The sulfidic surface sediments were dominated by *Epsilonproteobacteria* and *Deltaproteobacteria*. Sequences closely related to those found in microbial mats, hydrothermal sediments (Moyer et al., 1995; Teske et al., 2002), and at *in situ* colonizers at

sulfidic, basaltic systems (Alain et al., 2002; Lopez-Garcia et al., 2003; Moussard et al., 2006) confirmed that these microbial communities showed many similarities to those from basaltic systems. However, CARD-FISH results revealed that filamentous and vibrioid *Epsilonproteobacteria* were highly abundant in sulfur-mat covered sediments at Logatchev, in contrast to gammaproteobacterial mat-forming bacteria observed at basalt-hosted fields. Highly abundant *Epsilonproteobacteria* were as well found in plume or on surfaces of sulfur chimneys in basalt-hosted systems (Takai et al., 2005; Nakagawa et al., 2006). This suggests a significant ecological importance as primary producers in ultramafic and basaltic hydrothermal vent systems. Further biochemical and molecular investigations are necessary to determine the microbial communities in deeper layers, which are most probably methane- and hydrogen-metabolizing microorganism acting as a sink for hydrogen and methane.

Experimental Procedures

Study sites and sampling

Two samplings of the Logatchev hydrothermal field were performed on R/V Meteor cruise M64/2 (HYDROMAR II, 2005) and R/V M.S. Merian cruise MSM04-3 (HYDROMAR III, 2007) using remotely operated vehicles (ROV; M64/2: Quest MARUM, Bremen, Germany, MSM04-3: Jason II, Woods Hole, USA). The Logatchev hydrothermal vent field is located at the lower, eastern ridge-flank close to the axial valley at 15°N on MAR in about 3,000 m water depth. The Logatchev area includes two known vent fields of high temperature hydrothermal activity (SE: Quest and Irina II; NW: site A, Anna Louise, Irina and site B) and white mat sediments at Anya's Garden and site F (Gebruk et al., 2000). Four sediments were sampled in different areas of the Logatchev hydrothermal vent field. White mat sediments were taken at site F and AG, which were located about 100 m apart. The sediments were taken with push corers (AG) or a shovel (site F). Site F was characterized by a patchy distribution of white mat areas that covered more than 50 m^2 and clumps of vent mussels scattered within the white area. The second site was a 1 m^2 white patch within AG, a site with only dead mussels present. Shimmering water above these white mats was observed. The third sediment (Quest) was located a few meters from the active smoker Quest in between a mussel bed and a white covered area and sampled by push cores. Sediment without detectable hydrothermal influence was sampled 25 m from the nearest apparent hydrothermal activity.

In situ measurements and profiles

Temperatures were determined using a 0.5 m long lance with 8 temperature sensors spaced at 4 cm intervals and connected to an 8-channel logger (Brancker, Canada) close to the sampling sites. Temperature data reflect the measured *in situ* temperature after 5-10 min equilibration.

Microprofiles were recorded with a modified version of profiling instrument described by Gundersen and Jørgensen (1992) and Glud et al. (1994). The sensor package consisted of two glass pH microelectrodes with one common Ag/AgCl reference electrode (Amman, 1986), two Clark type H_2S microelectrodes with internal reference (Jeroschewski et al., 1996), three Clark-type O_2 electrodes with internal reference and guard (Revsbech, 1989), as well as one LM-35 (National Semiconductor) temperature probe in a needle shaped casing. The tip diameters of the electrodes were 20-50 µm. The temperature sensor had a 3 mm thick cylindrical shaft and a cone-tapered tip. The spatial resolution of the temperature sensor was better than 0.5 mm. Positioning as well as the activation of the profiler by pressing a mechanical switch was performed with the ROV. Once the profiler was activated, the electronic cylinder holding the microsensor array was lowered in increments of 0.1 mm for a total distance of 130 mm.

Mineralogy

Dry surface sediment samples (0-1 cm) from all four sites were powdered and pressed to tablets for X-Ray diffraction measurements on a Philips PW 1710 (IFM-GEOMAR, Kiel, Germany) with automatic divergence slit and monochromatic CoKα radiation. Data processing was performed using the freeware MacDiff (Petschick, 2001).

Sulfur fractionation

Isotopic measurements were carried out on a ThermoFinnigan Mat DELTA Plus mass spectrometer (Bremen, Germany) coupled with an elemental analyzer (Carlo Erba). Acid-volatile and chromium reducible sulfur from the surface layer of each sediment was extracted according to Canfield et al. (1986) and Newton et al. (1995). The evolved sulfide was precipitated as ZnS in 3% zinc-acetate solution. ZnS-preserved samples were acid distilled into a silver nitrate solution, and the precipitated Ag_2S was dried and converted to SO_2 during combustion with V_2O_5 at 1,050°C. Results are given in the common δ notation as per mill difference to the V-CDT-reference (Vienna Canyon Diablo Troilite). On the V-CDT scale the standard materials IAEA-S1 and IAEA-S2 show $\delta^{34}S$ values of -0.3‰ and 21.5‰, respectively. All replicate analyses of standards were repeatable in ±0.3‰.

Measurements of sulfate reduction rates

Sulfate concentration was determined for three subcores (1 cm diameter), which were sectioned in 1 cm layers, preserved in 20 ml of 20% (w/v) and frozen at -80°C until analysis. Thawed sediment samples were centrifuged at 4,500 r.p.m. at 4°C for 5 min. The preserved supernatant porewater (100 µl) was diluted 100-fold with distilled water and analyzed by non-suppressed ion chromatography with a Waters IC-Pak anion exchange column (50 mm × 4.6 mm) and a Waters 430 conductivity detector (Ferdelman et al., 1997). Sulfate reduction rates were measured in triplicates at room temperature within 5-8 hours after core recovery. In three subcores, 5 µl aliquots of carrier-free $^{35}SO_4^{2-}$-tracer solution in 4% NaCl (~100 kBq per injection) were injected at 1 cm intervals according to Jørgensen (1978). Incubation was terminated after 24 h by sectioning into 20 ml of 20% (w/v) zinc-acetate solution. The reduced ^{35}S was separated from non-reacted tracer by single-step reduction and distillation (Kallmeyer et al., 2004b) and sulfate reduction rates were calculated from the fraction of tracer reduced and the sulfate concentration.

Measurements of anaerobic oxidation of methane (AOM)

Methane concentration was measured in sectioned subcores (1 cm diameter) preserved with 2.5% NaOH in rubber-sealed glass vials. Methane was measured by injecting 100 µl of the headspace gas on a Hewlett Packard 5890A gas chromatograph with flame-ionization detector. Radiotracer incubations for AOM were performed in triplicates, injecting 10 µl of $^{14}CH_4$ (~0.24 kBq, dissolved in double-destilled H_2O) in 1 cm intervals, respectively. The subcores (1 cm diameter) were incubated for 24 h at room temperature, followed by cm-wise sectioning and fixation in 2.5% NaOH. The rates of methane oxidation were calculated from concentration of the reactant, its radioactivity and the radioactivity in the reaction product according to Treude et al. (2003).

DGGE

DNA was extracted from 0.5 g surface sediment sample (0-1 cm) following the protocol of the FastDNA SPIN Kit for Soil (Q·BIOgene, CA, USA). The amplification of the bacterial 16S rRNA genes for DGGE was performed with the primer set GM5F (GC-clamp at the 5´-end) and 907 RM (Teske et al., 1996) using a touchdown protocol (Muyzer et al., 1995). The reaction mixture of 100 µl included 10-100 ng DNA, 1 µM of each primer, 100 mM of dNTPs, 1 x buffer (Eppendorf, Hamburg, Germany), 1 x enhancer (Eppendorf, Hamburg, Germany), and 1.25 U of the Eppendorf-Taq DNA Polymerase (Eppendorf, Hamburg,

Germany). DGGE was carried out using a Bio-Rad D-Code system at 60°C and a constant voltage of 200 V for 3.5 h. The DGGE bands were visualized under UV light.

DNA extraction, PCR reaction and 16S rRNA clone library construction

DNA was extracted from 0.5 g surface sediment sample (0-1 cm) following the protocol of the FastDNA SPIN Kit for Soil (Q·BIOgene, CA, USA). Bacterial and archaeal 16S rRNA genes were amplified using the primer pair GM3/GM4 (Muyzer et al., 1995) and Arch21F/ Uni1390 (Delong, 1992; Zheng et al., 1996), respectively. The 50 µl reaction contained 10-100 ng DNA as template, 0.5 µM of each primer, 10 mM of dNTPs, 1 x buffer, 1 x enhancer, and 5 U of the Eppendorf-Taq DNA Polymerase (Eppendorf, Hamburg, Germany). PCRs were performed in ten replicates with 20 cycles to minimize PCR-bias. After 3 min at 94°C each cycle consists of 1 min at 94°C, 1 min at 42°C (*Bacteria*) or 57°C (*Archaea*), and 3 min at 72°C. The amplicons were pooled, purified using a PCR purification kit (QIAGEN, Hilden, Germany), and cloned using TOPO TA Cloning Kit for sequencing (pCR4-TOPO) (Invitrogen, Karlsruhe, Germany). Clones with a correct insert size of ~1,500 bp were sequenced using the vector primers M13 F and M13 R.

Sequence analysis

Sequences were imported into the ARB software package (Ludwig et al., 2004), aligned using the ARB FastAligner to the SILVA database (SSU Ref 0207_1_4_tree_silva) (Pruesse et al., 2007), and then refined manually. Phylogenetic trees of full-length sequences were calculated applying the maximum likelihood algorithm (PHYML), a 50% positional conservation filtering, and 100 bootstrap replicates. Partial sequences were added to the tree using the ARB parsimony tool. The software Distance-Based OTU and Richness was applied to ARB distance matrices generated with the Jukes-Cantor correction to estimate operational taxonomic units (Schloss and Handelsman, 2005).

Light microscopy and fluorescence *in situ* hybridization

Sediment samples were preserved in 4% paraformaldehyde for light microscopy and for FISH. Total cell numbers were determined by 4',6-diamidino-2-phenylindole (DAPI, 1 µg/ml) staining. Means were calculated based on a minimum of 1,000 DAPI-stained cells. CARD-FISH sediment samples (0.5 g) were fixed in 1.5 ml 4% paraformaldehyde for 2 h at 4°C, washed several times with 1 × phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.6), and finally stored in 1:1 mix of PBS/Ethanol at

-20°C. Samples were sonicated 2-5 × 20 s at low intensity using one-second sonication pulses (20% intensity) (Sonopuls HD70, Bandelin, Berlin, Germany) and filtered onto polycarbonate membrane filters (type GTTP, pore size 0.2 µm, Sartorius, Göttingen, Germany). FISH and CARD-FISH were performed according to previously published protocols (Pernthaler et al., 2001; Pernthaler et al., 2002). Probe hybridization parameters are listed in Table 3.

Nucleotide Sequence Accession Numbers

Bacterial 16S rRNA gene sequences reported in this study were deposited in the EMBL database under the accession numbers FN553922-553992 (F) and FN553993-554029 (F), FN554082-554165 (AG) and FN553519-553571 (AG), FN553585-553646 (Q) and FN553647-553677 (Q), FN553441-553518 (OC) and FN553762-553837 (OC) for full-length and partial sequences, respectively. Archaeal sequences were deposit under the accession numbers FN554030-554075 (F) and FN554076-554081 (F), FN553572-553584 (AG), FN553678-553733 (Q) and FN553734-553761 (Q), FN553838-553892 (OC) and FN553893-553918 (OC) for full-length and partial sequences, respectively.

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2 Metagenomic and functional analysis of the importance of sulfide as an energy source for primary production in an ultramafic hydrothermal vent field

Metagenomic and functional analysis of the importance of sulfide as an energy source for primary production in an ultramafic hydrothermal vent field

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Keywords: metagenomics, 454 pyrosequencing, sulfide turnover rates, microbial genomics, molecular ecology

Abstract

The ultramafic-hosted Logatchev field harbors hydrothermally influenced sediments, characterized by diffusion rather than advective processes. The surface sediments are sulfidic and are covered by dense epsilonproteobacterial white mats. Therefore, reduced sulfur compounds were proposed to be the dominant electron donors for primary production. In this study, we investigated the microbial metabolic capacity and present activity of the microbial communities of this surface layers by pyrosequencing, key genes detection and incubation experiments. The comparison between pyrosequencing and previously obtained CARD-FISH data of the same surface layer revealed that pyrosequencing represents a semi-quantitative method suitable for fast biodiversity and metabolic capacity screening. The detection of key genes for sulfur oxidation and sulfate reduction, such as *aprA* and *soxB*, suggested the presence of putative sulfur cycling microorganisms. Taxon-specific genes assigned by the tool PAOLA for pyrosequencing-derived sequences revealed that genes for sulfur oxidation were exclusively detected for *Gammaproteobacteria* and *Epsilonproteobacteria*. Furthermore, a higher presence of genes for denitrification affiliated to *Epsilonproteobacteria*, proposes that nitrate reduction, coupled to sulfur oxidation, could be an important process in the oxic-anoxic interface of these sediments. The importance of sulfide for this habitat was confirmed by the detection of sulfide consumption rates. Higher rates in the surface sediment in comparison to the overlying *Epsilonproteobacteria*-mat supported the relevance of *Gammaproteobacteria* besides *Epsilonproteobacteria* as sulfur-oxidizing primary producers. Our study shows that pyrosequencing allows the description of microbial communities and provides insights into characteristic metabolic capacities of taxonomic groups. The combination of a metagenomic approach and incubation experiments confirmed the importance of sulfide as an energy source in these surface sediments and provided information about taxonomic groups involved in sulfur cycling.

Introduction

Deep-Sea hydrothermal vents are among the most productive ecosystems on earth (Van Dover, 2000). Due to the lack in light, they rely almost entirely on chemolithotrophic primary production. A variety of electron donors can sustain chemolithotrophy at hydrothermal vent sites, but their availability depends on the composition of the underlying rock. Hydrothermal vent fields along the Mid-Atlantic Ridge are either hosted by basaltic rocks, or by ultramafic components, which are primarily composed of mantle-derived peridotite (Wetzel and Shock, 2000). Fluids of basalt- and ultramafic-hosted hydrothermal vent fields vary considerable with respect to temperature, pH, and the presence and concentration of energy sources (H_2, H_2S, CH_4, Fe(II)) as a result of seawater-rock reactions (Schmidt et al., 2007). Of these, sulfide is considered to be an important electron donor for primary production in both types of hydrothermal systems (Schauer et al., in revision). *Beggiatoa*- and *Epsilonproteobacteria*-dominated mats at basalt- and ultramafic-hosted hydrothermal systems suggest dominance of sulfur-oxidizing bacteria at hydrothermal vents (Jannasch and Wirsen, 1981; Gundersen et al., 1992; Schauer et al., in revision). Recent studies at geothermal springs have supported the importance of sulfide in habitats whit sulfide and hydrogen, as sulfide was the favored electron donor used by microbial mats (D'Imperio et al., 2008).

Hypotheses on the relevance of metabolic processes in a given habitat are often deduced from the diversity of the microbial community as determined by comparative 16S rRNA gene analysis and fluorescence *in situ* hybridization (FISH). Thereby it is assumed that the metabolic capabilities of detected taxa are similar to those of the closest cultivated relatives. Phylogeny may however not be reliable for predicting physiology. Even closely related bacteria might have very different metabolic repertoires, e.g. resulting from horizontal gene transfer events, enabling them to occupy differing ecological niches (Suen et al., 2007).

Cultivation-independent metagenomic analysis represents a useful approach to assess the metabolic potential of microbial communities (DeLong et al., 2006), and to identify new metabolic capabilities of known phylogenetic groups (Beja et al., 2000), or clusters of as yet uncultured species (Beja et al., 2002). Metagenomics was considered to offer the potential for a relatively unbiased view on microbial diversity and metabolic capacity (von Mering et al., 2007). However, traditional metogenomic employs methods that suffer from inherent bias, like primer-bias when targeting specific sequences, or PCR- and cloning bias in the amplification process. Within the past few years, high-throughput pyrosequencing of metagenomic DNA (Rothberg and Leamon, 2008) has proven to provide valuable information on the diversity (Sogin et al., 2006; Huber et al., 2007; Roesch et al., 2007, Gaidos et al.,

2009), gene content, and genetic variability within microbial communities (Biddle et al., 2008; Dinsdale et al., 2008; Gaidos et al., 2009; Simon et al., 2009). The large amounts of sequence data in such next-generation sequencing (NGS) metagenome studies provide informations that allow insights in the genetic mechanisms microorganisms employ in response to changing environmental conditions. A key challenge for the future is the interpretation of such genomes and metagenomes from an ecological perspective. Comparative metagenomics of environments already revealed habitat-specific fingerprints, suggesting that environmental conditions selects for a similar genetic repertoire in microbial communities, in particular for microorganisms occupying similar ecological niches (Tringe et al., 2005; Suen et al., 2007; Poretsky et al., 2009).

In this study, we used GS FLX Titanium technology to sequence metagenomes from a sulfur-rich microbial mat and the underlying sediment (0-1 cm) from the ultramafic-hosted Logatchev hydrothermal vent field. We validated the potential of pyrosequencing to provide an unbiased view on microbial diversity and metabolic capacity by a comparative analysis with sequences from a 16S rRNA clone library and a CARD-FISH study. As a result, the quantitativeness of pyrosequencing in conjunction with functional annotations allowed for the sulfur-mat sediments to indentify habitat- as well as taxon-specific genes for the classes *Gamma-*, *Epsilon-*, *Deltaproteobacteria*, and *Bacteroidetes*. Furthermore, the diversity of key genes such as *aprA* and *soxB* were investigated. The metabolic potential deduced from gene annotation of the microbial community at site F was confirmed by microbial sulfide and hydrogen consumption rate measurements.

Material and methods

Study site and sampling

The Logatchev hydrothermal vent field is located at the lower, eastern ridge-flank close to the axial valley at 15°N on the Mid-Atlantic Ridge in about 3,000 m water depth (Gebruk et al., 2000). The south-eastern vent field hosts two active smokers (Quest and Irina II) as well as sediment areas covered by sulfur-mats (Anya's Garden, site F). During the R/V M.S. Merian cruise MSM04-3 (HYDROMAR III, 2007) and the R/V M.S. Merian cruise MSM10-3 (HYDROMAR IV, 2009) sediment was sampled using remotely operated vehicles (Jason II, Woods Hole, USA; Kiel 6000, IFM-GEOMAR Kiel, Germany). Sediment samples were taken from Anya's Garden and site F, and the oceanic sediment. Sampling was performed by push-corers which were sliced in 1 cm layers and frozen at -80°C. For the analyses the surface layer (0-1 m) was used. Site F was characterized by a patchy distribution of white mat areas

that covered more than 50 m^2 and clumps of vent mussels scattered within the white area. In contrast, Anya's Garden was a 1 m^2 white patch with only dead but no living mussels. Oceanic sediments devoid of detectable hydrothermal influence were present 25 m apart from the nearest apparent hydrothermal activity. Different methods were applied to the tree different sediments (Table 1).

Table 1: Overview of applied methods to the three different surface sediment microbial communities.

Site	gene clone library	fosmid library	insert end-sequencing	pyro-sequencing	incubation experiment
F	*aprA*, *soxB*	~30 000	6773	1,152,840	H_2S, H_2
Anya's Garden	*aprA*, *soxB*	-	-	-	-
Oceanic sediment	*aprA*	-	-	-	-

Construction and analysis of *aprA*- and *soxB*-gene clone libraries

DNA was extracted from 0.5 g of each of the three surface sediment samples (0-1 cm) following the protocol of the FastDNA SPIN Kit for Soil (Q·BIOgene, CA, USA). Genes for the alpha-subunit of the dissimilatory adenosine-5'-phosphosulfate (APS) reductase (*aprA*) were amplified using the primers AprA-1-FW (TGGCAGATCATGATYMAYGG) and AprA-5-RV (GCGCCAACYGGRCCRTA) (Meyer and Kuever, 2007a). The *soxB*-genes were amplified using the primers soxB432F (GAY GGN GGN GAY ACN TGG) and soxB1446B (CAT GTC NCC NCC RTG YTG) (Petri R et al., 2001). The PCR reaction contained 10-100 ng DNA as template, 0.5 µM of each primer, 10 mM of dNTPs, 1 x buffer, 1 x enhancer, and 5 U of the Eppendorf-Taq DNA Polymerase (Eppendorf, Hamburg, Germany) in a 20 µl reaction volume. After initial 3 min at 94°C each cycle consisted of 1 min at 94°C, 1 min at 58°C (*aprA*) or 55°C (*soxB*) annealing, and 3 min elongation at 72°C. The amplicons were purified using a PCR purification kit (QIAGEN, Hilden, Germany), and cloned using TOPO TA Cloning Kit for sequencing (pCR4-TOPO) (Invitrogen, Karlsruhe, Germany). Clones with a correct insert size were sequenced using the vector primer M13 R (5'- GTTGTAAAACGACGGCCAGT -3') or the primer pair soxB432F/soxB1446B.

Sequences were translated and analyzed in terms of phylogeny using the ARB software package (Ludwig et al., 2004). Phylogenetic trees were calculated based on amino acid sequences using the Maximum Likelihood algorithm (PHYML) and a 25% positional conservation filter and 100 bootstrap replicates.

Fosmid library construction, screening and sequencing

Sediment from site F (0-1 cm) was used for fosmid library construction. DNA was extracted as previously described (Zhou et al., 1996), followed by gel purification (Plaque GP Agarose, Biozym, Hess. Oldendorf, Germany) to remove humic substances. The fosmid library was constructed using the EpiFOS CopyControl Fosmid Library Production Kit (Epicentre, Madison, WI) according to the manufacturer's instructions with the modification that the DNA was concentrated using MICROCON YM-100 columns (Promega, Mannheim, Germany). Size-selection was performed by pulsed-field gel electrophoresis (PFGE) to obtain appropriate DNA fragment lengths (~40 kb) for cloning.

Of approximately 30,000 obtained fosmid clones 2,304 were screened for bacterial *aprA*- and *hupL*-genes. The amplification of *aprA*-genes was performed using the above-mentioned primers and PCR conditions. The primer pair HUPLX1/HUPLW2 targeting the highly conserved N- and C-terminal regions of the large subunit of [NiFe] hydrogenases (Csáki et al., 2001) was used to amplify *hupL*-genes. The PCR reaction mixture contained the components above described and PCR condition using 58°C (*hupL*) as annealing temperature. Shotgun sequencing of selected fosmids was performed using Big Dye 3.1 chemistry and ABI3130XL capillary. In addition 6,582 fosmid insert end-sequences were determined. Open reading frames (ORFs) were predicted with 'borf' by Hanno Teeling.

ORF prediction and annotation of fosmids

The selected fosmids were analyzed using the gene prediction programs GLIMMER 3.02 (Delcher et al., 2007), MetaGene 1.0 (Noguchi et al., 2006), ZCURVE 1.02 (Guo et al., 2003), and MED 2.0 (Zhu et al., 2007). ORFs were predicted based on the consensus of all used prediction programs. Annotation was carried out using the software package GenDB (Meyer et al., 2003). For each predicted ORF, similarity searches against sequence databases (NCBI-nr, SWISSPROT) and protein family databases (Pfam, Prosite, InterPro, COG, KEGG), as well as signal peptide (SignalP v2.0, Nielsen et al., 1999) and transmembrane helix predictions (TMHMM v2.0, (Krogh et al., 2001) were conducted. Subsequently, all ORFs were subjected to automatic annotation using MicHanThi (Quast, 2006), followed by manually refinement.

Pyrosequencing

Genomic DNA was extracted from the surface sediment layer (0-1cm) of site F following the protocol of Zhou et al. (1996). A total of ~4 µg DNA was used for direct sequencing using the GS DNA Library Preparation Kit, following the instructions of the GS FLX Shotgun DNA Library Preparation Manual (Roche Diagnostics). Pyrosequencing resulted in 1,152,840 reads. The SP3 processing pipeline (MPI Berlin) was used for vector and quality clipping. The unassembled reads were de-replicated with a CD-Hit-based 454 replicate filter (Gomez-Alvarez et al., 2009). Pyrosequencing derived data were assembled by Roche Newbler Assembler (454 Life Science) using the standard parameters for de novo assembly and hybrid data.

ORF prediction, phylogenetic and metabolic assignment of pyrosequencing derived data

Pyrosequencing-derived sequences underwent an ORF prediction with MetaGene by Noguchi *et al*. (2006). All ORFs exceeding 150 bp were compared with BLASTP (Altschul et al., 1990) against the non-redundant NCBI protein database (as of October 28th, 2008) and with hmmpfam (Eddy 1996; Eddy 1998) against the Pfam database (release 22) (Sonnhammer et al., 1997; Sonnhammer et al., 1998). This leads to an over prediction of genes. Therefore, only a relative small part of all genes could be assigned to a metabolic function. Hits with good E-values (BLASTP: 10^{-15}, hmmpfam: 10^{-5}) were subsequently analyzed with three tools.

