Analysis of the microbial composition of the deep biosphere in four different sediments

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Dipl.-Biol. Anja Breuker
geboren am 28.01.1962 in Göttingen

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Referent: Prof. Dr. rer. nat. Axel Schippers
Koreferent: Prof. Dr. rer. nat. Marcus Andreas Horn
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Zusammenfassung

Ziel dieser Studie war die vergleichende Analyse der mikrobiellen Zusammensetzung der Tiefen Biosphäre in vier verschiedenen Sedimenten mit qualitativen und quantitativen Methoden. Es handelte sich hier sowohl um marine als auch terrestrische Sedimente, die sich hinsichtlich der Temperatur, des Gehaltes an organischem Kohlenstoff als auch anderer geochemischer Parameter unterschieden. Die mikrobiellen Lebensgemeinschaften wurden mit Hilfe der Gesamtzellzahlzählungen, der real-time-PCR (q-PCR), CARD-FISH und der 16S rRNA Gensequenzierung analysiert. Die Häufigkeiten von Bakterien und Archaeen zeigten deutliche Unterschiede. Mit Hilfe einer verbesserten DNA Extraktionsmethode wurde das erste Mal eine Dominanz von Archaeen im kalten, oligotrophen Sediment am Meeresboden des mittelatlantischen Rückens (North Pond) dokumentiert. Ähnlich häufig kamen die beiden Domänen im relativ kohlenstoffarmen Sediment des Flachmeeres von New Jersey vor. Bakterien dominierten in den hydrothermal beeinflussten marinen Sedimenten des Okinawa Beckens vor Japan ebenso wie in den terrestrischen Sedimenten nahe der Chesapeake Bay (Virginia, USA). In Übereinstimmung mit vorherigen Studien zeigte sich eine Abnahme der Gesamtzellzahlen mit der Tiefe und dem organischen Kohlenstoffgehalt. Dominante bakterielle Gruppen waren die *Proteobakterien*, das vorgeschlagene Phylum *'Atribacteria'* (OP9/JS1) und die Klassen *Anaerolineae* und *Caldilineae* der *Chloroflexi*. Die Häufigkeiten der *Geobacteraceen* (Eisen(III) und Mangan(IV) reduzierende Bakterien) korrelierten mit den Konzentrationen von reaktivem Mangan und Eisen im oberen Sediment. Die Analyse ergab neue Vertreter der Archaeen aus den Gruppen *'Bathyarchaeota'* (MCG), *'Hadesarchaea'* (SAGMEG), *'Lokiarchaeota',* (Untergruppe der MBG-B/DSAG Gruppe), *'Aenigmarachaeota'* (DSEG), *'Pacearchaeota'* (Teil der DHVE-6 Gruppe), *'Aigarchaeota', 'Thaumarchaeota',* der Terrestrial Hot Spring Group sowie verschiedener Gruppen der *Euryarchaeota*. Von diesen häufig in tiefen Sedimenten gefundenen Archaeen gibt es bis auf die *'Thaumarchaeota'* bisher keine kultivierten Vertreter. Die Zusammensetzung der hoch diversen Gruppe der *‘Bathyarchaeota‘* war tiefenabhängig. Im Hinblick auf die funktionellen Gene *cbbl* und *aprA* legen die Ergebnisse nahe, dass autotrophe Mikroorganismen neben heterotrophen und Sulfat reduzierenden Bakterien in der Biogeochemie der Sedimente wichtig sind. Die Tatsache, dass das funktionelle Gen *mcrA* kaum nachgewiesen werden konnte,

untermauert die Annahme bisher nicht beschriebener methanogener Mikroorganismen in der tiefen Biosphäre wie es kürzlich für die *'Bathyarchaeota'* diskutiert wurde. Die Ergebnisse unterstützen die These, dass Archaeen besser an kohlenstoffarme (und extreme) Habitate angepasst sind aber Bakterien im Allgemeinen dominieren.