BLASTP hits were processed with an adaption of the DarkHorse algorithm (Podell and Gaasterland 2007), as well as with the newly developed algorithm Kirsten (Kinship Re-establishment). In brief, DarkHorse performs rank-based reasoning on the taxonomic terms from BLASTP hits, calculates a so-called lineage probability index (LPI) for each hit and assigns the ORF to the hit with the highest LPI (see Podell and Gaasterland 2007 for details). Kirsten analyzes the BLASTP hits from the highest taxonomic rank (superkingdom) downwards. On each rank, all taxonomic terms are weighted by the sum of their BLASTP bit scores. As long as the term with the highest weight exceeds an adjustable threshold, that term is kept and the process continues to the next taxonomic level only with the hits that contain the term from the previous level.

CARMA infers the taxonomic affiliation of ORFs from their Pfam hits and was proposed by Krause et al. (2008). Here, a rewritten and improved version of the original algorithm was used (Huang et al., unpublished). The final taxonomic assignment for whole reads was done by means of a self-written logic named PAOLA (Huang et al., unpublished), which builds a weighted consensus of all three aforementioned tools. A self-written C++ library termed

phyloprint was used to map the taxonomic terms and their NCBI identifiers during the whole analysis (phyloprint currently contains 462,019 nodes of the NCBI taxonomy) (Waldmann, 2008). In addition, the SILVA pipeline was used (Pruesse at al., 2007) for all reads >250 bp of the de-replicated dataset to extract and select 16S rRNA and 23S rRNA genes. The phylogenetic analyses were performed with the ARB software package (Ludwig et al., 2004).

PAOLA was as well used for the metabolic assignment. ORFs were compared with BLASTP (Altschul et al., 1990) against the non-redundant NCBI database (as of October 28th, 2008). Hits with good E-values (BLASTP: 10^{-15}) were compared to the functional genes in the SEED platform (http://www.theseed.org, Overbeek et al., 2005). The descriptions of the best BlastP hits (E-values: 10^{-15}) were screened for key words of the 2009 SEED platform and subsequently assigned to subsystems.

Incubation experiments

Glass serum vials (58 ml for hydrogen or 117 ml for sulfide incubations) were filled with ⅓ sterile-filtered (0.22 µm) bottom seawater, so that the headspace always contained air. The surface sediment or only the overlying sulfur mat (0.5 g) was placed in the vial, closed with a gas-tight rubber stopper and crimped with aluminium seals. Control vials contained boiled sediment or solely seawater.

For sulfide consumption experiments, a Na_2S stock solution (10 mM) was added to the seawater to a final concentration below 20 µM. The initial concentration of dissolved sulfide was determined immediately after the addition (T0). All vials were incubated at 4°C on a slowly rotating table. Subsamples were taken at time intervals and fixed in zinc acetate (2%, w/v). The concentration of the resulting ZnS precipitate was measured at 663 nm by spectrophotometer (Trüper and Schlegel, 1964). The sulfide consumption rates reflected the sulfide removed from the seawater in the first hour per gram sediment (wet weight).

For hydrogen consumptions experiments, pure hydrogen gas (100%) was injected to the final concentration (15 000 ppm) in the headspace using a gas-tight syringe. All vials were placed up-side down and a H_2 stock solution was added to the seawater to a final concentration of 20 µM. Subsamples were taken at given time intervals with a gas-tight syringe from the headspace compensated through the inflow of sterile-filtered seawater from a second syringe. The H_2 concentration in the headspace was determined using a gas chromatograph (Thermo Trace GC Ultra, Thermo Scientific) equipped with two packed columns and a pulsed discharge detector (VICI). Recording and calculation of results was

performed using a PC operated integration system (Thermo Chrom Card A/D, Thermo Scientific).

Nucleotide sequence accession numbers

Sequences reported in this study were deposited at EMBL under the accession numbers xxx for xxx AprA sequences, under xxx for xxx SoxB sequences, and under xxx for fosmid sequences. The pyrosequencing dataset was deposited at xxx.

Results

Comparative analyses of microbial diversity and abundance retrieved by 454-pyrosequencing, clone and fosmid library sequencing and CARD-FISH

Pyrosequencing of the metagenome of the surface sediment layer from site F produced about 408 Mb of sequence with an average read length of 354 bp (Table 2). Of the total dataset, 34% were identified as technical replicates produced during emulsion PCR (Gomez-Alvarez et al., 2009). These replicates occurred independently of sequence lengths, as indicated by similar sequence length distribution profiles before and after replicates removal (Figure 1). The de-replicated non-assembled dataset was used for all further analysis. This dataset was compared with previous datasets of 16S rRNA clone library, CARD-FISH (Schauer et al., in revision), and insert end-sequences of a fosmid library retrieved from the same surface layer.

Table 2: Characterization of raw data and corresponding de-replicated pyrosequencing dataset from the sulfur-mat at site F.

	raw data	**de-replicated data**
Total number of reads	1,152,840	757,646
Total data, Mb	408	277,5
Total base pairs	407,654,843	268,318,916
Mean read, bp	354	354
Median read, bp	391	391
Number of predicted ORFs (metagene)	795,537	503,628
PAOLA		
Phylogeny BLASTP: 10^{-15}, hmmpfam: 10^{-5}	n.d.	135,250 (27%)[a]
Function BLASTP: 10^{-15}	n.d	11,602 (2.3%)[a]
16S rRNA gene	n.d.	369 (0.05%)[b]
23S rRNA gene	n.d.	714 (0.09%)[b]

[a] percentage of the total number of predicted ORFs (metagene)
[b] percentage of the total number of reads
n.d. not detected

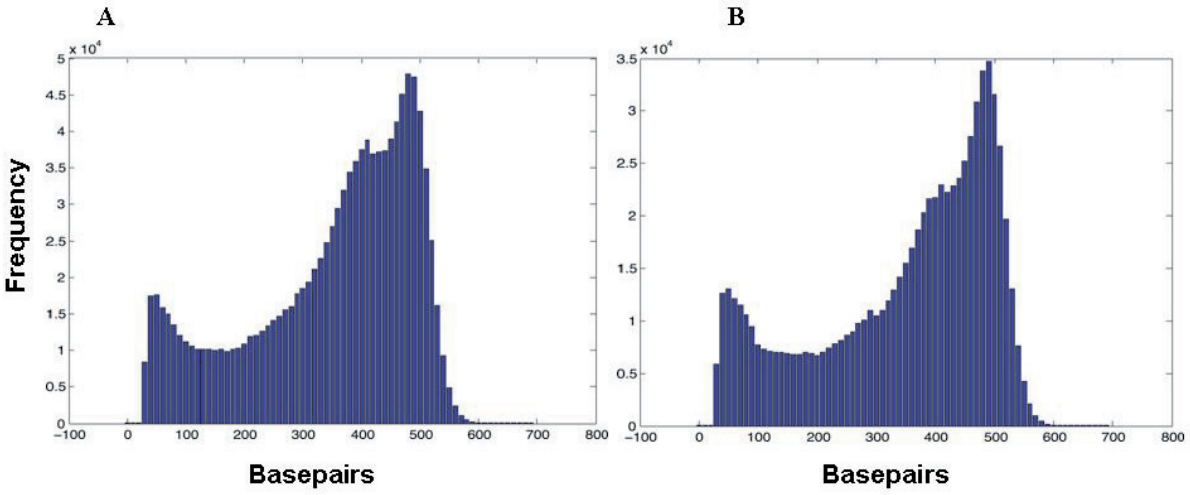

Figure 1: (A) Read length distribution of the pyrosequenced raw data (454 FLX Titaniium) and (B) the corresponding distribution of the de-replicated dataset of DNA from the surface sediment sample at site F (~3,000 m depth).

The analyses of pyrosequencing-derived 16S $rRNA_{454}$ (369 reads; 0.05%) and 23 $rRNA_{454}$ (714 reads; 0.09%) gene sequences revealed the dominance of *Bacteria*-related rRNA sequences (84% and 90%, respectively) (Figure 2). Archaeal $rRNA_{454}$ sequences accounted for 12% and 7% of the respective 16S and 23S $rRNA_{454}$ gene dataset, of which 93% and 100% were related to the phylum *Euryarchaeota*. Of the 503,628 predicted ORFs, 27% ORFs were phylogenetically assigned by the PAOLA software tool. The PAOLA-derived phylogeny confirmed the dominance of *Bacteria* (*Bacteria* 92%, *Archaea* 3%). The frequency of bacterial (80%-92%) and archaeal (3%-12%) sequences deduced from the pyrosequencing dataset were consistent with PAOLA-derived phylogeny for the insert end-sequences of the fosmid library, (*Bacteria* 80%, *Archaea* 3%), and with CARD-FISH counts (*Bacteria* 75%, *Archaea* 6%) (Figure 2).

All compared datasets revealed that most pyrosequencing-reads were related to *Proteobacteria* (51-55%), while CARD-FISH counts revealed the lowest abundance for this class (33%). Differences between CARD-FISH results and sequencing-based methods were detected for the abundance of *Bacteroidetes*. CARD-FISH counts revealed the highest abundance (21%), in contrast to 1%-5% detected by all other sequenced-based analysis, except for the 16S rRNA gene analysis (clone library, 14%). Further differences were detected for the class *Gammaproteobacteria* and *Deltaproteobacteria*. CARD-FISH counts revealed that *Deltaproteobacteria* (CARD-FISH=21%, seq. analyses=7%-11%) were the most abundant class, while all sequence-based analyses predicted the highest frequency for the *Gammaproteobacteria* (CARD-FISH=5%, seq. analyses=15%-28%). Moreover, CARD-FISH counts revealed 7% *Epsilonprotobacteria* compared to inconsistent results from sequence

based methods (PAOLA: 0.3%-3%; 16S and 23S rRNA$_{454}$ gene: 14%-20%). Therefore, CARD-FISH revealed distinctly different results at the phylum and class level in comparison to the sequence based methods.

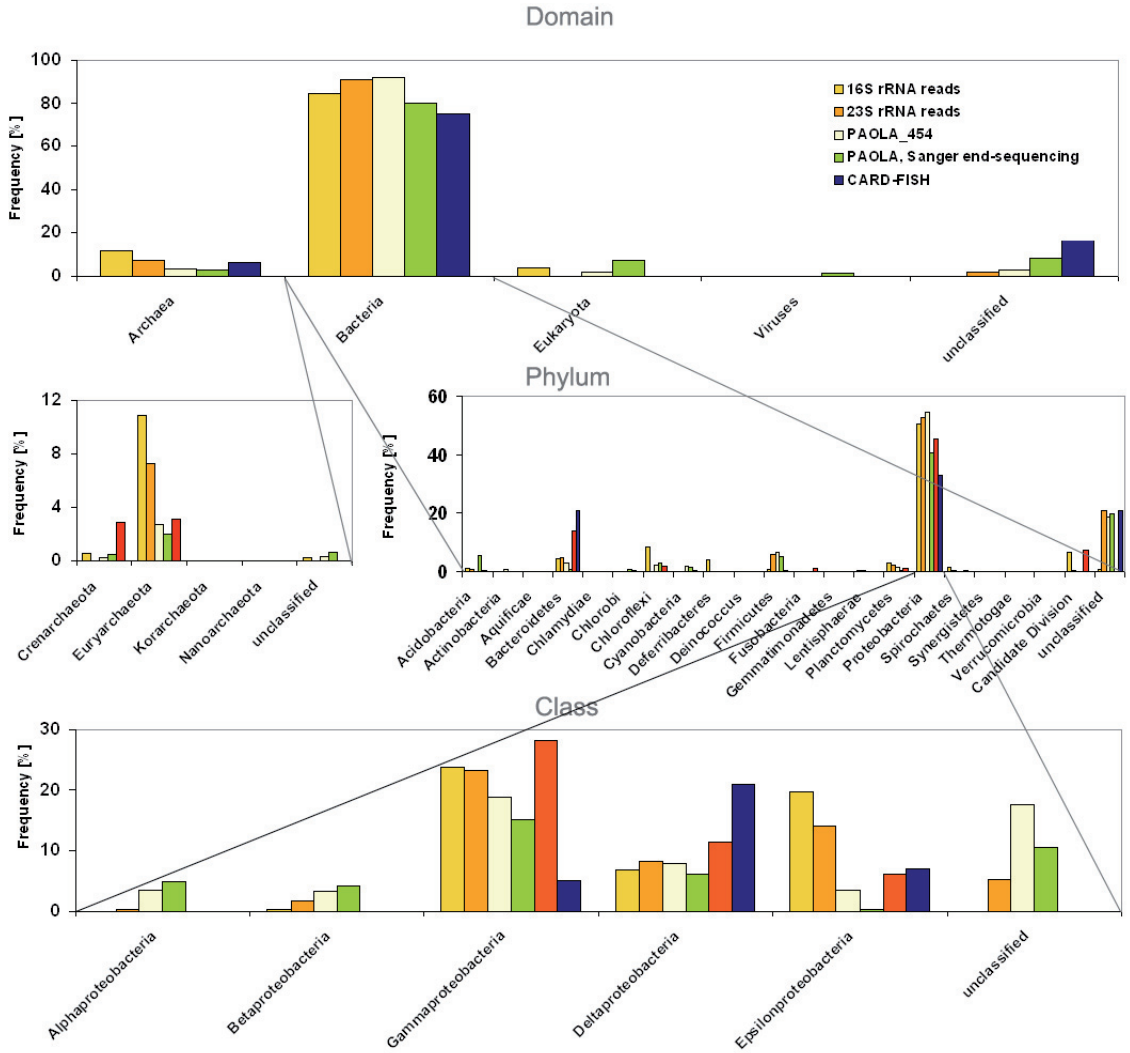

Figure 2: Phylogenetic comparison of the pyrosequencing-based dataset (16S and 23S rRNA$_{454}$ genes, PAOLA) with fosmid insert end-sequence data (PAOLA, Sanger end-sequencing) sequences from 16S rRNA gene clone libraries and CARD-FISH results. A phylogenetic assignment was done on the domain, phylum and class (only *Proteobacteria*) level. CARD-FISH counts were only available for *Bacteroidetes* and classes of the *Proteobacteria*.

The analyses of the pyrosequencing dataset allowed a further taxonomic breakdown. All three analyses (16S and 23S rRNA$_{454}$ gene, PAOLA$_{454}$) revealed that most sequences assigned to *Deltaproteobacteria* affiliated with the order *Desulfobacterales*, while epsilonproteobacterial sequences predominantly affiliated with the order *Campylobacterales* (Figure 3). According to the 16S rRNA$_{454}$ gene dataset, most epsilonproteobacterial sequences were related to the genera *Sulfurimonas* and *Sulfurovum*. Besides these two genera,

Campylobacter was predicted as a third genus of high abundance by PAOLA. In contrast to the few genera of high abundance that were detected within *Epsilonproteobacteria*, much higher diversity and less consistency was observed for *Deltaproteobacteria*. In general, data from all three methods became noisier and hence less consistent with increasing phylogenetic resolution (order to genus).

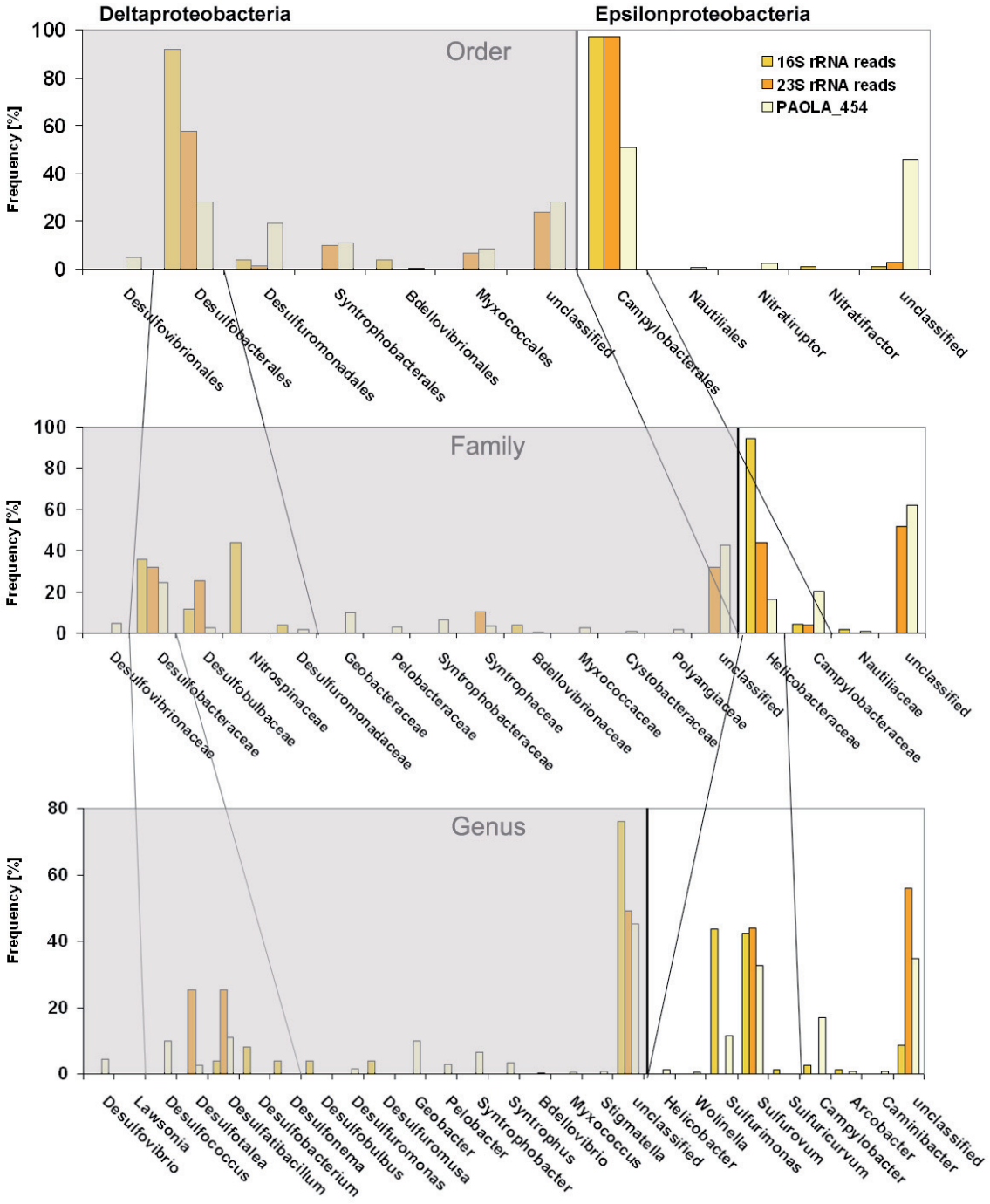

Figure 3: Phylogeny reconstruction based on total detected 16S and 23S rRNA genes as well as PAOLA predicted phylogeny of the 454-pyrosequenced DNA from the surface layer (0-1 cm) from site F.

Metabolic potential of the dominant microbial phyla in site F surface sediment

PAOLA could predict functions for 11,602 ORFs (2.3%; Table 1) that were mapped onto SEED subsystems as of 2009 (http://www.theseed.org). Subsystems represent groups of genes that act together, like genes constituting dedicated metabolisms or cellular structures. Half of the ORFs were assigned to the subsystems *Amino Acids Derivatives* and *Carbohydrates*. Other abundant subsystems were *Cell Wall* and *Capsule, Cofactors, Vitamins, Prosthetic Groups, Pigments* and *Protein Metabolism* (Figure 4). A comparison of ORFs that were

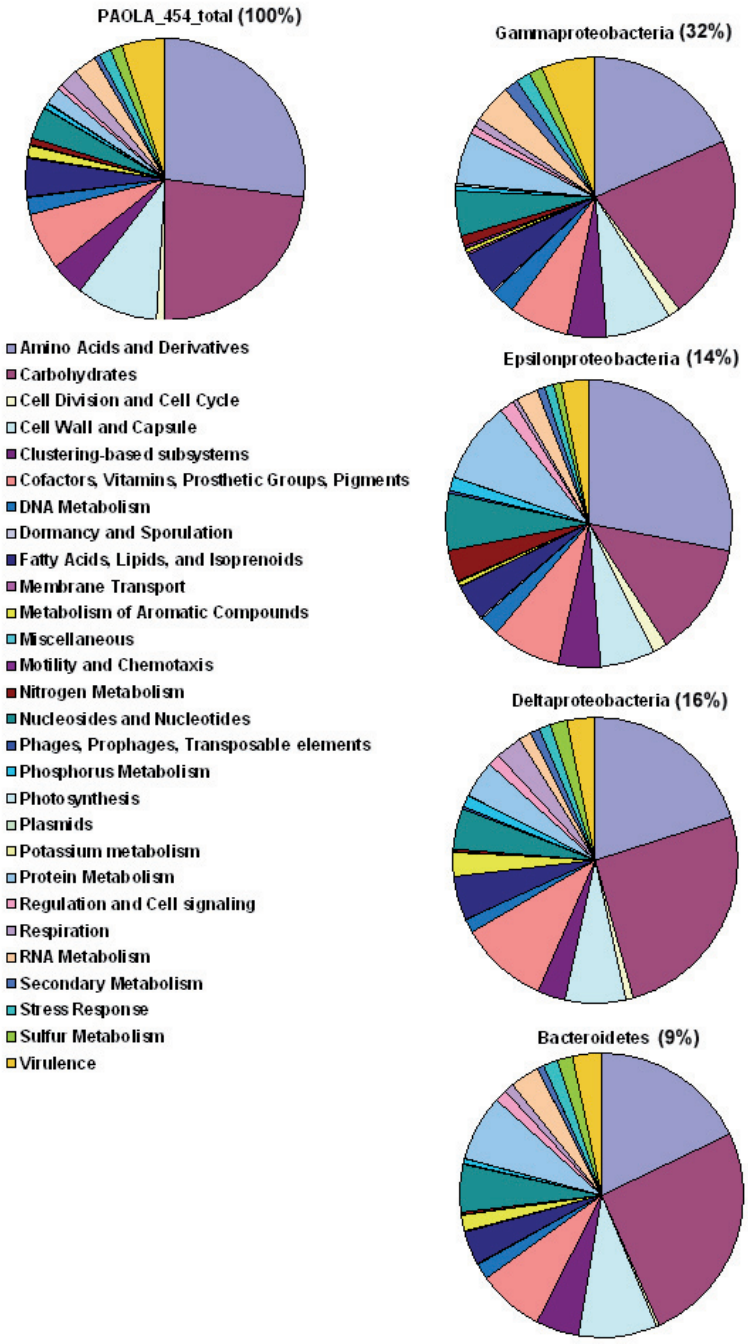

Figure 4: Mean percentage of reads similar to major metabolism of the total dataset and of reads phylogenetic related to *Gammaproteobacteria*, *Epsilonproteobacteria*, *Deltaproteobacteria*, and *Bacteroidetes*.

phylogenetically assigned to taxonomic bins of *Bacteroidetes, Gammaproteobacteria*, *Epsilonproteobacteria*, and *Deltaproteobacteria* exhibited similar patterns. Therefore, no considerable differences were detected between these classes with respect to their overall gene content. However, small differences were detected on a finer scale. Although *Sulfur Metabolism* genes were found in all four classes, genes for sulfur-oxidation pathways were restricted to *Gammaproteobacteria* and *Epsilonproteobacteria*. Further differences were detected for the *Nitrogen Metabolism* subsystem. Genes of this subsystem were only detected within *Gammaproteobacteria* and *Epsilonproteobacteria*, while *Epsilonproteobacteria* contained the highest number of genes for denitrification (Figure 5). Furthermore, the *Gammaproteobacteria* revealed the highest number of virulence genes. For the complete dataset, the Calvin-Benson cycle and the Serin-Glyoxylate cycle were the only identified metabolic pathways for CO_2-fixation. *Deltaproteobacteria*- and *Epsilonproteobacteria*-related genes affiliated to the Serin-Glyoxylate cycle while *Gammaproteobacteria* and *Bacteroidetes* exhibited genes involved in both pathways.

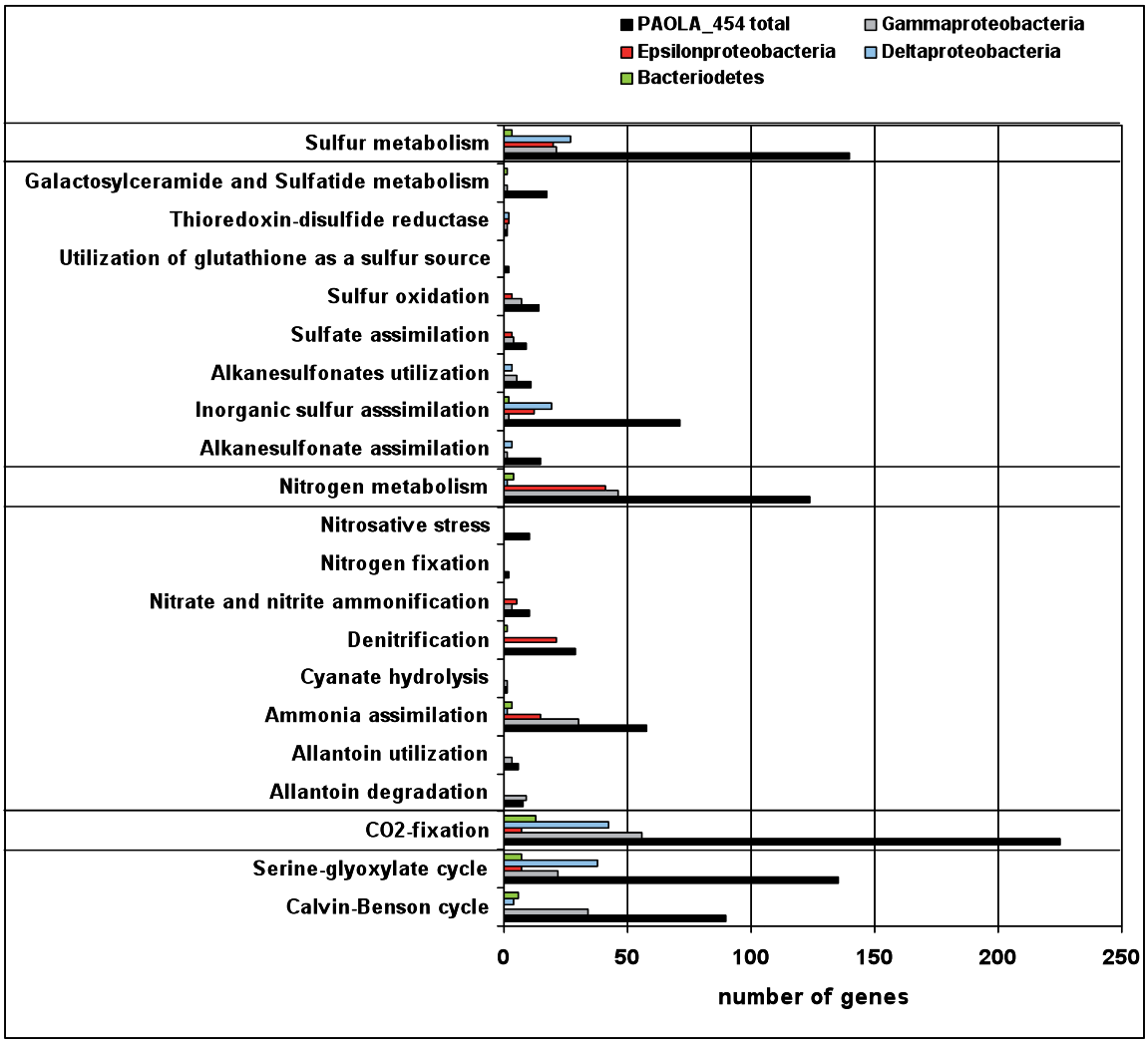

Figure 5: Number of genes similar to sulfur, and nitrogen metabolism as well as carbon fixation pathways of total reads as well as of *Gammaproteobacteria*, *Epsilonproteobacteria*, *Deltaproteobacteria* and *Bacteroidetes* related reads.

Key genes of the sulfur and hydrogen metabolism

The Screening of 2,304 clones of the fosmid library from the surface sediment at site F revealed 16 clones with an adenosine-5'-phosphosulfate reductase genes (*aprA*) and 20 clones with a [NiFe] hydrogenases (*hupL*). The complete sequences of three distinctly related *aprA* and four *hupL* carrying fosmids were determined. The genetic neighborhood of the a*prA*- and the *hupL*-genes on the fosmids was similar to those previously described (Mussmann et al., 2005; Nakagawa et al., 2007).

AprA-genes of the reductive and oxidative sulfur metabolism (Meyer and Kuever, 2007b) were detected by *aprA*-gene clone libraries constructed from the surface sediment of Anya's Garden and site F (Figure 6). The AprA sequences from site F were related to those of *Gammaproteobacteria*, *Betaproteobacteria*, and *Deltaproteobacteria*. The majority of these

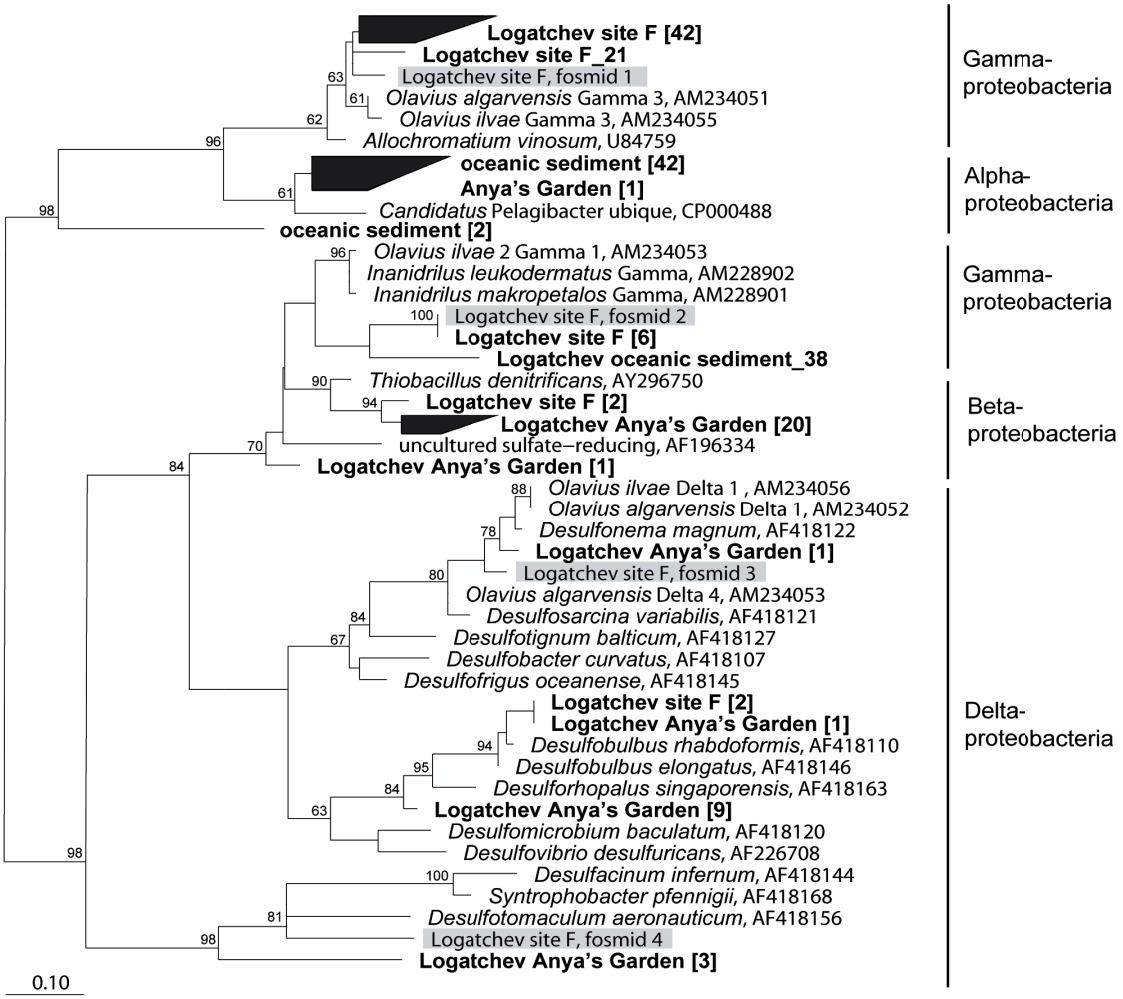

Figure 6: Phylogenetic affiliation of AprA from site F, Anya's Garden and oceanic sediment. The tree was calculated based on amino acid sequences (131) applying Maximum Likelihood algorithm with a 25% positional conservation filter and with 100 bootstrap replicates. Sequences identified on the fosmids are highlighted in grey and sequences deduced from the clone libraries in bold. Scale bar=0.10 estimated substitutions per site.

sequences affiliated with sequences of sulfur-oxidizing gammaproteobacterial symbionts (43 sequences *O. algarvensis* Gamma 3 symbiont, 6 sequences *O. ilvae* Gamma 1 symbiont). In contrast, AprA sequences from Anya's Garden clustered with *Alphaproteobacteria*, *Betaproteobacteria* and *Deltaproteobacteria*. Most sequences were related to *Thiobacillus denitrificans* (*Betaproteobacteria*, 20 sequences, >90% amino acid identity). While *Alphaprotebacteria* were represented by just one sequence in Anya's Garden sediments, the majority of AprA sequences retrieved from oceanic sequences affiliated with the novel class of alphaproteobacterial sulfur-oxidizing bacteria (SOB) (Meyer and Kuever, 2007b). In

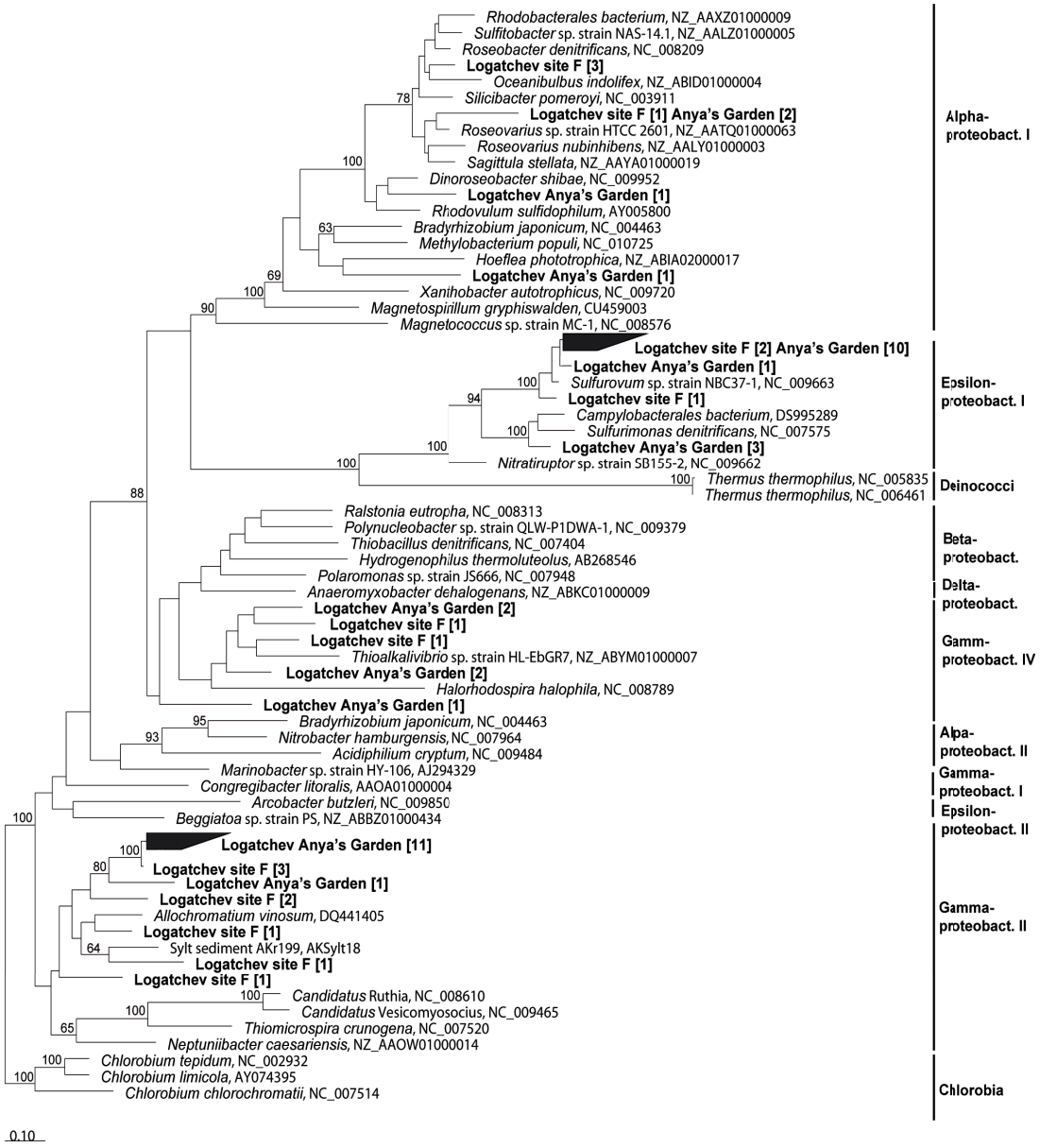

Figure 7: Phylogenetic affiliation of SoxB from site F and Anya's Garden. The tree was calculated based on amino acid sequences applying Maximum Likelihood algorithm with a 30% positional conservation filter and with 100 bootstrap replicates. Sequences retrieved from the clone library of the surface layer are in bold. Scale bar=0.10 estimated substitutions per site.

contrast to site F and Anya's Garden sediments, the oceanic sediments revealed only *aprA*-genes involved in sulfur oxidation.

SoxB-genes involved in sulfur oxidation related to those of *Alphaproteobaceria*, *Gammaproteobacteria*, and *Epsilonproteobacteria* were detected in site F and Anya's Garden surface sediments (Figure 7). Most sequences from both sites clustered to *Gammaproteobacteria*. Epsilonproteobacterial SoxB sequences from site F and Anya's Garden were affiliated with the genera *Sulfurovum* and *Sulfurimonas*.

Sulfide versus hydrogen consumption rates

Surface sediments (0-1 cm) as well as the overlying sulfur mat from site F were used in incubation experiments. Both samples showed sulfide consumption after one hour of incubation. Thereby, the surface sediment revealed higher sulfide consumption rates (10.2 ± 0.5 µmol h^{-1}(g wet weight)$^{-1}$) than the overlying white mat (2.4 ± 0.9 µmol h^{-1}(g wet weight)$^{-1}$) (Figure 8A). The sulfide concentrations in the surface sediment sample decreased continuously, and 90% of the sulfide was consumed within six hours. In contrast, the sulfide

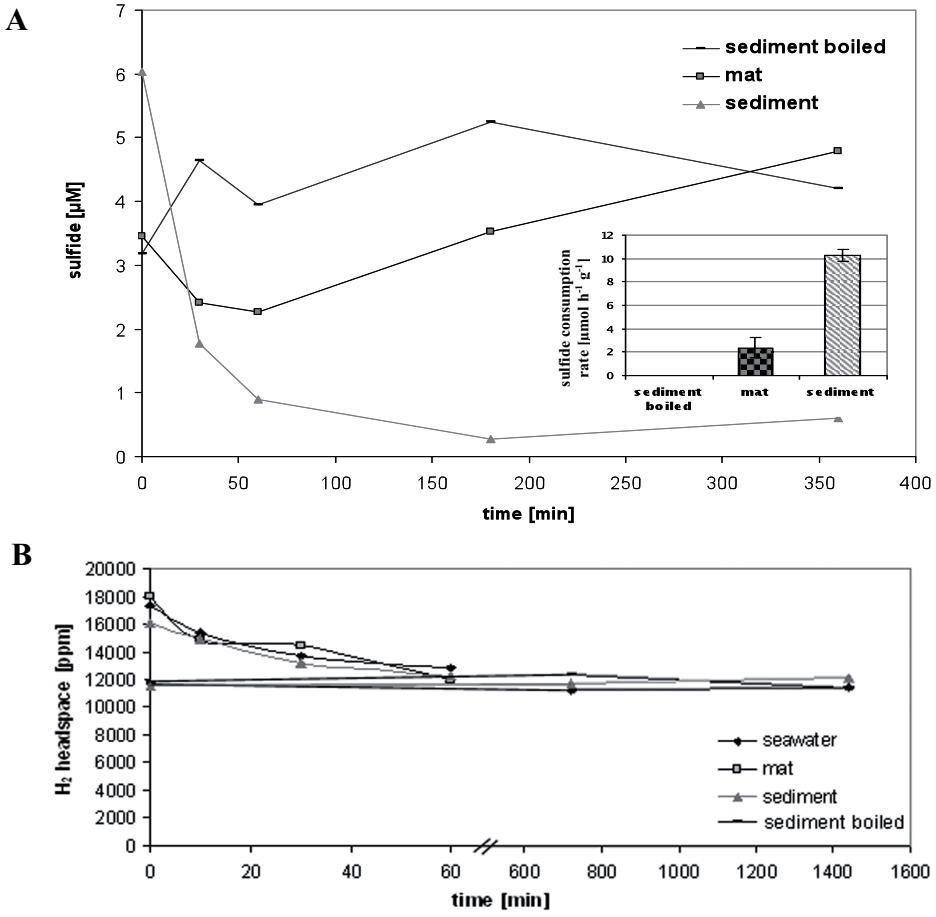

Figure 8: (A) Sulfide uptake and consumption rates in the surface sediment layer (0-1cm) and in the overlying sulfur-mat at site F and (B) hydrogen incubation experiments of the same samples. Each incubation was performed with 3 parallels and as control boiled sediment and/or seawater was used.

concentration in the mat sample increased after one hour, indicating sulfide production. Incubation experiments with hydrogen for one and for 24 hours did not show any hydrogen consumption for both samples, as hydrogen consumption rates were in the range of the controls (Figure 8B).

Discussion

How quantitative can a microbial community be assessed by pyrosequencing?

Microbial ecologists describe and quantify microbial diversities within given environments to understand how microorganisms interact with each other and with their environment to generate and to maintain such diversity (Xu et al., 2006). PCR-based techniques (fingerprint techniques; DGGE, T-RFLP, ARISA) and cloning-based techniques (e.g. 16S rRNA or metagenomics library) are widely used to assess diverstiy, although PCR (Polz and Cavanaugh, 1998; Sipos et al., 2007) and cloning (Feingersch and Beja, 2009; Temperton et al., 2009) are known to be biased. Likewise, hybridization techniques (FISH, micro-array) are used, whose accuracies are depend on the specificity and coverage of the applied probes. In case of FISH, this is dependent on the total number of known 16S and 23S rRNA gene sequences (Amann and Fuchs, 2008). High-throughput sequencing such as pyrosequencing, which addresses diversity across entire metagenomes, might be a complementary conceptual approach to assess diversity.

In order to determine how quantitative pyrosequencing data and hence the phylogenetic and metabolic reconstructions derived from such data are, we compared data from pyrosequencing, 16S rRNA clone libraries, fosmid insert end-sequences and CARD-FISH for one well characterized sediment sample. All compared methods revealed that *Bacteria* dominate the surface sediment community at site F and that *Archaea* were less abundant. However, the frequency of pyrosequencing reads assigned to *Bacteria* and *Archaea* differed from the CARD-FISH data (3.5%-12% to 6%, respectively).

As the group coverage of the probes used for the domain *Bacteria* and *Archaea* is very high (94% and 90% respectively) (Amann and Fuchs, 2008), the abundances of the detected and undetected parts (unclassified fraction) of the microbial community can be determined. However, FISH- and sequence-based studies generally miss both a part of the microbial community due to methodical limitations (e.g. permeabilizitaion issues or incomplete cell lysis). In contrast to FISH, Sequencing-based methods did not reveal the abundance of the undetected part. Therefore, it is likely that the abundance deduced from sequence-based

methods is overestimated for all levels (e.g. domain, phylum, class), as the abundances of the undetected part is not be taken in account.

At the phylum level, CARD-FISH results were only available for *Bacteroidetes* and classes of the *Proteobacteria*. CARD-FISH and pyrosequencing revealed for both phyla considerably different abundances. The group-level probes that were applied exhibit a lower group-coverage and more outgroup hits in comparison to the domain-level ones (Amann and Fuchs, 2008). Probes targeting *Bacteroides*, such as CFB319a (outgroup hits=5%, Amann and Fuchs, 2008) or the *Deltaproteobacteria* (DELTA495a=17%, DELTA495b=71% and DELTA495c=24%; Lücker et al., 2007) show many outgroup hits, indicating an overestimation of the corresponding abundances. In contrast, the *Gammaproteobacteria*-targeting probe GAM42a has a low group coverage (76%), so that the gammaproteobacterial part related to the *Xanthomonadaceae* family were missed by the CARD-FISH study (16S $rRNA_{454}$ reads=4%, 23S rRNA $reads_{454}$=0.2%, $PAOLA_{454}$=0.2%).

Pyrosequencing and fosmid insert end-sequencing indicated a low abundance of *Bacteroidetes*, which was in contrast to the 16S rRNA clone library and CARD-FISH study. Lower abundances of this phylum retrieved by pyrosequencing in comparison to FISH, were previously detected for microbial assemblage in the anoxic bottom waters of a volcanic subglacial lake (Gaidos et al., 2009). Furthermore, different abundances were detected by 16S clone library (0.2%) and CARD-FISH counts (13%) for particle associated *Bacteroidetes* in the Namibian upwelling system (Woebken et al., 2007). This indicates that the DNA extraction is incomplete for this phylum. Furthermore, the low GC content of some *Bacteroidetes* can lead to an underrepresentation in cloning-based methods (Temperton et al., 2009), which would explain the low frequency for *Bacteroidetes* determined for the fosmid insert end-sequencing data. However, as the CFB319a probe has many false negative hits and relative low group coverage (38%), abundances revealed by FISH techniques are likely overestimated.

A rather different picture was observed for *Epsilonproteobacteria.* The abundances of *Epsilonproteobacteria* determined by CARD-FISH were only about half as high as what was indicated by rRNAs abundances in the pyrosequencing dataset. The EPSY549 probe was shown to have a unique behavior as it showed increasing in fluorescence intensities with increasing formamide concentration (Lin et al., 2006). Therefore, really high formamide concentrations are required to detect all *Epsilonproteobacteria*. Although we used the proposed high formamide concentration of 55%, higher concentration could be necessary in order to detect the total epsilonprotoebacterial community. The rRNA genes retrieved from

the pyrosequencing dataset indicated much higher abundances of *Epsilonproteobacteria* than classification based on protein-coding genes with PAOLA. This indicates that for *Epsilonproteobacteria* not the extraction but the underrepresentation of this taxon in the public sequence databases is limiting for PAOLA classifications.

CARD-FISH and pyrosequencing have both their strengths and their weaknesses. At the domain-level, probes were shown to be less problematic than group-level probes, providing trustful quantitative data. Therefore, the comparison of pyrosequencing and CARD-FISH data at the domain level revealed that pyrosequencing retrieved data are semi-quantitative. At lower levels the limitations of CARD-FISH are increasing so that the quantities obtained from pyrosequencing are comparable to CARD-FISH data. An advantage of pyrosequencing data is that they reveal the richness of all known domains (*Bacteria*, *Archaea*, *Eukaryota*, and *Viruses*), phyla, class etc. without a priori knowledge. In order to achieve this by FISH methods, pre-selection of probes and time-consuming experiments are required. Therefore, pyrosequencing is currently a well-suited technique for fast biodiversity screenings that provides valuable insights on the overall community composition and indicates FISH probes that should be used for in-depths studies. Regarding the investigated site F, it is assumed that the combination of all methods covered largely the diversity in the habitat and led to the detection of all dominant groups that could be of interest in further analyses of metabolic pathways and group specific metabolic fingerprints.

Metabolic potential and energy sources in sulfur-mat sediments

The ultramafic-hosted Logatchev hydrothermal vent field emits fluids enriched in hydrogen, sulfide and methane. The dissolved hydrogen (19 mM), sulfide (2.5 mM) and methane (3.5 mM) concentration are all in the micromolar range (Schmidt et al., 2007). At site F, direct vent emission was not detected, but *in situ* profiles showed that fluid components diffuse towards the sediment-water interface along with the heat from deeper layers (Schauer et al., in revision). We investigated the genes captured within the metagenomes, in order to detect gene profiles that are selected by this environment and therefore provide insights into the environment's microbial ecology (Tringe et al., 2005).

In the surface layer of the sulfur-mat, high proportions of genes involved in amino acid biosynthetisis and inorganic sulfur assimilation were found. These subsystems were proposed to be an indicator for the presence of sulfur-utilizing microorganisms (Edwards et al., 2006). Hence, our metagenomic analysis is consistent with previous biogeochemical and molecular investigations characterizing the surface sediments as a sulfidic habitat. On the other hand, the

fraction of sulfur metabolism genes was not considerably higher than in non-sulfur influenced environments (Simon et al., 2009). That identified metabolic functional genes show little relevance to the geochemistry measurements has previously been reported by Biddle et al. (2008). One reason could be that many genes (98%) were unclassified since no close relatives were present in the non-redundant NCBI database. Moreover, analyses of all genes tend to underestimate the fraction of genes responsible for the energy metabolism, as this fraction of genes is much smaller in comparison to the fraction of genes used for the basic metabolism.

Therefore, we selected genes for the sulfur-, nitrogen and carbon cycle, used $PAOLA_{454}$ to cluster them to taxonomic bins ('taxobins') and determined the distribution of these selected subsystems for the four dominant classes - *Gammaproteobacteria*, *Epsilonproteobacteria*, *Deltaproteobacteria*, and *Bacteroidetes*. Similar overall gene distribution patterns were observed for all these classes, suggesting that at least on class-level, groups of genes relevant for a particular environment are enriched in all microbial groups in that environment (Tringe et al., 2005; Suen et al., 2009; Dinsdale et al., 2008). Genes involved in the nitrogen metabolism (1%) were in the range of the values determined for other metagenomes (lake in Antarctica, 1.5%; farm soil, 1.1%; hot springs 1.4%; glacier ice 1.3%).

However, differences in the distribution were detected for the metabolic pathways of each subsystem. *Epsilonproteobacteria* contained the highest number of genes for denitrification. This confirms the important role of this class in the oxic-anoxic interface (Brettar et al., 2006, Grote et al., 2007). Previous studies have verified their significant role in pelagic marine redoxclines of the Black Sea (Vetriani et al., 2003), the Cariaco Trench (Madrid et al., 2001) and the Baltic Sea (Grote et al., 2007), where oxygen deficiency and sulfide accumulation occurs. Therefore, chemolithoautotrophic oxidation of sulfur compounds coupled to nitrate reduction, such as denitrification, likely play an important role in the oxic-anoxic layer of this sediment.

Genes for the sulfur oxidation were exclusively found for *Gammaproteobacteria* and *Epsilonproteobacteria*, confirming their relevance as sulfur-oxidizing primary producers. The absence of genes of the reductive tricarboxylic acid (rTCA) cycle, which are generally used by *Epsilonproteobacteria* for CO_2-fixation (Nakagawa and Takai, 2008) indicates that besides *Epsilonproteobacteria* other groups like *Gammaproteobacteria* must play an important role in carbon fixation in the mat. The higher frequency of virulence genes in *Gammaproteobacteria* suggests that besides free-living forms they as well occur as symbionts as they are more abundant in the organism-associated microbes than in free-living microbes (Disdale et al., 2008).

The analysis of the selected pathways exhibit differences in the gene content for the four dominant groups. This suggests different responsibilities of each group in the different biogeochemical cycles present in the sulfur-mat microbial community. However, only for 2.3% a functional could be assigned. This could explain the absence of genes for the rTCA, which represents one of the principal carbon fixation pathways in deep-sea vent ecosystems (Campbell and Cary, 2004). To identify all genes involved in e.g. carbon fixation further investigations are necessary in order to reveal habitat- as well as taxon-specific genes in this environment.

Sulfide consumption and candidate sulfur oxidizing genes

Recent molecular and biogeochemical investigations (Schauer et al., in revision) and the present metagenomic analyses suggested that sulfide can serve as one energy source for the microbial community of the sulfur-mat at site F. Therefore, different genes for sulfur oxidation were analyzed from the sulfur-mat sediments at site F and AG. The analysis of *aprA*-genes revealed that these sulfur-mats consisted predominantly of different putative *Betaproteobacteria* and *Gammaproteobacteria* sulfur-oxidizers and putative sulfate-reducing *Deltaproteobacteria*. The presence of different *aprA*-genes confirmed that in these mats sulfur-based communities are present (Meyer and Kuever, 2007b). In contrast, non-hydrothermal sediment revealed putative alphaproteobacterial sulfur-oxidizing *aprA*-genes closely related to the SAR11 cluster, which forms together with the *Rosebacter* clade the dominant bacteriaplankton groups in the ocean. The reverse-acting dissimilatory APS reductase might be used for detoxification of sulfite, so that the product APS can be incorporated into the assimilatory sulfur metabolism (Meyer and Kuever, 2007b). The presence of *soxB*-genes related to *Epsilonproteobacteria* in the sulfur-mat sediments is consistent with the metagenomically retrieved data which revealed that both *Gammaproteobacteria* and *Epsilonproteobacteria* are capable of sulfur-oxidation.

Incubation experiments finally confirmed that sulfide is consumed by the surface sediment microbial communities, as sulfide consumption rates were detected. The sulfide consumption rates from the surface sediment community (0-1 cm, containing the overlying microbial mat) were fivefold higher than those of the overlying microbial mat. This raises the question to which extent which groups are involved in sulfide oxidation. Based on previous studies, it was hypothesize that highly abundant *Epsilonproteobacteria* in the overlying white mat are mainly responsible for sulfur oxidation. The *Epsilonproteobacteria*-mat revealed sulfide consumption rates comparable to those of the endosymbionts of the hydrothermal vent mussel

Bathymodiolus puteoserpentis from the same hydrothermal vent field (Zielinski et al., in prep), indicating a highly active community. However, highest rates of sulfide consumption detected for the surface layer suggested that sulfur oxidation is not limited to the overlying *Epsilonproteobacteria*-mat. Detected gammaproteobacterial *aprA*-genes and the presence of genes involved in sulfur oxidation in taxonomic bins related to *Epsilonprotebacteria* and *Gammaproteobacteria* propose that *Gammaproteobacteria* play as well an important role in the sulfur cycle in this sediment.

The detection of genes for uptake hydrogenases (*hupL*) suggested also chemolithotrophic primary production based on hydrogen consumption. Incubation experiment with hydrogen did not reveal detectable hydrogen consumption rates. Previous hydrogen consumption rates studies using sulfur-oxidizing symbionts showed that hydrogen consumptions rates increased with increasing dissolved hydrogen concentration (Zielinski et al., in prep.). However, even high dissolved hydrogen concentrations of 5-8 mM did not reveal any hydrogen consumption of sulfur-mat sediments at site F. This confirms that the deduction of metabolic capabilities from phylogenetic affiliation and of active metabolic processes from metabolic capablities always has to be evaluated by functional test such as incubations, and *in situ* measurements.

Conclusion

The comparison of pyrosequencing and CARD-FISH data from the same surface layer revealed that pyrosequencing is a well-suited technique for fast biodiversity screenings. The comparison of these methods at phylum and class level however revealed differences, so that FISH experiments should be used for in-depths studies. An advantage of pyrosequencing data is that the total diversity (*Bacteria*, *Archaea*, *Eukaryota*, and *Viruses*) at different taxonomic levels (phyla, class etc.) is investigated. Therefore, the combination of all methods covered largely the diversity in the habitat and led to the detection of all dominant groups that could be of interest for further analyses of metabolic pathways. The assignment to taxonomic bins by the tool PAOLA allowed the detection of taxon-specific genes. Taxonomic bins, as well as the detected diversity of the *aprA*- and *soxB*-genes, propose that members of *Epsilonproteobacteria* and *Gammaproteobacteria* represent active sulfur-oxidizing primary producers. In addition, metagenomic data proposed a potential importance of sulfide-oxidizing *Epsilonproteobacteria* in the oxic-anoxic layer as nitrate-reducers responsible for denitrification. Finally, incubation experiments confirmed the revealed metabolic potential for sulfur cycling of the microbial community in the surface sediment at the Logatchtev field.

High sulfide concentrations modulate the composition of active microbial communities at different hydrothermal vent fields, independently of the composition of the underlying rocks.

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3 Bacterial diversity and biogeography in deep-sea sediments of the South Atlantic Ocean

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ORIGINAL ARTICLE

Bacterial diversity and biogeography in deep-sea surface sediments of the South Atlantic Ocean

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Microbial biogeographic patterns in the deep sea depend on the ability of microorganisms to disperse. One possible limitation to microbial dispersal may be the Walvis Ridge that separates the Antarctic Lower Circumpolar Deep Water from the North Atlantic Deep Water. We examined bacterial communities in three basins of the eastern South Atlantic Ocean to determine diversity and biogeography of bacterial communities in deep-sea surface sediments. The analysis of 16S ribosomal RNA (rRNA) gene clone libraries in each basin revealed a high diversity, representing 521 phylotypes with 98% identity in 1051 sequences. Phylotypes affiliated with *Gammaproteobacteria*, *Deltaproteobacteria* and *Acidobacteria* were present in all three basins. The distribution of these shared phylotypes seemed to be influenced neither by the Walvis Ridge nor by different deep water masses, suggesting a high dispersal capability, as also indicated by low distance–decay relationships. However, the total bacterial diversity showed significant differences between the basins, based on 16S rRNA gene sequences as well as on terminal restriction fragment length polymorphism fingerprints. Noticeably, both geographic distance and environmental heterogeneity influenced bacterial diversity at intermediate (10–3000 km) and large scales (>3000 km), indicating a complex interplay of local contemporary environmental effects and dispersal limitation.
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Introduction

Biogeographic patterns in microbial communities are traditionally explained by two factors, the environmental heterogeneity and historical events (Martiny *et al.*, 2006; O'Malley, 2008). On the basis of the cosmopolitan hypothesis, 'everything is everywhere, but the environment selects' (Baas-Becking, 1934), environmental conditions have long been considered to have a strong influence on microbial biogeography. The effects of spatial distances (historical events) have been shown to affect microbial diversity in several studies (Papke *et al.*, 2003; Whitaker *et al.*, 2003; Martiny *et al.*, 2006; Ramette and Tiedje, 2007). The relative influences of environmental heterogeneity and historical events on microbial biogeography are still poorly understood. In marine habitats like the deep sea, microorganisms in the surface sediment may be assumed to disperse with oceanic currents. Bioirrigation by the activities of larger benthic organism as well as near-bed currents (Hughes and Gage, 2004; Queric and Soltwedel, 2007) influence the sediment-water interface exchange and consequently lead to the dispersal of particles and therefore of microorganism. Barriers to microbial dispersal could be physical (topography) or physiological conditions (temperature, pH or hydrostatic pressure).

In the eastern South Atlantic Ocean, the Cape Basin is separated from the Angola and Guinea basins by the Walvis Ridge that forms a barrier to the northward and southward flow of water below a depth of about 3000 m (Shannon and Chapman, 1991). Furthermore, the Cape Basin is dominated by Lower Circumpolar Deep Water arriving from Antarctica and the deepest part of the Angola and Guinea Basins are filled with North Atlantic Deep Water originating from the Arctic (Bickert and Wefer, 1996). Noticeably, the Walvis Ridge has been shown to function as a barrier for the dispersal of some crustacean species of *Peracarida* (Brandt *et al.*, 2005), but it is not known whether this physical barrier also affects microbial dispersal.

To analyze whether different deep water masses associated with the physical barrier of the

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Walvis Ridge have significant structuring effects on microbial diversity, the bacterial diversity in three deep-sea basin surface sediments was determined by 16S ribosomal RNA (rRNA) gene sequencing and the community fingerprinting method terminal restriction fragment length polymorphism (T-RFLP). The relative contribution of environmental heterogeneity and of historical events on microbial biogeography were assessed for this data set in concert with earlier published data on basaltic lavas in the Pacific Ocean (Santelli *et al.*, 2008), shallow permanently cold sediment of the Arctic Ocean (Ravenschlag *et al.*, 1999) and Antarctic continental shelf sediment (Bowman and McCuaig, 2003).

Materials and methods

16S ribosomal RNA gene clone libraries construction
Sediment sampling was performed on the DIVA II cruise by a multicorer (Barnett *et al.*, 1984) in water depths ranging from 5032 to 5649 m. The sediment cores were sliced on board in layers of 2 cm and the layers were subsampled top-to-bottom by sterile 1- to 2-ml syringes at 4 °C. After storage at −80 °C, DNA was extracted from 0.5 g of the surface sediment sample (0–2 cm) of the Cap, Angola and Guinea I areas (Figure 1, Table 2) after the protocol of the FastDNA SPIN Kit for Soil (Q-BIOgene, Carlsbad, CA, USA). Bacterial 16S rRNA genes were amplified using the primer pair GM3/GM4 (Muyzer *et al.*, 1995). The 100-μl reaction contained 30 ng DNA as template, 0.5 μM of each primer, 10 mM of dNTPs, 1 × buffer (Eppendorf, Hamburg, Germany) and 5 U of the Takara-Taq DNA polymerase (TAKARA, Dalian, China). PCRs were performed in 10 replicates with 20 cycles to minimize PCR bias. Final extension was performed 60 min at 60 °C to increase 3′-A-overhang. The amplicons were pooled and purified with a PCR purification kit (Qiagen, Hilden, Germany). Cloning of the amplicons was performed using TOPO TA Cloning Kit for sequencing (pCR4-TOPO, Invitrogen, Karlsruhe, Germany). Clones with a correct insert size of ∼1500 bp were

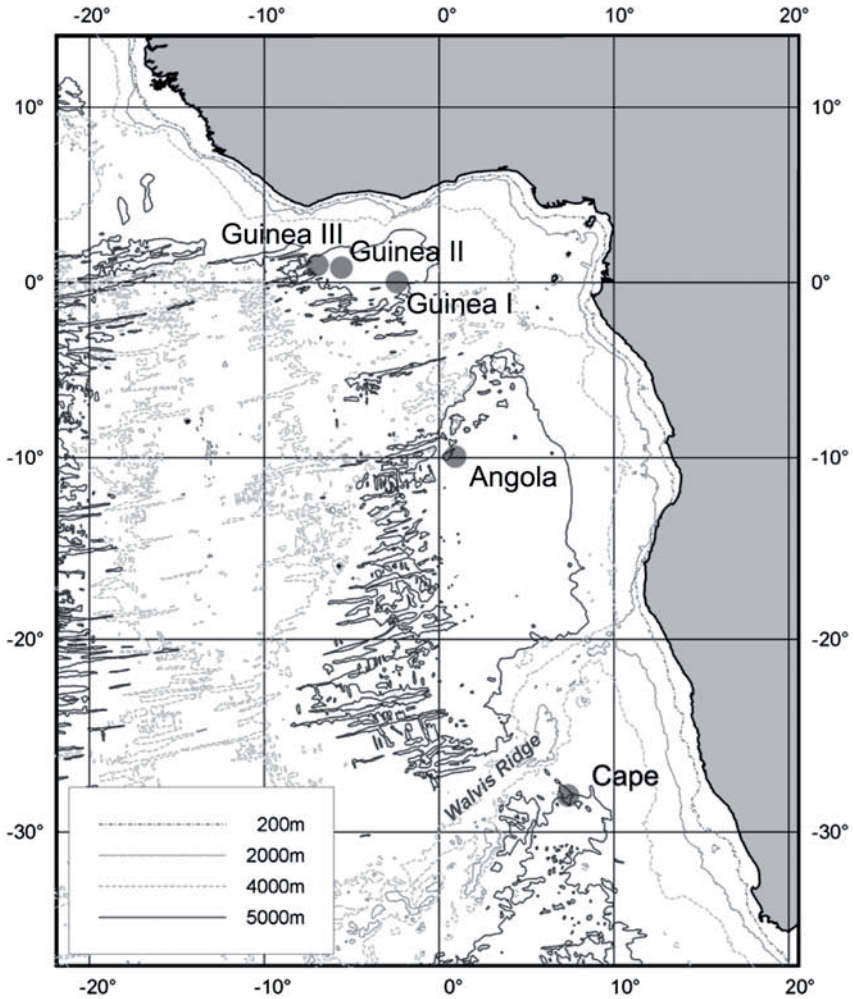

Figure 1 Sampling areas in the South Atlantic Ocean as well as the Walvis Ridge that separates the Cape Basin from the Angola and Guinea basins. For the 16S ribosomal RNA (rRNA) gene approach surface sediment (0–2 cm) of the Cape, Angola and Guinea I areas were used and for the terminal restriction fragment length polymorphism (T-RFLP) analysis 3–5 surface sediments of the Cape, Angola and Guinea I–III areas were analyzed.

sequenced using the vector primers M13 F (5′-GGAA ACAGCTATGACCATG-3′) and M13 R (5′-GTTGTAA AACGACGGCCAGT-3′).

Phylogenetic and sequence analyses

The quality of the obtained sequences was manually checked using Sequence Analysis 5.2 (Applied Biosystems, Weiterstadt, Germany). Full-length sequences were assembled with Sequencher (Gene Code, Ann Arbor, MI, USA). No chimeras were detected with Bellerophon (Huber *et al.*, 2004) and CHECK_CHIMERA (Maidak *et al.*, 1996). Sequences were imported into the ARB software package (Ludwig *et al.*, 2004) and aligned using the ARB FastAligner, then refined manually. The ARB software package was used to generate phylogenetic trees of 810 full-length sequences using the maximum likelihood algorithm with a 50% positional conservation filter and with 100 bootstrap replicates. Sequences reported in this study were deposited at EMBL under the accession numbers AM997284–AM997988 for 705 full-length sequences and under AM997989–998333 and AM997283 for 346 partial sequences.

The software distance-based OTU and richness (DOTUR) was applied to ARB distance matrices generated with the Jukes-Cantor correction to estimate operational taxonomic units (OTU), rarefaction curves of observed OTUs, richness estimators and diversity indices (Schloss and Handelsman, 2005). A sequence identity of 98% was used to define OTUs, as this cut-off roughly corresponds to the species level (Rossello-Mora and Amann, 2001; Stackebrandt and Ebers, 2006). The statistical tool $\int$-LIBSHUFF was applied to genetic distance matrices to determine whether differences in library composition were because of chance or to biological effects, and significances were assessed by Monte Carlo permutations and further corrected for multiple comparisons (Schloss *et al.*, 2004). The statistical tool SONS (Schloss and Handelsman, 2006) was used on full-length 16S rRNA gene sequences to calculate Chao1 shared richness estimates, the J_{class} index for the ratio of shared to total number of OTUs, and θ_{yc} for the estimated similarity in community structure between any two communities.

Terminal restriction fragment length polymorphism

Terminal restriction fragment length polymorphism analyses included three to five samples of surface sediments (0–2 cm) from several cores of each area, Cape, Angola and Guinea I–III (Figure 1, Table 2). Genomic DNA was extracted from 0.5 g sediment samples using the FastDNA Spin Kit for Soil (Q-Biogene, Irvine, CA, USA). PCR amplification of the 16S rRNA gene was carried out using the fluorescently labelled primers 27F (FAM, 5′-AGAGTTTGA TCCTGGCTCAG-3′) and 907R (HEX, 5′-CCGTCAAT TCCTTTRAGTTT-3′), targeting all bacteria as well as 558F (FAM, 5′-ATTGGGTTTAAAGGGTCCG-3′) (Abell and Bowman, 2005a, b) and 1390R (HEX, 5′-GACGGGCGGTGTGTACAA-3′) (Zheng *et al.*, 1996), targeting the class *Flavobacteria*. Undigested and digested amplicons were identified by capillary electrophoresis to verify the absence of false-positive fragments in the undigested control and the completeness of the digestion. PCRs were carried out in a total volume of 25 µl, including 12.5 µl PCR Master Mix (Promega GmbH, Mannheim, Germany), 1 µM forward and reverse primer, and 5–24 ng DNA template. PCR reactions were carried out in triplicates and purified on Sephadex columns (Sephadex G-50 Superfine, Amersham Biosciences AB, Uppsala, Sweden). PCR amplicons (70-120 ng) were digested in a total volume of 10 µl at 37 °C for 3 h using 5 U of the restriction enzyme *AluI* (Fermentas, Burlington, Canada) for bacterial amplicons and 5 U of the enzyme *MspI* (Fermentas) for *Flavobacteria* amplicons. The two restriction enzymes were chosen based on high numbers of unique terminal restriction fragments assessed with *in silico* analyses using enzyme restriction power analysis (http://mica.ibest.uidaho.edu/) as well as on best performance in laboratory experiments (that is, producing maximum numbers of terminal restriction fragments (TRFs)). After heat inactivation (65 °C, 25 min) and purification on Sephadex columns, detection of TRFs was performed on a ABI Prism 3130 XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) equipped with a 80-cm capillary, a POP-7 polymer and the filter set DS-30. The ROX-labelled MapMarker 1000 (Eurogentec, Seraing, Belgium) served as a size standard between 50 and 1000 bp. The fragment profiles were visualized and automatically analyzed with GeneMapper v. 3.7 Software (Applied Biosystems), using standardized settings with a peak detection cut-off set to 30 fluorescence units. The 5′-end labelled TRFs were used as they produced a higher number of fragments in comparison with 3′-end TRFs (Suzuki *et al.*, 1998; Osborn *et al.*, 2000).

A binning procedure was applied to the GeneMapper output to compensate for slight peak shifts between runs and for TRF size calling imprecision, in order to avoid artificial, technically derived differences between profiles (Hewson and Fuhrman, 2006). The technical variability of peak size calling in different replicates including runs conducted on different days was determined as of ± 0.25 bp (window size of 0.5 bp). The binning function included two different starting points (50 and 50.25 bp) and the binning strategy yielding higher correlation between all samples was selected for further statistical analyses. The binning window was adjusted to 1 bp for samples amplified with *Flavobacteria* primers, because a window frame of 0.5 bp did not yield higher resolution. The computation was carried out with the Interactive Binner function (Ramette, 2009 http://www.ecology-research.com). The output consisted of a table of TRFs with

corresponding relative fluorescence intensities, which are the individual peak area divided by the total area of peaks in a given profile. Master profiles were generated by building a consensus table of the binned TRF profiles for all samples from one basin, averaging the respective relative fluorescence intensities values of all samples. A TRF was considered present if it appeared in one or more PCR parallels, therefore including all natural and technical variability at this level of analysis.

Statistical analyses
Non-metric multidimensional scaling (nMDS) and analysis of similarity (ANOSIM) were carried out with the program PAST (Paleontological Statistics, ver. 1.47, http://folk.uio.no/ohammer/past). Simple and partial Mantel tests were used to determine the significance and correlation coefficients between genetic-, spatial- and environmental distance matrices, using the R package vegan (http://vegan.r-forge.r-project.org/) (Legendre and Legendre, 1998; Mantel, 1967). Spatial dissimilarities based on geographic distances between sites and environmental dissimilarities (temperature, salinity, pH, Eh, TOC, Chl *a* and grain size; Table 1) were used to explain genetic dissimilarity. To determine the strength of the relationship between genetic and geographic distance linear models were fitted and slope coefficients were calculated with their 95% confidence intervals.

Results and discussion

Bacterial biomass and richness in sediments of the South Atlantic Ocean
The cell numbers of the suboxic surface sediments (0–2 cm) in three eastern South Atlantic Ocean basins were $3.4–3.7 \times 10^9$ cells g^{-1} sediment (Table 1). The abundances were in the range found in other deep-sea sediments (9.2×10^8 cells g^{-1} (Deming and Colwell, 1982), 1.5×10^9 cells g^{-1} (Guezennec and Fiala-Medioni, 1996) and 5×10^8 cells g^{-1} (Harvey *et al.*, 1984)). The 16S rRNA gene libraries showed a high diversity with up to 20 different phyla in the Cape Basin and 17 phyla in the Angola and Guinea basins (Figure 1). Earlier described deep-sea (Bowman and McCuaig, 2003; Polymenakou *et al.*, 2005, 2009; Xu *et al.*, 2005) and shallow sediments (Ravenschlag *et al.*, 1999) had also found a large diversity, which may be based on a weak and symmetric competition (Grant, 2000). The reciprocal Simpson's indices for all sites were above 50 (Table 2), suggesting evenly distributed diversity profiles as typical dominance profiles show $1/D$ values below approximately 50 (Zhou *et al.*, 2002). Total richness estimates (Chao1) (Table 2) and rarefaction curves (Supplementary Figure S1) based on a 98% sequence identity showed that Cape, Angola and Guinea basin surface sediments contained an equal bacterial richness at a significance level of 0.05.

Both analyses predicted a lower richness for the South Atlantic sediments in comparison to the Antarctic sediments and a higher richness in comparison to the Arctic sediment. The library-based equality of richness was supported by the T-RFLP analysis, as basin-specific master profiles showed a comparable OTU richness (167, 190 and 182 TRFs for the Cape, Angola and Guinea Basin, respectively) (Figure 4a).

Bacterial diversity of the 16S ribosomal RNA genes
The clone libraries contained 521 phylotypes with 98% identity in 1051 sequences, containing 705 full-length sequences. Applying a 100% identity threshold revealed 230 sequences, which were present at least twice, with a majority of 176 sequences (18 OTUs) present in all deep-sea sediments. The bacterial communities were dominated by *Proteobacteria*, which accounted for 64, 58 and 63% of all sequences in the Cape, Angola and Guinea Basin, respectively, with the class *Gammaproteobacteria* representing 45, 37 and 40% of all sequences in the respective basins (Figure 2). The class *Gammaproteobacteria* comprised 116 phylotypes (98% identity, 427 sequences), of which 39 phylotypes (138 sequences) were related to known cultivated species. These belonged mainly to families of psychrophilic microorganisms including *Enterobacteriaceae, Alteromonadaceae, Oceanospirillaceae* and *Legionellaceae* (Figure 3a). Among

Table 1 Sediment data (Türkey and Kröncke, in preparation) and cell numbers of microbial communities in the South Atlantic Ocean

Basin	*Depth (mbsl)*[a]	*Temp. (°C)*	*Salinity (‰)*	*pH*	*Eh (mV)*	*TOC (%)*	*Chl a (μg g⁻¹)*	*Grain size (%)*		*Cell counts (cell g⁻¹)*	*MPN (cells ml⁻¹)*
								<63μm	*>63 μm*		
Cape	5032	1.14	34.6	7.74	177	0.83	0.017	92.89	6.87	3.5×10^9	1.22×10^4
Angola	5649	ND	ND	7.72	96	0.9	0.069	83.84	16.4	3.4×10^9	2.67×10^5
Guinea I	5063	2.1	34.9	7.77	183	0.72	0.264	84.23	15.23	3.7×10^9	2.67×10^4
Guinea II	5225	ND	ND	7.76	132	0.77	0.301	84.99	14.46	ND	ND
Guinea III	5525	2.1	34.5	ND	ND	0.76	0.152	86.45	13.34	ND	ND

Abbreviation: ND, not detected.
[a]Meters below sea level.

Table 2 Sampling sites of sediments used for 16S rRNA gene sequencing or for T-RFLP analysis with corresponding richness and diversity indices for bacteria

Sampling area	*Latitude*	*Longitude*	*Depth (mbsl)*[a]	*Stations*[b] *T-RFLP*	*Stations*[b] *16S rRNA gene seq.*	*No. of clones*[c]	*OTU DOTUR 0.02*	*Richness estimator Chao1*[d]	*Simpson 1/D*	*Study*
Cape	28°06′42″ S	7° 20′48″ E	5032	33, 34, 37, 38	33	342 FP	202	466 (369, 620)	77	This study
						228 F	**145**	**508 (351, 785)**	**53**	
Angola	9°56′00″ S	0° 53′48″ E	5649	46, 48, 50	46	354 FP	183	256 (227, 305)	77	This study
						219 F	**126**	**369 (259, 570)**	**59**	
Guinea I	0°00′00″ S	2°25′06″ W	5063	56, 58, 59, 60, 61	60	355 FP	203	369 (308, 465)	125	This study
						258 F	**155**	**489 (348, 735)**	**91**	
Guinea II	0°50′00″ N	5° 35′00″ W	5225	74, 75, 76, 77, 79	—	—				This study
Guinea III	0°37′12″ N	6° 28′06″ W	5525	95, 97, 99	—	—				This study
Antarctic continental shelf	66°31′86″ S	143°38′30″ E	761	—	MERTZ 0–2 cm	590 P	322	899 (713, 1175)	125	Bowman and McCuaig, 2003
Arctic ocean Svalbard	79°42′81″ N	11°05′18″ E	218	—	Station J	123 P	84	125 (104, 167)	167	Ravenschlag *et al.*, 1999
East Pacific	9°28′48″ N	104°13′48″ W	2516	—	EPR	**370 F**	**239**	**601 (475, 796)**	**200**	Santelli *et al.*, 2008
Rise	−9°50′38″ N	−104°17′86″ W	−2674							
Hawaii	18°52′17″ N	155°14′53″ W	888	—	PV	**472 F**	**276**	**764 (597, 1017)**	**167**	Santelli *et al.*, 2008
	−18°58′31″ N	−155°53′42″ W	−1714							

Abbreviations: DOTUR, distance-based OTU and richness; OTU, operational taxonomic units; rRNA, ribosomal RNA; T-RFLP, terminal restriction fragment length polymorphism.
[a]Meters below sea level.
[b]For details see cruise report DIVA II (M63/2).
[c]Number of full-length (F) and partial (P) sequences, full-length sequences and values calculated from them are presented in bold.
[d]Chao1 richness with lower and upper bound of 95% confidence interval.

these phylotypes 11 OTUs (12 sequences) clustered with the NOR5/OM60 clade that includes '*Congregibacter litoralis*' strain KT71, the first marine aerobic anoxygenic phototrophic *Gammaproteobacteria* in culture (Fuchs *et al.*, 2007; Yan *et al.*, 2009). Three phylotypes (5 sequences) were related to free living (*Thiothrix*) and endosymbiotic sulfur oxi-'dizers and methylotrophic bacteria. A large portion of 77 phylotypes (289 sequences) clustered distinctly from cultured species to JTB255/BD3-6 (38 phylotypes, 192 sequences), BD7-8/MERTZ (10 OTUs, 36 sequences), JTB23/Sva0091 (18 OTUs, 34 sequences) (Figures 3a and b) and to Cret-1F, BD1-1, PWP and South Ionian groups (11 OUT, 27 sequences). These groups included only 16S rRNA gene sequences that originated from other deep-sea or permanent cold marine habitats (Kato *et al.*, 1999; Li *et al.*, 1999; Ravenschlag *et al.*, 1999; Urakawa *et al.*, 1999; Bowman and McCuaig, 2003; Polymenakou *et al.*, 2005; Xu *et al.*, 2005; Zhao and Zeng, 2005).

The *Alpha-*, *Beta-* and *Deltaproteobacteria* accounted together for 18 to 23% of all sequences in the libraries. *Deltaproteobacteria* (11 to 14%) outnumbered *Alphaproteobacteria* (6 to 8%) and *Betaproteobacteria* (1 to 3%) (Figure 2). Other groups with a sequence abundance of over 5%, which occurred in all three basins, were the phyla *Chloroflexi* (1, 10 and 4% for Cape, Angola and Guinea basins, respectively), *Planctomycetes* (6, 4 and 10%), *Acidobacteria* (4, 7 and 5%) and *Bacteroidetes* (10, 4 and 6%).

Bacterial diversity comparison

The proportion of bacteria present in two or three basins was high in the 16S rRNA gene sequences analyses (23%) and in the T-RFLP analyses (58%) (Figure 4a). A third of the fragments (93 TRFs) was detected in the sediments of all basins and represented 82% of the total relative fluorescence intensities. Among the 16S rRNA gene sequences, a shared membership of 19 OTUs (98% identity) was found in all three basins with the statistical tool SONS. The manual assignment in ARB confirmed the small fraction of OTUs detected in all three basins (29 OTUs, 347 sequences), but provided additional information regarding the sequence abundance and identity of each OTU. These were dominated by *Gammaproteobacteria* (76%; Figure 4b). In this class, the common members were related to marine heterotrophic aerobic and facultative anaerobic microorganisms (*Alteromonadaceae* and *Oceanospirillaceae*), photoheterotrophic aerobic bacteria (NOR5/OM60 clade) (Fuchs *et al.*, 2007), and to groups consisting of uncultivated bacteria

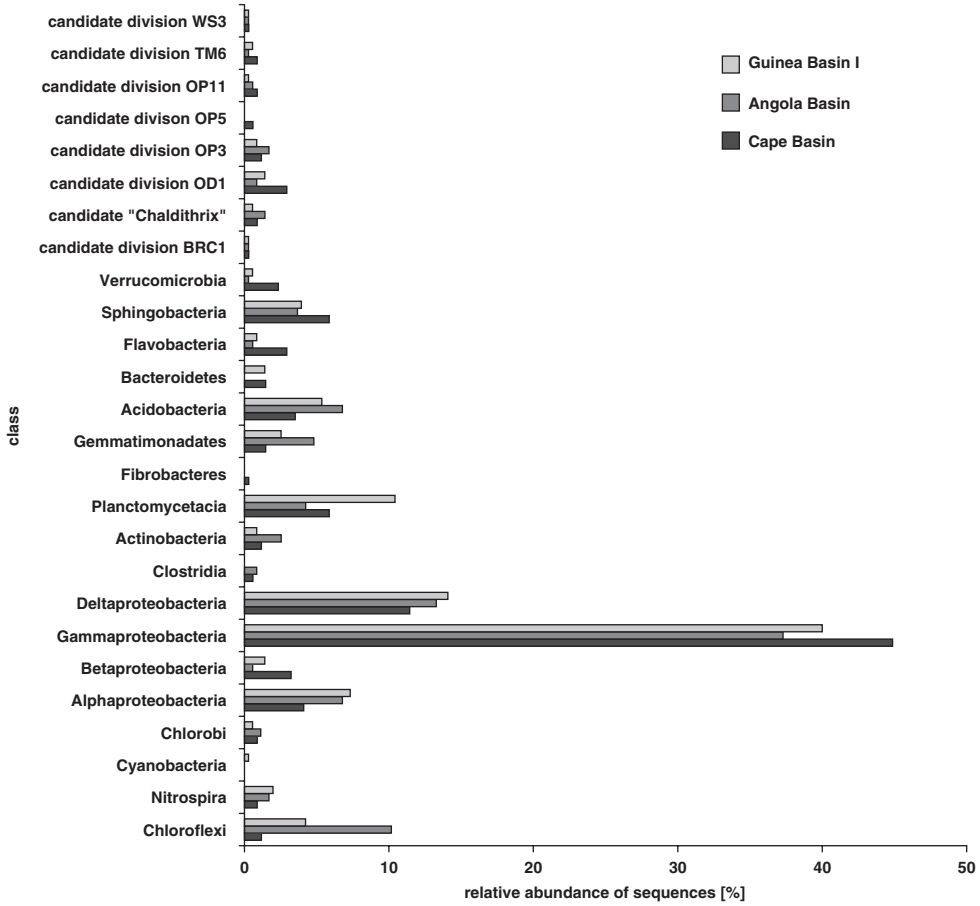

Figure 2 Bacterial diversity in the Cape Basin (342 sequences), Angola Basin (354 sequences) and Guinea Basin (355 sequences). All detected classes in the domain *Bacteria* are shown.

(JTB255/BD3-6, JTB23/Sva0091/BD3-1, BD7-8/MERTZ, Gret-1F and South Ionian).

Phylotypes present in two of three basins belonged to the *Gamma-* and *Deltaproteobacteria* and to the *Chloroflexi*. A major group of *Chloroflexi*-OTUs were restricted to Angola and Guinea basin sediments (7 OTUs, 21 sequences). The $\int$-LIBSHUFF analyses revealed no significant difference between the Angola and Guinea libraries as well as Cape and Guinea libraries (using a minimum *P*-value of 0.0012) (Supplementary Table S1). Thus, common phylotypes dominate the communities of these basins. The largest number of TRFs covered by two basins was found for the Angola and Guinea basins (30 TRFs, 30.5 relative fluorescence intensity) (Figure 4a). High chlorophyll *a* contents were detected in the Angola and Guinea surface sediments indicating a large fraction of fresh, recently arrived organic carbon (Table 1, Türkay and Kröncke, in preparation). This probably originated from a primary productivity in the surface waters that can be linked to the discharge of nutrients from the Congo and the Niger Rivers into the Angola and Guinea basins, respectively (Schefuss *et al.*, 2004).

Angola and Cape basins showed significantly different communities ($\int$-LIBSHUFF test, $P = 0.008$) and significantly different *Flavobacteria* T-RFLP profiles (Figure 5b) (analysis of similarity, *R* values 0.869, $P < 0.001$) (Supplementary Table S2). These differences were consistent with a different chlorophyll *a* content as well as a different sediment particle size in the Cape Basin (Table 1) (Etter and Grassle, 1992), indicating that environmental factors seem to influence bacterial communities in deep-sea sediments of the eastern South Atlantic Ocean. It is, however, needed to also take spatial parameters into account in this analysis to strengthen our interpretation concerning environmental or spatial effects on the observed community shifts.

Biogeography: environmental and historical factors

In the eastern South Atlantic Ocean the Walvis Ridge separates the Cape Basin from the Angola and Guinea basins below a depth of about 3000 m and causes different deep water masses in these basins. The dominance of common phylotypes in the 16S rRNA gene libraries and T-RFLP master profiles suggested that microbial dispersal may not be influenced by the Walvis Ridge or by the presence of different water masses. This was

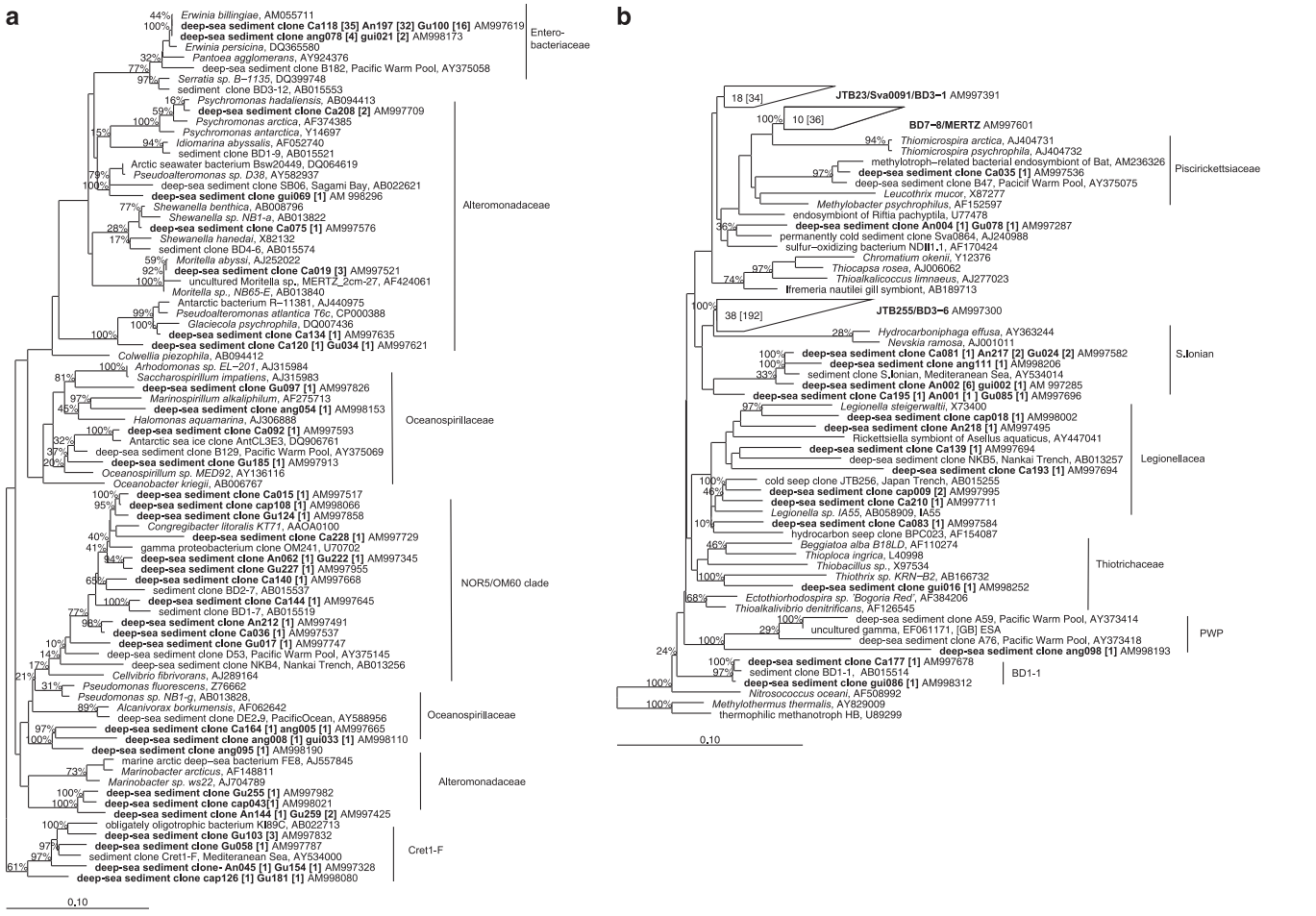

Figure 3 Phylogenetic tree based on 16S ribosomal RNA (rRNA) gene sequences of the class *Gammaproteobacteria* showing position of (**a**) marine heterotrophic aerobic and facultative anaerobic microorganisms and photoheterotrophic aerobic (NOR5/OM60 clade) bacteria and (**b**) potential auto- or mixotrophic sulfur oxidizers and bacteria that inhabit various geographic regions (JTB255/BD3-6). The tree was calculated using the maximum-likelihood algorithm with a 50% positional conservation filter and with 100 bootstrap replicates. The bar represents 10% estimated sequence divergence. Full-length sequences (Ca, An and Gu), partial sequences (cap, ang and gui), the number of OTUs in a cluster and the corresponding number of sequences (squared bracket) are shown.

supported by the significantly similar distance–decay relationships of the TRFs in the pairwise comparison (Cape/Angola, slope coefficient 6.9×10^{-5} and 95% confidence interval (3.4×10^{-5}, 10.3×10^{-5}); Angola/Guinea, slope coefficient 8.7×10^{-5} and 95% confidence interval (1.5×10^{-5}, 15.9×10^{-5})). Phylotypes common in the communities of the South Atlantic Ocean and the Pacific, Antarctic and Arctic Oceans sediments (Supplementary Table S1) indicated that some microorganisms disperse effectively over a huge distance and therefore are cosmopolitan, at least at the resolution of 16S rRNA genes that is insufficient for the classification of microorganisms into species (Konstantinidis and Tiedje, 2005).

To get more information regarding the amount of spatial structure present, we analyzed the relative relationships between genetic diversity and geographic distances. The 16S rRNA gene and TRFs based distance–decay relationships for the South Atlantic Ocean and for all sites were all very low (0.003 to 0.07) (Table 3), as also found in taxa-area relationships for soil and salt marsh communities (0.03 to 0.074) (Green *et al.*, 2004; Horner-Devine *et al.*, 2004; Fierer and Jackson, 2006), suggesting high dispersal rates and low extinction rates because of vast population sizes (Connor and McCoy, 1979).

From the clustering of TRF profiles by basins, as shown by non-metric multidimensional scaling (Figure 5a) associated with large, significant R values for all pairwise comparisons between the deep-sea basins (analysis of similarity, 0.586 to 0.999, $P<0.001$) (Supplementary Table S2), and from significant differences between the South Atlantic Ocean communities to all other communities ($\int$-LIBSHUFF tests, Supplementary Table S1), it seemed obvious that communities were structured either by the contemporary environment, spatial distances (historical events) or by a combination of both (Martiny *et al.*, 2006; Ramette and Tiedje 2007).

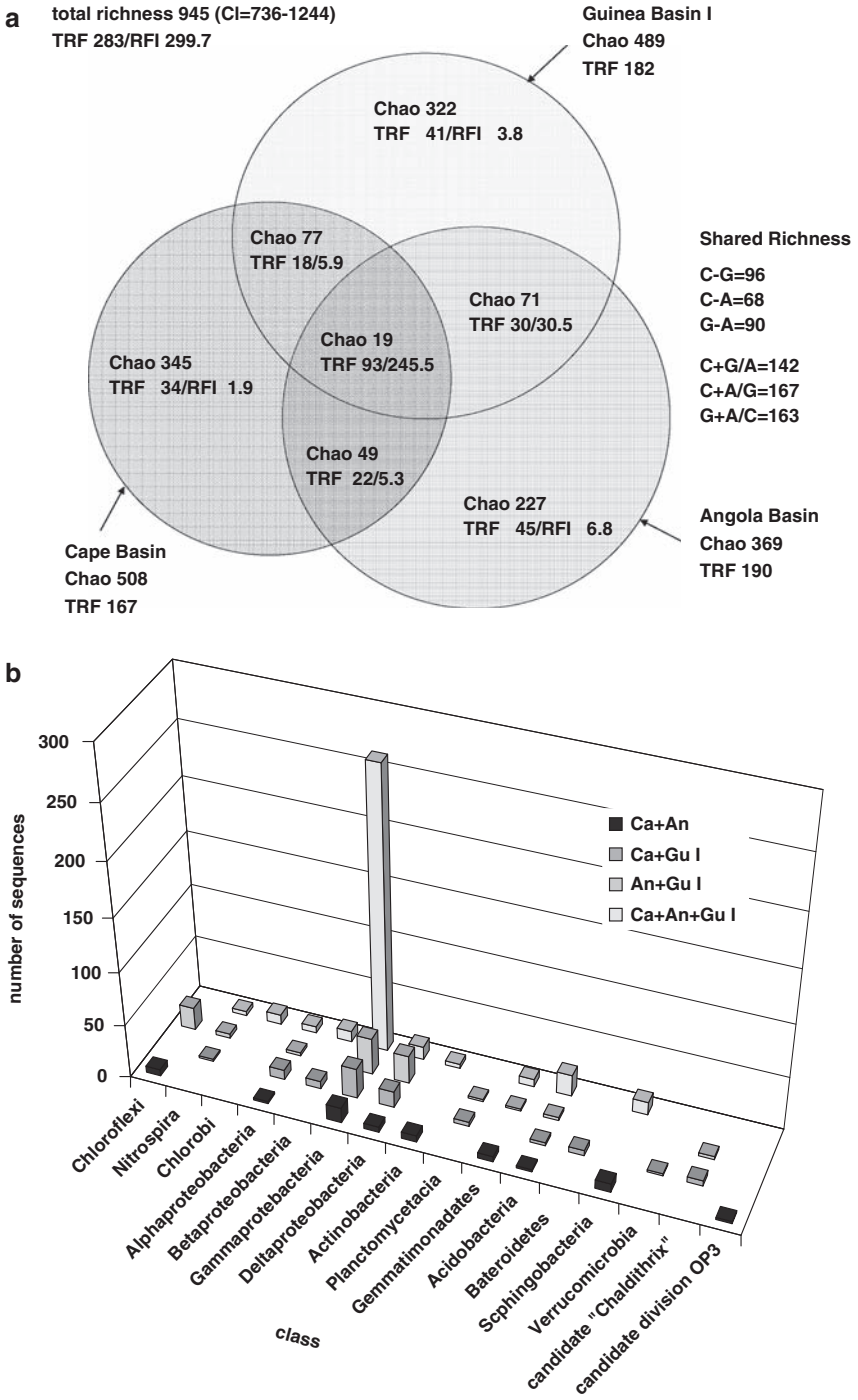

Figure 4 (**a**) Shared and basin-specific SONS generated $OTU_{0.02}$ and terminal restriction fragment (TRF) and corresponding relative fluorescence intensities (RFI) values for the three deep-sea basin of the South Atlantic Ocean. (**b**) Phylogeny of the shared OTUs between three and two deep-sea basins and the corresponding number of sequences.

To disentangle the relative influence of environmental heterogeneity and spatial distance on the distribution of microbial deep-sea sediment communities, we used a combination of simple and partial Mantel tests. For distances of 0–1200 km T-RFLP results showed a comparable influence of both factors (environment $r=0.636$, $P<0.001$, geography $r=0.651$, $P<0.001$) (Table 2) (Figure 6b). But environment ($r=0.588$, $P<0.001$) overwhelmed any effect of geographic factors ($r=0.278$, $P=0.009$) for intermediate distances (1200–3500 km), as also supported by significant partial Mantel tests (Table 3). A higher correlation between spatial and genetic distance for small spatial scales (<200 m) was reported for other microbial groups in soil (Cho and Tiedje, 2000), suggesting the existence of

Figure 5 (**a**) Non-metric multidimensional scaling (nMDS) plot using Bray–Curtis distance for the data set derived from amplification with bacterial primers 27F and 907R and subsequent digestion with *AluI*. Stress: 0.15. (**b**) nMDS plot using Bray–Curtis distance for the data set derived from amplification with *Flavobacteria*-specific primers 517F and 1457R and digestion with *MspI*. Stress: 0.07.

endemic taxa, as genetic distance increases with spatial distances.

Mantel tests for 16S rRNA gene sequences revealed that both environment and geography ($r=0.008$, $P=0.006$ and $r=0.024$, $P=0.001$, respectively) had an influence on the bacterial diversity of the South Atlantic. Significant correlations between genetic and geographic distances (Mantel's coefficient $r=0.013$, $P=0.001$) (Table 3, Figure 6a) were as well observed for all deep-sea and coastal sediments.

When compared in more detail, the two methods showed different results for the relative influence of both factors on microbial biogeography. These detected differences reflect different levels of similarity, saturation and resolution of each method and sampling effort, for example, the T-RFLP analyses including Guinea I versus Guinea I+II+III (Table 3). Indeed, the analysis of 16S rRNA gene sequences

Table 3 Slope coefficients and Mantel *r* statistics for genetic distance matrices derived from 16S rRNA gene sequences or T-RFLP

	Slope coefficient (genetic versus geographic distance)		*Simple Mantel tests*			*Partial Mantel tests*		*Number of samples*
			Geography[a]		*Environment*	*Geography*	*Environment*	
	$\times 10^{-5}$	*Log*$_{10}$ *transformed*	*Untransformed*	*Log*$_{10}$ *transformed*				
South Atlantic, 0–1200 km distance								
T-RFLP	28.28 [21.34, 35.22][b]	0.074 [0.053, 0.095]	0.651 (<0.001)*	0.593 (<0.001)*	0.657 (<0.001)*	0.054 (0.306)	0.131 (0.134)	14
South Atlantic, 0–3500 km distance								
16S rRNA gene	0.19 [0.17, 0.22]	0.0036 [0.0028, 0.0044]	0.024 (0.001)*	0.012 (0.001)*	0.008 (0.006)*	0.031 (0.002)*	−0.021 (0.981)	3
T-RFLP (Guinea I)	7.5 [5.38, 9.62]	0.076 [0.063, 0.090]	0.698 (0.004)*	0.841 (0.001)*	0.886 (0.001)*	−0.138 (0.86)	0.768 (<0.001)*	11
T-RFLP (Guinea I+II+III)	2.6 [1.24, 3.96]	0.055 [0.042, 0.068]	0.278 (0.009)*	0.532 (0.001)*	0.599 (<0.001)*	−0.227 (0.992)	0.573 (<0.001)*	19
All sites, 0–18 000 km distance								
16S rRNA gene	0.02 [0.019, 0.022]	0.0030 [0.0027, 0.0032]	0.013 (0.001)*	0.013 (0.001)*	NA	NA	NA	7

Abbreviations: NA, no environmental parameters available for all comparisons; rRNA, ribosomal RNA; T-RFLP, terminal restriction fragment length polymorphism.
For simple and partial Mantel tests, *P*-values based on 1000 permutations are indicated in parentheses with * when the *P*-values are still significant after Bonferroni correction for multiple comparisons.
[a]For simple Mantel tests, geographic distances were either left untransformed or log10 transformed to allow for comparisons with previously published studies.
[b]Lower and upper bounds of 95% confidence interval for the slope coefficients are indicated within square brackets.

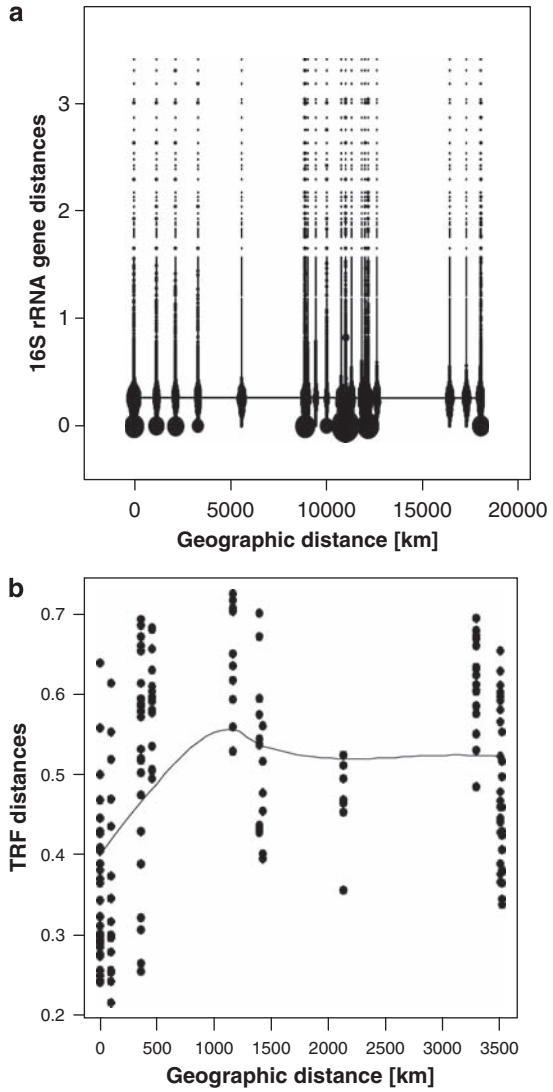

Figure 6 Scatterplots with loess curve presenting (**a**) genetic dissimilarity plotted against geographic distance for genetic dissimilarity of distance matrices derived from 16S ribosomal RNA (rRNA) gene sequences of samples of the South Atlantic and (**b**) for terminal restriction fragment length polymorphism (T-RFLP) profiles of samples from five different stations in the South Atlantic (Cape, Angola, Guinea I, Guinea II and Guinea III).

provides information regarding randomly chosen phylotypes ('sampling communities') where the finding of an OTU is proportional to its abundance in the clone library (Bent and Forney, 2008). In contrast, the fingerprinting method T-RFLP screens for all OTUs present above the detection threshold of the method ('screening' communities; Bent and Forney 2008), typically $>6 \times 10^2$–10^3 DNA fragment copies per ml samples (Ramette 2009), but does not provide clear taxonomic distinction (Dunbar *et al.*, 2001).

Although high dispersal rates were detected for some groups in deep-sea sediments, both T-RFLP and 16S rRNA-based analyses suggest barriers for the dispersal of microorganisms in the deep sea. The influence of both factors at intermediate scales was already shown by other studies (Green *et al.*, 2004; Reche *et al.*, 2005; Yannarell and Triplett, 2005), but our study suggest an effect of both factors for large scales as well, as shown for soil microbial communities (Fierer and Jackson, 2006). Although the small size, high dispersal rates, large population size and low extinction rates of microorganisms suggest a low effect of geographic barriers on microorganisms (Staley and Gosink, 1999; Beja *et al.*, 2002; Finlay, 2002; Ramette and Tiedje, 2007), our study shows that the distribution of microorganisms in deep-sea sediments is limited at intermediate (10–3000 km) and large scales (>3000 km).

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III General conclusions

1 Discussion

1.1 Microbial diversity

Most deep-sea floors are covered with permanently cold sediments. Microorganisms in these sediments are responsible for the mineralization of the organic carbon that reaches the deep-sea floor from the photic zone. The eastern South Atlantic Ocean is known for its high productivity (Berger and Wefer, 1996). However, the microbial diversity in the sediments of the three abyssal plains (Cape, Angola and Guinea basins) in the eastern South Atlantic Ocean was so far not investigated.

Phylogenetical analyses of 16S rRNA gene sequences revealed a highly diverse bacterial community for all three deep-sea basins and therefore confirmed previously described rich diversities of other marine and deep-sea sediments (Ravenschlag et al., 1999; Bowman and McCuaig, 2003; Polymenakou et al., 2005; Hongxiang et al., 2008). The analysis of the microbial community structures within the South Atlantic Ocean revealed shared phylotypes in all three deep-sea basins. Most sequences belonging to this shared community were affiliated with *Gammaproteobacteria*. In this class, the common-to-all basin members were related to marine heterotrophic aerobic and facultative anaerobic microorganisms (*Alteromonadaceae* and *Oceanospirillaceae*) and photoheterotrophic aerobic (NOR5/OM60 clade) bacteria. These microorganisms utilize a broad range of organic compounds (Fuchs et al, 2007). *Acidobacteria* represented another common-to-all basin phylum that inhabits a large variety of habitats (soil, freshwater habitats, marine sediments, marine sponges, acidic mining lakes, hot springs). Their broad distribution suggests an important ecological role (Quaiser et al., 2003). They have great metabolic versatility (Quaiser et al, 2003) and, according to metagenomic analyses, they are capable to degrade aged organic matter like recalcitrant organics (Quaiser et al, 2008). These heterotrophic microorganisms of the families *Alteromonadaceae* and *Oceanospirillaceae* and of the class *Acidobacteria* could be involved in the first step of aerobic remineralization of complex organic carbon in deep-sea sediments. Dissimilatory sulfate reduction (DSR) is responsible for the anaerobic degradation of organic matter in marine sediments, which forms the basis of the biological sulfur cycle (Heinrichs and Reeburgh, 1987; Jørgensen 1982, Widdel 1988). Sequences affiliated with sulfate-reducing heterotrophic *Deltaproteobacteria* were detected in all three basins. This group could use the first fermentation products for further anaerobic remineralization. In

addition to heterotrophic bacteria, members related to the group JTB23/Sva0091/BD3-1 and BD7-8/MERTZ, which are potential auto- or mixotrophic sulfur oxidizers were present. Reduced sulfur compounds are end products of the sulfate reduction. Therefore, chemolithoautotrophic bacteria are likely able to oxidize reduced sulfur compounds for the fixation of CO_2.

Significant differences in bacterial composition were only detected between the Angola and Cape basins (Table 7). In the Angola and Guinea basins, high frequencies of *Chloroflexi*-related sequences were found, in contrast to the Cape Basin. The detected chlorophyll *a* contents for the Angola and Guinea basins revealed fresher organic carbon and therefore a higher macromolecular fraction in these sediments compared to the Cape Basin. Furthermore, the supply of terrigenous materials by the rivers could transport human organic solvents and degreasing agents like tetrachloroethenes (PCE) and trichloroethene (TCE) to the deep-sea surface sediments of the Angola and Guinea basins. Members of the genus *Dehalococcoides* of the phylum *Chloroflexi* can utilize chloroethenes as substrates by a dehalorespiration, where chloroethenes are transformed to nontoxic products (Hendrickson et al., 2002). Therefore, presence of *Chloroflexi*-related sequences in these two basins can be linked to a

Table 7: Comparison of bacterial community structures of different deep-sea floors by the statistical tool ∫-LIBSHUFF (*P*-value of significance=0.0005).

	Cape	**Angola**	**Guinea I**	**OC**	**Quest**	**AG**	**site F**	**EPR**	**Hawaii**	**Arctic**
Angola	0* 0.0015	-								
Guinea I	**0.002** **0.4134**	**0.0328** **0.1096**	-							
OC	0* 0.647	0* 0.7984	0* 0.4752	-						
Quest	0.0003* 0.262	0* 0.368	0* 0.4317	**0.012** **0.0006**	-					
AG	0* 0*	0* 0*	0* 0*	0* 0*	0* 0*	-				
site F	0* 0.002	0* 0*	0* 0.0002*	0* 0*	0* 0*	0* 0.0212	-			
EPR	0* 0*	0* 0*	0* 0*	0* 0*	0* 0.0668	0* 0*	0* 0*	-		
Hawaii	0* 0*	0* 0*	0* 0*	0* 0*	0* 0.6739	0* 0*	0* 0*	0* 0*	-	
Arctic	0* 0.062	0* 0.0254	0* 0.0352	0* 0*	0* 0.008	0* 0*	0* 0*	0* 0*	0* 0*	-
Antarctic	0* 0.4082	0* 0*	0* 0.0006	**0.8679** **0.7868**	0* 0.7736	**0.0084** **0.6524**	0* 0.5874	0* 0*	0* 0*	**0.4637** **0.4640**

significant *P*-level are indicated by an asterisk (*)
both *P*-values representing not significant differences are indicated in bold
OC=oceanic sediment
AG=Anya's Garden

higher primary productivity in the surface waters due to the nutrient supply from the Congo and the Niger Rivers (Schefuss et al, 2004).

This phylogenetic analysis provided first insights into the bacterial diversity and distribution of bacterial communities in the three eastern South Atlantic deep-sea basins. The proposed roles of the bacterial groups in the mineralization process of organic carbon have to be further investigated, as phylogenetic studies are not reliable for predicting physiology. However, the detected phylogenetic groups provided hypotheses, helping to design further experimental setups and studies.

In contrast to permanently cold sediments, hydrothermal vents represent rich areas of very high productivity with dense communities of unusual animals. Chemolithoautotrophic microorganisms represent the primary producers in these systems. Most discovered hydrothermal systems are located far from continents, where sedimentation rates due to photosynthetic productivity are very low. Therefore, seabed focused investigation of microbial diversity concentrated mostly on solid surfaces such as hydrothermal precipitates (chimney structures) and vented rocks (Takai et al., 2006). Studies of microbial diversity in sediments of hydrothermal systems far from continents were so far limited to one study of the peridotite-hosted Rainbow hydrothermal vent field (Nercessian et al., 2005). Although these heterogeneous sediments represent a mixture of pelagic sediments, iron oxides and oxidized fragments of the dead chimneys, no apparent sign of hydrothermal activity was detected. Hydrothermally influenced sediments were so far only investigated from the coastal near basalt-hosted hydrothermal field of the Guaymas Basin (Gulf of California section of the EPR) (Teske et al., 2002).

The present thesis represents the first investigation of the microbial diversity in hydrothermally influenced sediments of a peridotite-hosted hydrothermal vent field (Logatchev). The white mats on top of the sediments at site F and Anya's Garden resemble those at the Guaymas Basin and therefore clearly differ from the surrounding pelagic sediments. Our analysis of the hydrothermally influenced sediments covered by white mats showed that *Epsilonproteobacteria* and *Deltaproteobacteria* dominated the microbial communities of the surface layers. We found many sequences which were closely related to those, found in microbial mats, hydrothermal sediments (Moyer et al., 1995; Teske et al., 2002), and on *in situ* colonizers in sulfidic, basaltic systems (Alain et al., 2002; López-Garcia et al., 2003a; Moussard et al., 2006). Therefore, the investigated microbial communities of the peridotite-hosted Logatchev field showed many similarities to those of basalt-hosted systems. The detected high abundance of *Epsilonproteobacteria* is consistent with previous studies,

where *Epsilonproteobacteria* were found in different deep-sea hydrothermal vent habitats such as mats on the surface of chimneys (Longnecker and Reysenbach, 2001; Brazelton et al., 2006; Nakagawa et al., 2006), colonizers and animals, vent fluids, plume, and symbiotic associations with vent animals (Reysenbach et al., 2000; Corre et al., 2001; López-Garcia et al., 2003a, Alain et al., 2002; Petersen et al., 2009; Takai et al., 2005). Therefore our study supports the hypothesis that *Epsilonproteobacteria* establish themselves as the primary colonizers at hydrothermal vent systems (Campbell et al., 2006).

However, so far only filamentous *Gammaproteobacteria* (e.g. *Beggiatoa*, *Thiothrix*) were known to appear in such dense populations, forming white mats on top of sediments. We detected filamentous and vibrioid mat-forming *Epsilonproteobacteria* dominating white mat communities. The mat at site F was dominated by filamentous, while the mat at Anya's Garden was dominated by vibrioid *Epsilonproteobacteria*. Therefore, each site exhibited their specific epsilonproteobacterial community. The statistical comparison of the microbial communities from site F and AG confirmed that these two microbial community structures differ significantly from each other (Table 7).

As expected, the bacterial community structures of the hydrothermally influenced sediment differ significantly from those of the permanently cold sediments of the South Atlantic Ocean (Table 7). However, the bacterial communities from permanently cold sediments from the South Atlantic Ocean were also significant different from the bacterial communities of cold sediments at the Mid-Atlantic Ridge (OC=oceanic sediments). The comparison of microbial communities from the oceanic sediment and Quest did not show significant differences. This confirmed that sediments without a white mat at the Logatchev field were not hydrothermally influenced and resembled more oceanic, pelagic sediments. This analysis confirmed that white mat sediments harbor vent specific bacterial groups that differ significantly to those found in non-hydrothermally influenced sediments.

When we compared our investigated bacterial communities with bacterial communities of other deep-sea floor studies, most comparisons revealed significant differences in the structure of the microbial community between the sites. However, significant differences were not observed between the microbial community from the surface sediments from an Antarctic continental shelf area (761 m depth) (Bowman and McCuaig, 2003), the microbial communities from Arctic sediments (Svalbard, 218 m depth; Ravenschlag et al., 1999), and the bacterial communities in the oceanic sediment at the Logatchev hydrothermal vent field. Surprisingly, the bacterial communities from the cold adapted sediments from an Antarctic continental shelf area showed as well no significant differences to those from the

hydrothermally influenced sediments at Anya's Garden (Table 7). The comparison of the estimated richness for all investigated site as well as other studies showed, that the highest bacterial richness was found for this Antarctic sediment community (Figure 23). This high bacterial richness could explain that this bacterial community showed the most similarities to other investigated microbial communities.

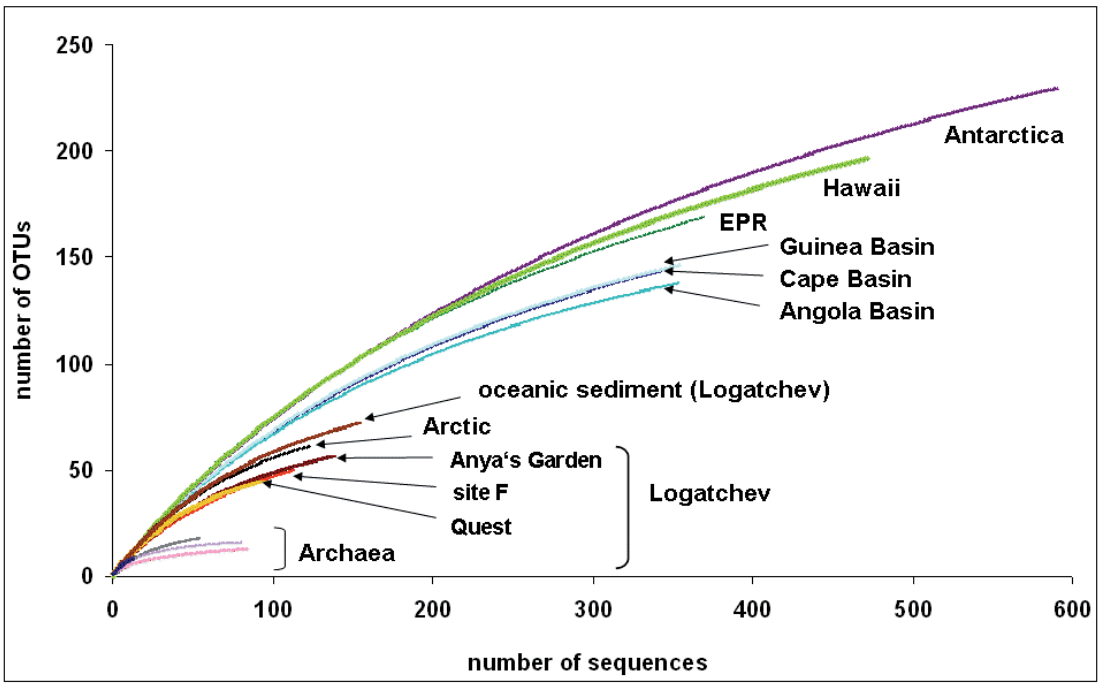

Figure 23: Rarefaction analyses of the relative bacterial richness of different communities compared at a genetic distance of 0.02.

Estimated bacterial richness in permanently cold sediments was always higher than that in hydrothermally influenced sediments. The archaeal community showed the lowest estimated richness of all studies. This indicates that habitat condition (environmental heterogeneity) and domain affiliation lead to different richness patterns. Previous studies showed that major environmental variables have an influence on microbial composition (Ward et al., 1998; Broughton and Gross, 2000). Some major environmental variables influencing the microbial composition of the investigated habitats are likely nutrient status and predation. Temperature can be assumed to have a minor influence on the microbial composition as temperatures of 2.4°C in the hydrothermally influenced surface layer are in the range of those found in typical permanently cold deep-sea sediments. Furthermore, our results support the assumption that a higher environmental heterogeneity potentially allows for higher microbial diversity by allowing the coexistence of a larger number of bacterial taxa and produce a more uniform

community composition (Zhou et al., 2002). Permanently cold deep-sea floors, in contrast to hydrothermal systems, are suggested to exhibit habitat variability and patchy disturbance leading to heterogeneous conditions (Snelgrove and Smith, 2002). In heterogeneous environments, intermediate disturbance has been shown to result in a diversity peak (Buckling et al., 2000). The amount of energy available in an ecological system is thought to be the key determinant of diversity (Jessup et al., 2004; Kassen et al., 2000). Whereby, primary productivity was as well shown to have both an increasing and decreasing effect on bacterial richness depending on the different taxonomic groups of bacteria (Horner-Devine et al. 2003). Our results suggest that increasing energy decreases diversity and that this affects more archaeal affiliated taxa than bacterial taxa.

The ecologist John Lawton (1999) has argued that, although the details of individual organisms and ecological systems matter, ecologists would profit most from trying to uncover underlying patterns, rules and laws. The comparison of microbial diversities of different deep-sea floors revealed habitat and taxon related patterns. Findings of previously conducted microbial model system experiments give good explanations for the detected patterns (Jessup et al., 2004). However, a good fit of species diversity to a theoretical model does not distinguish pattern from process (Alonso et al., 2006). Rapidly increasing information about the genetic pool provides the opportunity to understand ecological processes at all scales of biological organization. Understanding the mechanisms underlying detected patterns will improve our understanding on the different mechanisms controlling species diversity from ecological to evolutionary spatio-temporal scales.

1.2 Microbial activity

At hydrothermal vent fields, due to venting of hydrothermal fluids to the ocean, heat and mass is transferred from the mantle to the ocean. It has been estimated that 25-30% of the earth's total heat flux is transferred from the lithosphere to the hydrosphere by the circulation of seawater through oceanic spreading centers (Lowell, 1991; Stein and Stein, 1994). Besides high-temperature venting, off-axis diffuse fluid flow plays an important role in transferring energy from the crust and mantle to the oceans. The emerging chemical and thermal environments are inhabited by dense communities. Previous studies revealed that chemolithoautotrophic microorganisms use this chemical energy and are therefore responsible for the high primary production at these sites (Van Dover et al., 2002). However, little is known about what kind of pathways are used and how these microorganisms interact with

their environments. Furthermore, total fluxes and turnover rates are so far unknown which are necessary to reveal the energy transfer from the mantle to the microorganisms.

During this thesis, a combination of *in situ* rate measurements, *ex situ* laboratory experiments as well as metagenomic analyses was performed to gain more information about the potential and present activity of free-living microbes at the ultramafic-hosted Logatchev hydrothermal field. Before this study, *in situ* measurements of hydrothermally influenced sediment were not available. However, it was assumed that hydrothermally influenced sediments are heated by diffuse outflow of active flowing fluids and are therefore heated as a result of advective processes (Karl, 1995). Temperature and microprofiles obtained during this study revealed a conductive heating of the sediments likely by underlying hydrothermal fluids. Therefore, fluids slowly pass through the sediments in a diffusive process until they reach the surface layer. Hydrothermally influenced habitats controlled by diffusive processes were so far unknown, as previous studies only reported environments which are directly exposed to the fluid flow. Therefore, our study provides first insights on hydrothermal vent environments controlled by diffusive transport of fluid components.

Systems controlled by diffusion exhibit nearly constant concentration gradients between the sediment surface and the reactive layer so that everywhere the same diffusive flux is present. Such a concentration gradient is referred to as being in steady state, allowing quantitative evaluation of steady state concentration profiles. Therefore, the detected oxygen profiles in sediments controlled by diffusion at the Logatchev field enable us to calculate oxygen consumption rates for these surface sediments. Both mat covered sediments revealed oxygen consumption rates similar to those in eutrophic coastal, estuarine marine (Jørgensen, 2001) and cold seeps sediments (Niemann et al., 2006). These high oxygen consumption rates confirmed the presence of an active microbial community in the first centimeter of these sediments.

Furthermore, *in situ* microprofiles revealed that only sulfide was present in the upper sediment layers of the typical ultramafic fluid components such as methane, hydrogen and sulfide. This indicates that the other components are consumed in deeper layers. *Ex situ* laboratory rate experiments revealed for the white-covered surface layers high sulfate-reduction rates (SRRs), comparable to those measured in the sulfate methane transition zones in coastal and margin sediments (Knittel and Boetius, 2009). Therefore, sulfate reduction represented an important metabolic pathway in these *Epsilonproteobacteria*-mat covered sediments, as previously detected for *Beggioatoa*-mat covered hydrothermal locations. Measured rates of sulfate reduction (SR) were tenfold higher than rates of anaerobic oxidation

of methane (AOM). Thus, SR rates found in the surface layer cannot be completely fuelled by AOM, which is again similar to the detected processes present at basalt-hosted sedimented hydrothermal sites in the Guaymas Basin (Kallmeyer and Boetius, 2004). Consequently, sulfate-reducing bacteria are a dominant part of the active microbial communities in anoxic marine sediments independent of the origin of the sediments. Although sulfate is not the energetically most favourable electron acceptor for anoxic sediments, the high concentration of sulfate in seawater (29 mM) makes it a dominant electron acceptor for marine sediments (Schulz and Zabel, 2006).

In contrast to the near coastal Guaymas Basin, Logatchev did not show high sedimentation rates due to high biological productivity in surface waters and a large terrigenous input. Therefore, the active sulfate-reducing microbial community cannot be fuelled by deposited organic material. We propose that instead chemolithoautothrophic bacteria produce enough biomass to support secondary sulfate reducers. The detected availability of sulfide in the surface layer and the presence of *aprA*-genes related to putative sulfur-oxidizers *Betaproteobacteria* and *Gammaproteobacteria* as well as *soxB*-genes affiliated with *Epsilonproteobacteria* which can be used for sulfur oxidation indicate that sulfide-oxidizing bacteria represent another part of the active microbial communities in these sediments. *Ex situ* incubation experiments finally confirmed that sulfide is consumed by the microbial community in the surface layer. The measured sulfide consumption rates were comparable to those of the endosymbionts of the hydrothermal vent mussel *Bathymodiolus puteoserpentis* from the same hydrothermal vent field (Zielinski et al., in prep), indicating a highly active community. As sulfur oxidation was not limited to the overlying *Epsilonproteobacteria*-mat, it is likely that besides *Epsilonprotebacteria* other groups are responsible for sulfur oxidation. As *aprA*-genes affiliated with *Gammaproteobacteria* and *Betaproteobacteria* were detected in the surface sediments, it is likely that these groups are also involved in sulfur oxidation. Pyrosequencing represents another well-suited technique for fast biodiversity and metabolic capacity screenings, as revealed by a compararive analysis of pyrosequencing data and data from 16S rRNA clone libraries, fosmid insert end-sequences and CARD-FISH for one well characterized sediment sample. Pyrosequencing based metagenomic analysis supported that several groups are involved in sulfur oxidation as genes for sulfur-oxidation pathways were found for *Gammaproteobacteria* and *Epsilonproteobacteria*. The detection of genes for denitrification proposes that this may be another potential important pathway in the oxic-anoxic interface of this sediment. *Epsilonproteobacteria* contained the highest number of genes for denitrification (Brettar et al., 2006; Grote et al., 2007). Previous studies have

verified the significant role of *Epsilonproteobacteria* in pelagic marine redoxclines of the Black Sea (Vetriani et al., 2003), the Cariaco Trench (Madrid et al., 2001) and the Baltic Sea (Grote et al., 2007), where oxygen deficiency and sulfide accumulation occurs. Therefore, *Epsilonproteobacteria* are likely potentially important chemolithoautotrophic sulfide oxidizer and nitrate reducer responsible for denitrification processes at the oxic-anoxic interface in the hydrothermally influenced sediment.

So far our analysis of the active microbial community revealed highly active sulfate-reducing and sulfur-oxidizing bacteria in the surface sediment of the ultramafic-hosted Logatchev field. As sulfide is available in the surface layer of these sediments, it is not surprising to find active sulfur cycling bacteria as previously found in basalt-hosted systems. The present sulfide in the surface layer originated from hydrothermal fluids which were located below the sediment layer in conduits carrying these fluids. Besides sulfide, fluids at the Logatchev field are enriched in methane and hydrogen. Therefore, the question remains how methane and hydrogen influence microbial compositions in peridotite-hosted hydrothermal systems. Further investigations of deeper layers are necessary in order to detect active microbial communities which are acting as a sink for hydrogen and methane. Furthermore, comparative metagenomics could be used to identify genetic patterns typical for this habitat, which were selected by the evolutionary pressure of this ecological niche. The combination of environmental niche specific conditions and functional attributes would provide biological information in an ecological context and therefore improve our understanding of the responses of living organisms to their natural environments.

1.3 Microbial biogeography

The distribution of microorganisms and the factor controlling it at different scales are important for the understanding of the vast microbial diversity and for niche specific interaction of microorgasnims with their environment. The biogeography of macro- and microorganisms can be determined by two factors, the environmental heterogeneity (EH) and spatial distance (SD). The geographical impact on the biogeography via barriers is more accepted for macro- than for microorganisms (Richardson, 1981). The small size, dispensability, population size and low extinction rates of microorganisms make the presence of geographical barrier and therefore geographical restriction questionable (Staley and Gosink`s, 1999, Finlay, 2002). Most of the studies searching for the geographical isolation of prokaryotic populations and their divergence have been directed toward genetic characters (Whitaker et al., 2003; Green and Bohannan, 2006; Martiny et al., 2006; Ramette and Tiedje,

2007). Ribosomal RNA-based approaches (Amann et al., 1995), genomics, and currently metagenomics are the major sources of information for understanding microbial diversity.

Present studies of microbes inhabiting extreme environments, such as hot springs (hyperthermophilic archaeon *Sulfolobus*, and thermophilic *Synechococcus, Cyanobacteria*) or salt lakes (halophilic *Salinibacter ruber*, and haloalkaliphilic *Thioakalivibrio*) revealed that spatial distance has a significant influence on the microbial composition for intermediate (10-3000 km) and large scales (>3000 km) (Whitaker et al., 2003; Papke et al., 2003; Pagaling et al., 2009; Rosello-Mora et al., 2008). We investigated microbial community compositions of the Logatchev hydrothermal vent field in order to determine the influence of EH and SD on microbial communities in an extreme environment for small scales (>10 km). In contrast to the present studies regarding large scale biogeography, our study showed that SD was not a significant factor effecting microbial composition on small scales. At small scales microbial biogeography was primarily controlled by EH (Table 8). This is consistent with studies of salt marsh sediments and grassland soils, where microbial compositions were exclusively influenced by EH at small scales (Horner-Devine et al., 2004; Kuske et al., 2002). Therefore, microbial communities do not seem to be dispersal limited at small scales, not even extremophiles which are adapted to extreme physical or geochemical conditions.

In contrast to hydrothermal systems, the uniform conditions of the permanently cold deep-sea suggest that the distribution of deep-sea microbes is as well determined by EH for small, intermediate, and large distances. Fierer and colleagues (2006) revealed that soil bacteria across North and South America (large, continental-scale) were largely attributed to soil pH, with higher diversity observed in neutral soils. This study indicates that in contrast to extreme environments, microbial compositions of moderate environments are controlled by EH. Thus, microbial biogeography in moderate environments differs fundamentally from the biogeography of eukaryotes. To test which effect EH and SD have on the biogeographic patterns of deep-sea microbes, we investigated microbial communities of surface deep-sea sediments from the South Atlantic Ocean, which were separated by intermediate and by large distances. Our results revealed an effect of both factors for intermediate and large scales (>3000 km) (Table 8). Therefore, in contrast to the habitat soil, there seem to be dispersal barriers in the deep sea for microbial communities. Other studies as well showed an influence of both factors at intermediate scales (10-3000 km), but our analysis revealed for the first time that EH and SD have an effect on microbial biogeography for large scales (>3000 km).

In summary, an effect of SD on the microbial diversity of the Logatchev hydrothermal vent field and therefore for small distances was not detected, but the investigation of permanently

cold sediment microbial communities revealed that at intermediate and large scales both factors (EH and SD) influence the microbial distribution of deep-sea floors communities. Therefore, our results are in contrast to previous findings of extreme and moderate habitats, which were either affected by SD or EH (Martiny 2006; Fierer et al., 2006). We suggest that deep-sea floors represent environments which harbor a mixture of microbes present at extreme and moderate environments. The cultivation of obligate barophilic microbes of deep-sea habitats (Kato und Bartlett, 1997) supported that extremophiles are one part of deep-sea populations. Pressure likely represents a barrier for their dispersal, so that SD likely has an influence on their distribution. This part of a deep-sea community can be geographically isolated from other populations leading to divergent evolution and to an evolvement of specific, divergent species (allopatric origin) (Whitaker, 2006).

The distribution of the part of the population which is not dispersal limited depends on their rate of dispersal and their capability to adapt to new environments (migration-selection balance) (Whitaker et al., 2006). Sloan and colleagues (2007) have shown that the bigger the population size, the higher the migration rate is. Adaptations are introduced through random mutation, genetic exchange among individuals of the same population (recombination), horizontal gene transfer (HGT) of genetic material from other species in the environment and rare migration in microbial populations. It has been suggested that HGT is the primary mechanism through which *Bacteria* and *Archaea* acquire adaptive alleles (Konstantinidis and Tietje, 2005; Coleman et al., 2006). Therefore, HGT can provide the capability for the microorganism to adapt to different conditions (Thomas and Nielsen, 2005). Goddard and colleagues (2005) revealed that microbial species that recombine frequently have a greater rate of adaptation over clonal population in harsh environments where selection is strong. Differences in the rate of adaption would explain why some extreme hyperthermophilic *Archaea* are dispersal limited (Whitaker et al., 2003; Papke et al., 2003) while other *Archaea* show no influence of spatial distance on their distribution (Pagaling et al., 2009). Understanding the environmental factors that promote or limit HGT events will provide a better understanding of bacterial barriers and their biogeography. The general use of slowly evolving 16S rRNA genes makes it difficult to recognize such events in the evolutionary history of a species (incipient speciation) (Stackebrandt et al., 2002).

Therefore, it is preferable to use whole genome information to get a better understanding of the structure and rearrangement of the microbial genomes in a community (Konstantinidis et al., 2006). New sequencing technologies as pyrosequencing allow the study of cultured microbes and of microbial communities in their natural contexts without necessarily requiring

the culturing of the individual organisms. Further genomic information will increase our understanding of evolutionary parameters that lead to geographical barriers in some microbial species, but not in others. Comparative genomics will identify differences in microbial biochemical pathways, energetics, and the mechanisms of metabolic regulation and therefore the identification of trait-based biogeography (Green et al., 2008). In a second step, functional transcriptomics and proteomics may be considered, representing the real interaction of the microbes with its environment (Singh and Nagaraj, 2006b; Rossello-Mora et al., 2008). As microbes represent more than two-thirds of the metabolic and genetic diversity of the planet, there is no reason to expect a single concept or definition to apply to all microbes and therefore a clear-cut microbial biogeography.

Table 8: Slope coefficients and Mantel r statistic for genetic distance matrices derived from 16S rRNA sequences or T-RFLP.

	Slope coefficient (genetic and geographic distance)		Mantel			Partial Mantel		No. of samples
	$\times 10^{-5}$	$\log_{10}$ transformed	SD^{+}	log10 transf.	$EH^{\sim}$	SD^{+}	$EH^{\sim}$	
Logatchev, 0-200m distance								
16S rRNA	-1379.63	-0.031	-0.010	-0.009	0.063	-0.059	0.085	4
	(-2160.33, -590.93)[a]	(-0.050, -0.011)[a]	**(0.692)**	**(0.699)**	(<0.001)*	**(0.999)**	(<0.001)*	
South Atlantic, 0-1200 km distance								
T-RFLP	28.28	0.074	0.651	0.593	0.657	0.054	0.131	14
	(21.34, 35.22)[a]	(0.053, 0.095)[a]	(<0.001)*	(<0.001)*	(<0.001)*	**(0.306)**	**(0.134)**	
South Atlantic, 0-3500 km distance								
16S rRNA	0.19	0.0036	0.024	0.012	0.008	0.031	- 0.021	3
	(0.17, 0.22)[a]	(0.0028, 0.0044)[a]	(0.001)*	(0.001)*	(0.006)*	(0.002)*	**(0.981)**	
T-RFLP (Guinea I)	7.5	0.076	0.698	0.841	0.886	-0.138	0.768	11
	(5.38, 9.62)[a]	(0.063, 0.090)[a]	(0.004)*	(0.001)*	(0.001)*	**(0.86)**	(<0.001)*	
T-RFLP (Guinea I+II+III)	2.6	0.055	0.278	0.532	0.599	-0.227	0.573	19
	(1.24, 3.96)[a]	(0.042, 0.068)[a]	(0.009)*	(0.001)*	(<0.001)*	**(0.992)**	(<0.001)*	
All sites, 0-18000 km distance								
16S rRNA	0.02	0.0030	0.013	0.013	n.a.	n.a.	n.a.	7
	(0.019, 0.022)[a]	(0.0027, 0.0032)[a]	(0.001)*	(0.001)*				

[+] spatial distance; [~] environmetnal heterogeneity
* $P < 0.05$ for Bonferroni corrected P-level of significance for 1000 permutations
[a] lower and upper bound of 95% confidence interval, n.a. .no environmental parameters available

2 Outlook

In 1987 Carls Woese wrote ‘A revolution is occurring in biology’, referring to the impact of the increasing capacity to sequence nucleic acids. This revolution allowed a new phylogenetic classification of prokaryotes based on the 16S rRNA gene. Furthermore, it enabled the detection of the uncultured part of the microbial community. Consequently, microbial studies began to focus on microbial diversity, evolution, abundance and distribution leading to an increase in microbial ecology focused studies. Thereby, species is used as the fundamental unit of biological classification and is critical for describing, understanding and comparing biological diversities at different levels among ecological niches (Xu et al., 2006). However, what constitutes a species remains controversial, as there is not a widely accepted theoretical species concept for microbes in contrast to animals and plants.

About 20 years later, microbial ecology is again undergoing a revolution due to sequencing improvements (next-generation technologies; Rothberg and Leamon, 2008; Prosser et al., 2007). Instead of investigating the diversity of a single gene within microbial communities, whole genomes and metagenomes are now accessible within a reasonable time. However, this sequencing revolution brings again new challenges to the scientific field, which resembles those 20 years ago. Woese already reported that ‘Microbiology is consequently being inundated with sequence information, which accumulates so rapidly that the reading and entering of data are becoming major concerns’. Today again, automated DNA sequencing technology is so rapid that analysis has become the rate-limited step (Suen et al., 2007). The potential of the ongoing revolution will just be realized if scientists learn from the previous revolution. Therefore, to gain more information about the microbial communities’ interaction with the environment, microbial studies have to be interpreted from an ecological perspective. Thus, data about the environmental conditions (metadata) are of equal importance. As proposed by Woese, more studies should ‘be conceptualized more in a comparative way’ and ‘lead to a close relationship between geologist and the evolutionist’ or between ecologists and the bioinformatic scientists. Thereby, sequencing derived information ‘have to be accepted for what they minimally are: hypotheses, to be tested and either strengthened or rejected on the basis of other kinds of data’ (Woese, 1987).

Taking this into account, sequencing based studies will increase our understanding of traditionally microbial ecology areas like diversity, activity and biogeography. A metagenomically focused study is particularly favourable in habitats which are difficult to achieve so that sampling of the habitat is limited. The investigated deep-sea environments

(Logatcehv field on the MAR, deep-sea basins of the South Atalantic Ocean) in this thesis represent such environments. The presented 16S rRNA gene based investigation already provided first insights into microbial diversity, potential activity, and biogeography in different deep-sea habitats. Further metagenomic studies will provide higher resolution analyses, which can be used to test hypotheses obtained from the present study.

Moreover, the capability to analyse whole genomes and therefore the evolutionary history will provide more information about processes such as the horizontal gene transfer (HGT). Understanding HGT as well as the interactions of microbes with viruses will lead to an understanding of microbes as predominantly cooperative instead of organisms dominated by individual characteristics (Goldenfeld and Woese, 2007). In this way, a prokaryotic community can be thought of as a single evolving genome assemblage, with the environment, rather than the species, defining the organisms that inhabit the assemblage (trait-based diversity, ecotype; Cohan et al., 2007). Therefore, metagenomic approaches allow answering of traditional microbial ecology questions from a niche-adapting genomic (ecotype) perspective. To identify ecotypes, metagenomic data have to be broken down into fundamental units such as protein domains to identify niche specific patterns (Suen et al., 2007). Furthermore these units can be used for a correlation to environmental heterogeneity and spatial distance to gain more information about the biogeographic patterns of functional instead of phylogenetic groups.

As this kind of data can be used for comparative analyses, sampling at different time points and different scales will reveal temporal and spatial variability. The identification of niche specific pattern and therefore typical protein domain in the white mat covered sediments from the Logatchev field will reveal typical genetic patterns for sulfidic influenced hydrothermal sediments. A comparative metagenomic study with deeper layers, which are likely influenced by methane and hydrogen, will provide further information about the vertical distribution of ecotypes in these sediments. This detected pattern will support present hypotheses or lead to new hypotheses. Therefore, comparative metagenomic studies should be seen as the first step of understanding the relationship between microbes and environment which provides hypotheses, which have to be validated (Woese, 1987). This allows a fruitful interplay between quantitative predictions and experimental tests. Further *in situ* and *ex situ* experiments should be used to verify the hypotheses and ideally reveal total fluxes and turnover rates. These rates can be used for the calculation of the dynamics of energy and carbon transport through different trophic levels. The metagenomic dataset generated in this study has to be further analysed to get new insights into the role of the microbial community

in the different biogeochemical cycles that are relevant at the Logatchev field. Furthermore, revealing habitat specific patterns in an ecological context in comparison to other studies will lead to a better understanding of the relationship between microbial diversity, environmental heterogeneity and spatial distance providing further information about the factors controlling microbial diversity and distribution.

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VI Appendix

List of additional publications with differing subjects, created in cooperation with other groups:

Amelia-Elean Rotaru, Regina Schauer, Christina Probian, Marc Mussmann, and Jens Harder. Candidate Division OP3 cells in limonene degrading methanogenic cultures. Manuscript submitted for publication in *International Microbiology*.

R.S. during the thesis: conceived and edited the manuscript R.S: did the 16S rRNA sequencing and analyses A-E.R.: performed FISH and CARD-FISH experiments, cultivation studies, conceived and wrote the manuscript C.P.: helped with the cultivation M.M.: designed FISH probes J.H.: developed the manuscript, conceived and edited the manuscript

1 Candidate Division OP3 cells in limonene degrading methanogenic cultures

Candidate Division OP3 cells in limonene degrading methanogenic cultures

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Abstract

Limonene, a widespread monoterpene, served as organic carbon and electron donor source for methanogenic enrichment cultures. For the first time a methanogenic community involved in the degradation of an abundant monoterpene was characterized by sequencing and cloning of *Bacteria* and *Archaea* 16S rRNA gene sequences. The *Bacteria* were represented by *Deltaproteobacteria, Bacteroidetes, Firmicutes* and Candidate Division OP3, and the *Archaea* by *Methanoculleus* and *Methanosaeta*. The composition was determined with phylotype specific probes in catalyzed reporter deposition – fluorescence *in situ* hybridization experiments (CARD-FISH). The *Bacteria* in the enrichment cultures were dominated by members of *Deltaproteobacteria* and Candidate Division OP3. The probe specific for Candidate Division OP3 hybridized to small cocci encountered either solitary or attached to larger cells. Our study is the first visualization and quantification of cells of this candidate phylum in a well defined, stable enrichment culture.

Introduction

Limonene, like other monoterpenes, is produced in the plastids of plants by fusion of two isoprene units to form geranyl-diphosphate and a cyclization reaction, catalyzed by a limonene-synthase (Kreuzwieser et al., 1999; Hyatt et al., 2007). Limonene, like other mono-, di- and sesqui-terpenoids, is a secondary metabolite (Newman & Chappell 1999). Secondary metabolites are non-essential for the basic metabolic functions of plants, but they help establish complex ecological interactions, from symbiotic to pathogenic, between plants and microorganisms, fungi, insects or humans (Singer et al., 2003).

The main natural pools of terpenes including limonene are forest soils and freshwater sediments where dead vegetation is transported and deposited. Limonene has an enantiomer specific orange or herbal odour, which lead to its extensive use as scenting agent in cleaning and sterilizing products and as food flavour. Today, besides toluene, limonene is the major indoor volatile organic carbon and it was proposed as biomarker for modern sewage recharge in urban groundwater (Barrett et al., 1999).

In anaerobic environments, such as underground waters, anoxic soils or anaerobic wastewater treatment plants, limonene is degraded in the absence of oxygen. The two well described facultative anaerobes *Thauera terpenica* and *Castellaniella defragrans* can utilize limonene as sole energy source under denitrifying conditions (Foss & Harder 1998; Foss et al., 1998). However, limonene degradation in the presence of other electron acceptors besides oxygen and nitrate has not been demonstrated yet.

In this study we used monoterpene-utilizing methanogenic enrichment cultures (Harder and Foss, 1999) to establish and investigate the methanogenic degradation of limonene. The methanogenic community was studied by 16S rRNA gene libraries and fluorescence *in situ* hybridization (Amann et al., 1995). Unforeseen was the discovery of members of Candidate Division OP3, a phylum without any cultivated representatives (Hugenholtz et al., 1998). For the first time, we visualized and quantified members of this candidate phylum in a stable enrichment culture.

Materials and Methods

Source of organisms and cultivation

Limonene degrading enrichments were initiated from methanogenic enrichment cultures established on R-(-)-alpha-phellandrene, (+)-2-carene, (-)-alpha-pinene, or (+)-sabinene (Harder & Foss, 1999). All resulting methanogenic, sediment-free enrichment cultures were grown on 2-5% limonene in 30 ml 2,2,4,6,8,8-heptamethylnonane (HMN) and 300 ml aqueous medium as previously described (Harder & Foss, 1999). Cell density increase was determined by optical density measurements at 660 nm.

Chemical analysis

All samples for chemical analysis were taken with N_2-flushed hypodermic needles and syringes. Limonene concentrations were measured by gas-chromatographic head-space analysis on a Shimadzu 14B gas chromatograph (200°C, isothermal) adapted from Rotaru et al. (2010). Methane was measured by head-space analysis on the same instrument at an isothermal temperature of 110°C. Fatty acids were detected by high performance liquid chromatography as previously described (Rotaru et al., 2010). The standards consisted of the following fatty acids (detection limit - 0.1 mM): succinate, lactate, formate, acetate, propionate and butyrate.

Clone library construction and sequence analysis

The genomic DNA of 5 ml cell culture was extracted with the QIAGEN genomic kit (Qiagen, Germany). 25 ng of high-molecular weight DNA were used to amplify the 16S rRNA gene with bacterial primers 8F and 1492R (Hicks et al., 1992; Kane et al., 1993) and with archaeal primer pairs 21F-958R and 21F-1492R (Stahl et al., 1988; DeLong, 1992). The reaction mix consisted of 2 µM of each primer, 0.2 mM dNTPs, 0.04 U Red-Taq polymerase (Sigma, Germany) and 1×PCR buffer in 50 µl. After an initial denaturing step of 4 minutes at 94°C,

the polymerase was added at 80°C. The 32 cycles involved a denaturing step for 1 minute at 94°C, 1 min primer annealing at 42°C for *Bacteria* and 58°C for *Archaea*, and 1 min elongation at 72°C. The A-overhang for cloning was introduced by a final elongation at 60°C for 60 min. The amplicons were purified and cloned into the pGEM-T Easy vector (Promega, USA) for *Bacteria* and the pCR4-TOPO vector (Invitrogen, Germany) for *Archaea*. The recombinant plasmids were transformed into *E. coli* DH5α and *E. coli* TOP10 for *Bacteria* or *Archaea*, respectively. Inserted genes were amplified with the vector primers M13F (5'-GGAAACAGCTATGACCATG-3') and M13R (5'-GTTGTAAAACGACGGCCAGT-3'). We analyzed the diversity of clones by amplified rDNA restriction analysis (ARDRA). PCR products were purified, and aliquots of 1 μg of the amplified insert were digested with 7.5U of the restriction endonucleases BsuRI and RsaI (Fermentas) for 3 hours at 37°C. The resulting fragments were analyzed on a 3% agarose gels, and restriction patterns within each group were manually compared. Amplicons of 16S rRNA genes were sequenced using the BigDye 3.0 chemistry and analyzed on a 3130 XL Genetic Analyser (Applied Biosystems, Germany). Sequences were cleaned of vector data with Sequence Analysis 5.2 (Applied Biosystems) and assembled into contigs with the Sequencer software (Gene Codes, USA). The nearly complete 16S rRNA gene sequences were aligned with the ARB-Silva software package (Ludwig et al., 2004; Pruesse et al., 2007). A maximum parsimony phylogenetic tree was calculated excluding the influence of highly variable positions. The *Bacteria* tree was reconstructed using sequences longer than 1300 bp, whereas the *Archaea* tree was reconstructed with sequences longer than 800 bp.

Probe design and visualization of cells by CARD-FISH

Probes Eub-338(VI) (5' - GCAGCCTCCCGTAGGAGT - 3') and OP3-565 (5' - TACCTGCCCTTTACACCC - 3') were designed for a group of 16S rRNA sequences within the Candidate Division OP3 using the ProbeDesign tool of the ARB software (Ludwig et al., 2004; Pruesse et al., 2007). Probes were tested *in silico* against the RDP II database vs.9.48 (http://rdp.cme.msu.edu/probematch/search.jsp). Probe OP3-565 showed 1.2 weighted mismatches to *Thialkalivibrio halophylus* (DSM 15791) and *Marinitoga piezophila* (DSM 14283). These two microorganisms served as control for hybridization experiments. Probes were synthesized with a horse radish peroxidase (HRP) modification at the 5'-end and used for hybridization experiments performed by as previously described (Pernthaler et al. 2002). We applied the following probes: Eub-338(I) (Amman et al., 1990) in equimolar mix with the newly designed probe Eub-338(VI) specific for the 16S rRNA of Candidate Division OP3

clones; Arch-916 (Stahl & Amann, 1991); CF-319a (Manz *et al*., 1996); Delta-495a in equimolar mix with its competitor (Loy *et al*., 2002); Pla-46 (Neef *et al.*, 1998) MX-825 (Raskin *et al.*, 1994) and OP3-565 (this study). All samples were counterstained with 4',6-6 diamidino-2-phenylindole (DAPI), 1 µg/ml, as reference for relative cell counts. As control probe we used Non-338 (Wallner *et al.,* 1993), which gave one signal for 300 DAPI stained cells.

Nucleotide sequence accession numbers

The EMBL-EBI accession numbers of 16S rRNA gene sequences retrieved from our methanogenic enrichment culture thriving on limonene are FN646432 to FN646495.

Results and discussions

Cultivation on limonene under methanogenic conditions

We initiated limonene utilization by the methanogenic enrichment cultures (Harder & Foss, 1999) by transfers on limonene as primary organic carbon and energy source in 1999. Small amounts of acetate (2 mM) and cysteine (1 mM) were supplemented to sustain the methanogenic community during the initial growth phase. Cultures produced methane for up to three years. With 5% limonene in HMN (v/v) and 10% inoculum (v/v) and one transfer per year, we obtained stable enrichment cultures thriving on limonene. The cultures finally approached a maximum cell density in six months, whereas control cultures incubated only with acetate and cysteine showed no growth during incubation (Fig. 1A). Limonene consumption was observed after four months in all enrichment cultures, whereas sterile controls did not show any limonene loss during incubation (Fig. 1B). As expected, methane production in cultures with supplementary acetate (Fig. 1C) showed a steady methane increase during cultivation. In these cultures, acetate was no longer detected at the end of the incubation (data not shown). On the other hand, cultures incubated solely with limonene, in the absence of acetate, started methane production with a delay of 3 months (Fig. 1C). In these cultures we detected internal acetate build-up within the first two months followed by a sudden decrease during the third month of incubation (Fig. 1D).

The stoichiometry was calculated for culture duplicates (Table 1) incubated with different amounts of monoterpene in the presence or absence of 2 mM acetate. Control cultures supplemented with acetate in the absence of limonene produced one-tenth of the methane formed in the least active enrichment culture. The enrichment cultures showed a high carbon recovery as methane, which was 45% to 75% of the expected methane produced by complete

mineralization of the limonene consumed (Table 1). In addition to limonene degradation to methane gas, the absence of acetate or other organic acids as final fermentation products suggest the following stoichiometry: $C_{10}H_{16}$ + 6 H_2O → 7 CH_4 + 3 CO_2. This is an overall exergonic process, with a free energy change of ΔG_0'= -348 kJ mol^{-1} limonene (calculated after Mavrovouniotis, 1991).

Limonene degradation is likely initiated by fermenting bacteria. The transient acetate produced advocates for a syntrophic interaction initiated by the acetate and H_2 releasing reaction: $C_{10}H_{16}$ + 10 H_2O → 5 $C_2H_4O_2$ + 8 H_2 (ΔG_0'= + 156 kJ mol^{-1}). Since the process is endergonic it can only take place if the metabolic products are used to fuel the metabolism of other microorganism (Schink & Stams, 2006). Hence, both acetate and H_2 may be consumed in methanogenic reactions: (1) $C_2H_4O_2$ → CH_4 + CO_2 (ΔG_0'= - 49 kJ mol^{-1}) and (2) 4 H_2 + CO_2 → CH_4 + 2 H_2O (ΔG_0'= - 131 kJ mol^{-1}). The transient acetate accumulation in cultures incubated without additional acetate points to a less active methanogenic population at the beginning of the cultivation which correlates well to the 3 months of lag phase in methane production (Fig. 1C).

Microbial community composition

Different metabolic groups are expected to be involved in the methanogenic degradation of limonene in these syntrophic enrichment cultures. Yet it was unknown which microorganisms are involved (Widdel et al., 2006; Grossi et al., 2008), thus we investigated the community composition with molecular tools.

Microscopic observations showed the presence of numerous morphotypes, from small cocci and vibrios to thin and long, thick filaments (Figure 1D - inset). Using the 16S rRNA gene we could identify the phylogeny of the microorganisms. Amplified rRNA gene restriction analysis on 327 *Bacteria* clones and 141 *Archaea* clones and partial sequencing of 100 randomly selected clones directed the full sequence analyses of representative 16S rRNA genes. Phylogenetic analyses were performed on 35 *Bacteria* and 28 *Archaea* 16S rRNA gene sequences (Fig. 2 and 3). 16S rRNA gene sequences with more than 98.5% identity were regarded as one operational taxonomic unit (OTU). The microbial community composition was evaluated during mid-exponential growth on limonene by CARD-FISH.

Bacterial microbial community

Bacterial 16S rRNA gene sequences affiliated to *Deltaproteobacteria*, Candidate Division OP3, *Bacteroidetes* and *Firmicutes* (Fig. 2). The *Bacteria* general probe Eub-338 (I) matched

in silico all sequences from our *Bacteria* clone library, except the Candidate Division OP3 phylotype. Application of the *Bacteria* probes Eub-338 II & III did not increase the *Bacteria* abundance (data not shown). Moreover, none of these probes matched the 16S rRNA gene sequence of the Candidate Division OP3 phylotype. Therefore we designed a novel *Bacteria* probe, Eub-338 (VI), which matches positions 338-355 on the 16S rRNA gene of Candidate Division OP3 sequences. In equimolar amounts together with probe Eub-338, they targeted 40% of all DAPI stained cells (Fig. 4).

The *Deltaproteobacteria* phylotypes had as close relative (92%) the sulfate reducer *Desulfovirga adipica* (Fig. 2) (Tanaka et al., 2000). The *Deltaproteobacteria* cells were curved rods and highly represented in the enrichment cultures (12%). They likely play a role as syntroph in the complex degradation.

The Candidate Division OP3 phylotype showed 84% sequence identity (Fig. 2) to uncultured bacteria from pinyon juniper forest soil samples collected form a depth of 10-15 cm (Dunbar et al., 2002). Candidate Division OP3 16S rRNA gene sequences have been solely recovered from anoxic habitats such as anoxic sediments of Yellowstone Hot Spring (Hugenholtz et al., 1998), anoxic water body of the Cariaco basin (Madrid et al., 2001), or 700 m deep in the Antarctic continental shelf (Bowman & McCuaig, 2003). Two recent studies retrieved Candidate Division OP3 16S rRNA gene sequences from mesophilic and anoxic chemostats thriving on propionate and butyrate (Shigematsu et al., 2006; Tang et al., 2007). In this study, we visualized for the first time Candidate Division OP3 cells in a stable enrichment culture with the help of the newly designed probe OP3-565. It is specific for the OP3-phylotype found in our *Bacteria* 16S rRNA gene clone library. This specific probe detected 18 % of all DAPI-stained cells. The cells of Candidate Division OP3 were small and round-shaped (Fig. 4E). They were found either as single cells or attached to larger cells. The same cell morphology was revealed by probe Pla-46 which targets in silico the OP3 phylotype as well. It detected 13% of all DAPI-stained cells. The high abundance of Candidate Division OP3 members during exponential growth on limonene suggests that they play an important role in the degradation of this monoterpene, either by breaking down limonene and/or utilizing the primary degradation products. Probes Delta-495a and OP3-565 together detected almost the entire bacterial population as defined by the general *Bacteria* probes.

Another surprising finding in the *Bacteria* 16S rRNA gene library was the presence of *Bacteroidetes,* a phylum which contains numerous uncultured microorganisms. The six phylotypes identified were distantly related to *Prolixibacter bellariivorans* (81 to 89% identity) and *Alistipes putredinis* (84.1%). The genus *Prolixibacter* is represented by

facultative anaerobes which ferment sugars by mixed-acid fermentation (Holmes et al., 2007), whereas genus *Alistipes* includes strictly anaerobic microorganisms that form succinate as major metabolic product (Rautio et al., 2003). All *Bacteroidetes* sequences were targeted *in silico* by probe CF-319a, but we observed only a small population of *Bacteroidetes* (1%) in the enrichment cultures. The high abundance of *Bacteroidetes* in the 16S rRNA gene library and the low percentage of detected *Bacteroidetes* could be explained by a selective amplification of their 16S rRNA (Suzuki & Giovannoni, 1996). This group most likely scavenges residual organic material from dead cells.

Archaeal microbial community

The *Archaea* 16S rRNA gene sequences revealed *Euryarchaeota* affiliating with the orders *Methanomicrobiales* and *Methanosarcinalles* (Fig. 3). The general *Archaea* probe Arch-915 targeted *Methanosaeta*-like filaments and rods with flat ends. Staining of the *Methanosaeta*-like filaments was heterogeneous with Arch-915 as well as with MX-825: the probe signal was visible on particular parts of the filaments (Fig. 4C and 4D). Kubota et al. (2008) reported the same phenomenom: hybridization of *Methanosaeta concilii* filaments with Arch-915 was heterogeneous and incomplete, independent of the permeabilization procedures used. Arch-915 hybridized to 33% of the limonene-degrading microbial community. including an underestimation of the abundance of filamentous *Archaea*.

Methanosarcinales phylotypes (Fig. 3) were either related to to *Methanosaeta* sp. AMPB-Zg (>99% identity) or to *Methanosaeta concilii* (89% to 100% identity). Genus *Methanosaeta* comprises slowly growing acetoclastic methanogens with high affinity to acetate (Jetten et al, 1990). The transient formation of acetate and the detection of *Methanosaeta* species (1%), demonstrates the role of acetate as transfer metabolite during syntrophic limonene degradation. The second methanogenic group encountered in the *Archaea* clone library belongs to *Methanomicrobiales*. Their 16S rRNA gene affiliated with *Methanoculleus* (91.6% identity) (Zellner et al., 1998) a hydrogenotrophic methanogen with complex nutritional requirements (Zellner et al., 1998; Whitman et al., 2006). Its presence is an indication for hydrogen as intermediate in limonene degradation.

Conclusions

This is the first depiction of a syntrophic community thriving under methanogenic conditions on a naturally abundant monoterpene - limonene. The microbial community was represented by microorganisms spanning through the kingdoms of *Bacteria* and *Archaea*. Bearing in mind

the phylotypes encountered, limonene was most likely degraded by *Bacteria* to acetate and H_2 which were ultimately consumed by methanogenic *Archaea*. A significant portion of the microbial community consists of cells of the so far uncultured phylum, Candidate Division OP3. These small round cells were often attached to other bacteria and their high abundance implies they have an important role in limonene degradation under methanogenic conditions.

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Tables

Table 1 Limonene consumption and methane production in cultures thriving on limonene in the presence or absence of acetate (2 mM).

Cultures and conditions[1]	Initial limonene added (mmol)	Final limonene (mmol)	Methane produced (mmol)	Methane (theoretical) formed from:			Methane recovery
				Limonene[2]	Acetate[2]	Cysteine[2]	
1a) Cells (limonene)	4.6	2.6	9.7	14.0	-	0.3	65%
1b) Cells (limonene)	4.6	2.4	8.2	15.4	-	0.3	50%
1c) Cells (no limonene)	-	-	0.1	-	-	0.3	-
1d) Sterile control (limonene)	4.2	4.2	-	-	-	-	-
2a) Cells (limonene, acetate)	10	7.8	12.3	15.4	0.6	0.3	75%
2b) Cells (limonene, acetate)	10	6.3	12.0	25.9	0.6	0.3	45%
2c) Cells (no limonene, acetate)	-	-	0.9	-	0.6	0.3	-
2d) Sterile control (limonene, acetate)	12.6	10.7	-	-	-	-	-

[1] In cultures 1a to 1c, the inoculum was 1.3% of a concentrated cell suspension (20×) whereas in cultures 2a to 2c was 10%. [2] The theoretical methane formed from limonene was calculated according to the reaction $C_{10}H_{16} + 6\ H_2O \rightarrow 7\ CH_4 + 3\ CO_2$, whereas the methane formed from acetate and cysteine was calculated from the equations: $C_2H_4O_2 \rightarrow CH_4 + CO_2$ and $C_3H_7NO_2S + H_2O \rightarrow 5\ CH_4 + 7\ CO_2 + 4\ NH_3 + 4\ H_2S$.

Figure legends

Figure 1*:* Limonene degradation in methanogenic enrichment cultures. Black filled symbols show duplicate enrichment cultures incubated with 10 mmol limonene in a media supplemented with 0.6 mmol acetate and 0.3 mmol cysteine. The gray symbols show duplicate cultures incubated with 4.6 mmol limonene in the absence of acetate. (A) Cell densities increased in cultures supplied with different amounts of limonene (●,●). Inoculated controls did not grow in the absence of the hydrocarbon (empty symbols). (B) Consumption of limonene in cultures incubated with different amounts of monoterpene (■,■) in contrast to physical loss in sterile controls (□,□). (C) Methane production in cultures incubated with different amounts of limonene (▲,▲) versus a control enrichment culture (△) incubated in the absence of the monoterpene to determine the background methane production induced by the components of the media (2 mM acetate and 1 mM cysteine). The control enrichment in the absence of limonene, in media without acetate, did not produce methane (data not shown). (D) The transient development of acetate was observed in duplicate cultures incubated with limonene as sole organic electron acceptor. In cultures augmented with 0.6 mmol acetate complete consummation of this fatty acid was detected at the end of the incubation (data not shown). D-inset is a phase contrast micrograph of an enrichment culture. The scale bar = 5 μm.

Figure 2: Maximum parsimony tree of *Bacteria* 16S rRNA gene sequences retrieved from limonene degrading enrichment cultures. *Crenarchaeota* was used as outgroup. The phylotypes obtained in this study are emphasized in bold, and in paranthesis is shown the number of full 16S rRNA gene sequences with an identity above 98.5%. Related sequences of representative microorganisms can be acessed at NCBI using the tags given in parenthesis. The scale bar is 10 substitutions per 100 nuclotides.

Figure 3: Maximum parsimony tree of *Archaea* 16S rRNA gene sequences retrieved from limonene degrading enrichment cultures. *Crenarchaeota* was used as outgroup. The phylotypes obtained in this study are emphasized in bold, and in paranthesis is shown the number of full 16S rRNA gene sequences with an identity above 98.5%. Related sequences of representative microorganisms can be acessed at NCBI using the tags given in parenthesis. The scale bar is 10 substitutions per 100 nuclotides.

Figure 4: Microscopic immages of samples from limonene degrading enrichment cultures. The left pannels represent samples stained by HRP-labeled probes, which catalyze the deposition of Alexa-594 tyramides. The right pannels are the corresponding microscopic fields as visualized in blue by a DNA stain, DAPI. (A) *Bacteria* cells stained with Eub-338 (I and VI) and (B) the cells stained with DAPI. (C) *Archaea* cells stained with Arch-915, and (D) the corresponding DAPI signals. (E) Cells stained with a Candidate Division OP3 specific probe, OP3-565 and (F) the cells stained by DAPI. In the magnified field is an superimposed image of signals obtained with OP3-565 and DAPI, which resulted in a pink signal for cells that were stained by both dyes.

Figures

Figure 1

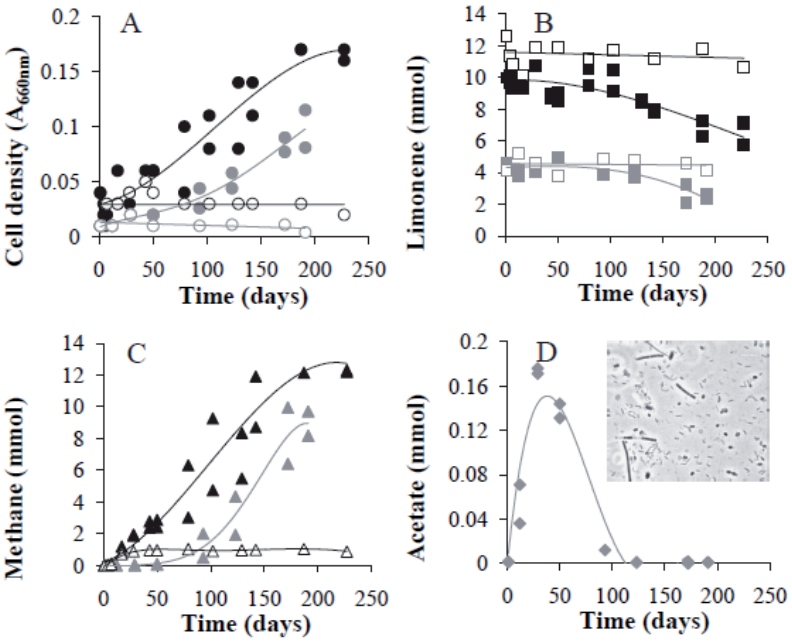


Figure 2


Crenarchaeota
Clone ridge flank crustal fluid (DQ513071)
Clone Yellowstone hot spring (AF027088)
Clone deep-sea sediment Pacific Nodule Province (AJ567574)
Clone trickling filter biofilm (AJ224540)
Clone pinyon-juniper forest soil (AF507707)
Clone sphagnum bog (AY775535)
Clone propionate-degrading methanogenic chemostat (AB232821)
Clone LiM-11E9 [5]
Clone biofilm at hydrothermal vent orifice (AM712351)
Clone sediments hydrothermal field (AB252431)
Clone sulfide- and sulfur-rich zodletone spring (EF602488)
Clone subseafloor sediments (EU386021)
Clone Hawaiian Archipelago Lake Kauhako (AY344402)
Candidate Division OP3
Prosthecobacter debontii (U60014)
Prosthecobacter fusiformis (U60015)
Verrucomicrobium spinosum (X90515)
Pelagicoccus litoralis (AB286017)
Pelagicoccus mobilis (AB286015)
Opitutus sp. VeSm13 (X99392)
Opitutus terrae (AJ229235)
Verrucomicrobia
Lentisphaerae
Clone LCFA methanogenic chemostat (AB244309)
Clone LiM-14F6 [8]
Clone LiM-14G10 [1]
Clone biofilm subsurface acid mine drainage (AY082469)
Clone LiM-14B6 [1]
Cytophaga sp. AN-BI4 (AM157648)
Prolixibacter bellariivorans (AY918928)
Clone LiM-15G7 [1]
Clone biofilm coking wastewater (DQ988269)
Clone microcosm with polychlorinated dioxins (AB234244)
Alistipes putredinis (L16497)
Bacteroides sp. DSM 12148 (AJ518876)
Rikenella microfusus (L16498)
Odoribacter splanchnicus (L16496)
Clone LiM-11A12 [2]
Clone DCM degradating environment (EU214534)
Clone anaerobic 1,2-dichloropropane mixed culture (AJ249109)
Flexibacter canadensis (M62793)
Clone LiM-15A3 [1]
Clone paper mill wastewater anaerobic reactor (AY426460)
Clone LiM-14D12 [3]
Cytophaga sp. Dex80-37 (AJ431253)
Cytophaga sp. BHI60-95B (AJ431254)
Clone inactive deep-sea hydrothermal vent (AB100013)
Bacteroidetes-Chlorobi group
Syntrophomonas sp. MGB-C1 (AB021306)
Clone LiM-15G5 [1]
Syntrophomonas cellicola (DQ288691)
Syntrophomonas wolfei subsp. saponavida (DQ666176)
Syntrophomonas palmitatica (AB274039)
Clone organic waste degrading packed-bed reactor (AB274497)
Firmicutes
Desulfovibrionales
Desulfuromonadales
Syntrophobacter fumaroxidans (X82874)
Syntrophobacter pfennigii (X82875)
Clone acidophilic methanogenic enrichment sphagnum peat bog (AF524857)
Syntrophobacter wolinii (X70905)
Clone LiM-14A12 [11]
Syntrophobacter sp. (AJ237605)
Clone LiM-13G9 [1]
Clone LCFA methanogenic enrichment cultures (DQ984663)
Clone LCFA methanogenic enrichment cultures (DQ459216)
Deltaproteobacteria
10%

Figure 3

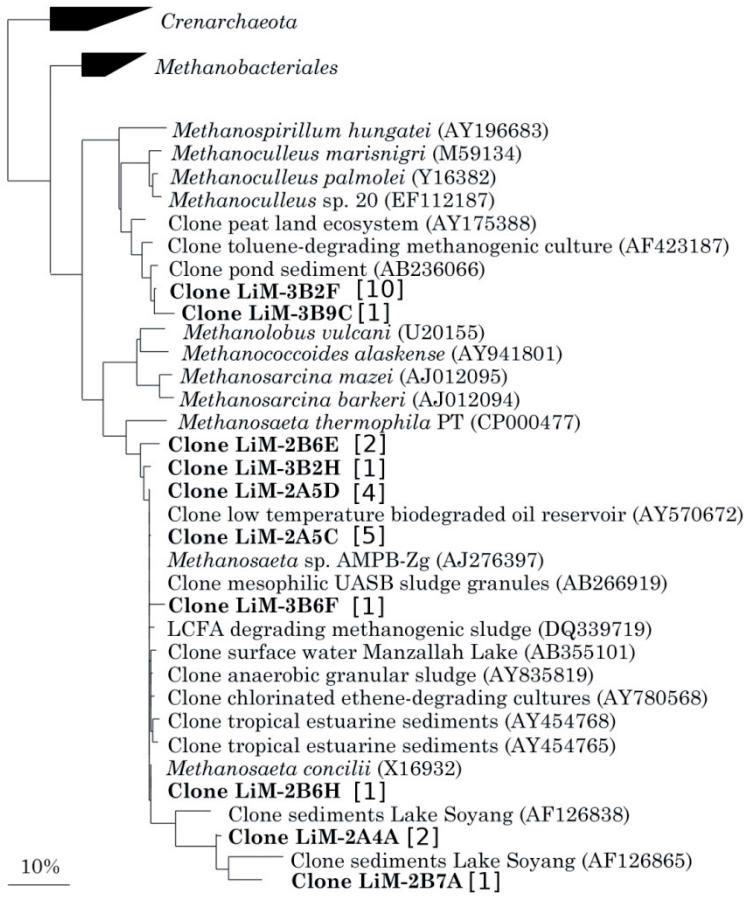
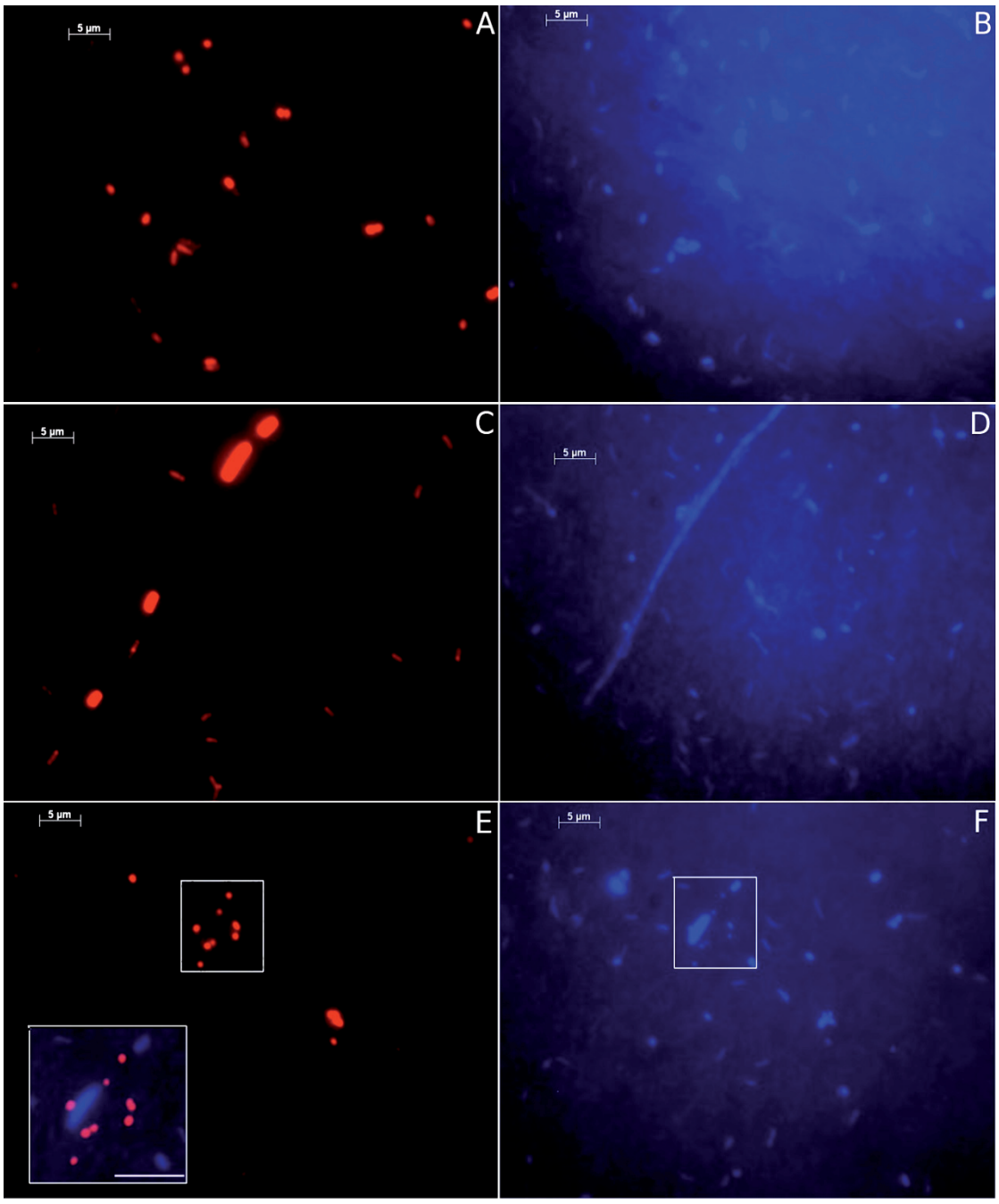

Crenarchaeota
Methanobacteriales
Methanospirillum hungatei (AY196683)
Methanoculleus marisnigri (M59134)
Methanoculleus palmolei (Y16382)
Methanoculleus sp. 20 (EF112187)
Clone peat land ecosystem (AY175388)
Clone toluene-degrading methanogenic culture (AF423187)
Clone pond sediment (AB236066)
Clone LiM-3B2F [10]
Clone LiM-3B9C [1]
Methanolobus vulcani (U20155)
Methanococcoides alaskense (AY941801)
Methanosarcina mazei (AJ012095)
Methanosarcina barkeri (AJ012094)
Methanosaeta thermophila PT (CP000477)
Clone LiM-2B6E [2]
Clone LiM-3B2H [1]
Clone LiM-2A5D [4]
Clone low temperature biodegraded oil reservoir (AY570672)
Clone LiM-2A5C [5]
Methanosaeta sp. AMPB-Zg (AJ276397)
Clone mesophilic UASB sludge granules (AB266919)
Clone LiM-3B6F [1]
LCFA degrading methanogenic sludge (DQ339719)
Clone surface water Manzallah Lake (AB355101)
Clone anaerobic granular sludge (AY835819)
Clone chlorinated ethene-degrading cultures (AY780568)
Clone tropical estuarine sediments (AY454768)
Clone tropical estuarine sediments (AY454765)
Methanosaeta concilii (X16932)
Clone LiM-2B6H [1]
Clone sediments Lake Soyang (AF126838)
Clone LiM-2A4A [2]
Clone sediments Lake Soyang (AF126865)
Clone LiM-2B7A [1]
10%

Figure 4



Forschung ist immer ein Spiel gegen die Zeit.

Richard Powers (2009)