Schlagworte: Tiefe Biosphäre, Mikrobielle Diversität, Archaeen, Sedimente, Sulfatreduktion

Keywords: deep biosphere, microbial diversity, archaea, sediments, sulfate reduction

Summary

Aim of this study thesis was the comparative analysis of the microbial composition of the deep biosphere in four different sediments. These were either marine or terrestrial and differed in temperature, organic carbon content as well as in various other geochemical parameters. The microbial communities were analyzed via total cell counting, real-time PCR (qPCR), CARD-FISH and 16S rRNA gene sequencing. The abundances of *Bacteria* versus *Archaea* varied strongly. Using an improved DNA extraction protocol, for the first time a dominance of *Archaea* was found for the cold oligotrophic sediment of a marine basin at the mid-Atlantic ridge (North Pond). A similar abundance of the two domains occurred in the relative organic lean sediments of the shallow shelf of New Jersey, and *Bacteria* dominated in marine sediments influenced by hydrothermal fluids in the Okinawa Trough off Japan as well as in the terrestrial sediments in the Chesapeake Bay area, VA, USA. Total cell counts decreased with sediment depth and with decreasing organic carbon content in agreement with previous studies. Dominant bacterial taxonomic groups were *Proteobacteria*, the candidate phylum *'Atribacteria'* (OP9/JS1) and the classes *Anaerolineae* and *Caldilineae* of the *Chloroflexi*. The abundance of *Geobacteraceae* (Fe(III)- and Mn(IV)-reducers) correlated with concentrations of reactive manganese and iron in the uppermost sediments. The analysis revealed new members of the *Archaea* belonging to the taxonomic groups *'Bathyarchaeota'* (MCG), *'Hadesarchaea'* (SAGMEG), *'Lokiarchaeota',* (section of the MBG-B/DSAG group), *'Aenigmarchaeota'* (DSEG), *'Pacearchaeota'* (section of the DHVE-6 group), *'Aigarchaeota'*, *'Thaumarchaeota',* the Terrestrial Hot Spring Group and different euryarchaeotic groups. These groups are often found in deep subsurface sediments but lack except the *'Thaumarchaeota'* cultured representatives. The highly diverse dominant *'Bathyarchaeota'* revealed a stratification dependent composition. Concerning the functional genes *cbbl* and *aprA*, the results suggest that autotrophic microorganisms could be relevant in addition to heterotrophs and sulfate reducing bacteria for the sediment biogeochemistry. The fact that the functional gene *mcrA* of methanogens was hardly detected argues for not yet described methanogens in the deep biosphere as recently discussed for the *'Bathyarchaeota'*. The results support the hypothesis that *Archaea* are better adapted to organic lean (and extreme) environments but *Bacteria* dominate in general.

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Abbreviations

16S rRNA gene	encoding gene for the small subunit (16S) of ribosomal RNA
AMP	adenosine 5'- monophosphate
AODC	acridine orange direct counts
aprA	encoding gene of adenosine 5' - phosphosulfate reductase subunit A
APS	adenosine 5'- phosphosulfate
ATP	adenosine triphosphate
bp	base pairs
CARD-FISH	catalyzed reporter deposition fluorescence in situ hybridization
cbbl	encoding gene of the large subunit of the ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo) enzyme
CBIS	Chesapeake Bay impact structure
CPR	Candidate Phyla Radiation
DHVE	Deep Hydrothermal Vent Euryarchaeotic Group
DIC	dissolved inorganic carbon
DNRA	denitrification and dissimilative nitrite reduction to ammonia
DOC	dissolved organic carbon
DPANN	superphylum of the *Archaea* (includes the proposed *'Diapherotrites'*, the proposed *'Aenigmarchaeota'*, the proposed '*Nanoarchaea'* and the proposed *'Nanohaloarchaea'*)
DSAG	Deep Sea Archaeal Group
DSEG	Deep Sea Euryarchaeotic Group
dsrA	encoding gene of the α-subunit of dissimilatory sulfite reductase
dw	dry weight
ICDP	International Continental Scientific Drilling Program
IODP	International Ocean Discovery Program or International Ocean Drilling Program
MBG-B	Marine Benthic Group B
MBG-D	Marine Benthic Group D
(m)bsf	(meters) below seafloor
MCG	Miscellaneous Crenarchaeotic Group
mcrA	encoding gene of methylcoenzyme M reductase subunit A

MG (1 α)	Marine Group (1 α)
NCBI	National Center of Biotechnology Information
ODP	Ocean Drilling Program
OECD	Organization for Economic Co-operation and Development
qPCR	quantitative PCR
RuBisCo	ribulose-1,5-bisphosphate carboxylase/oxygenase
Pg	Petagram (10^{15} g)
SAG	single-cell amplified genome
SAGMEG	South African Gold Mine Group
SMTZ	sulfate methane transition zone
TACK	superphylum of the *Archaea* (includes the proposed *'Thaumarchaeota',* the candidate phylum '*Korarchaeota',* the phylum *Crenarchaeota*, the proposed *'Aigarchaeota'* and the recently discovered *'Lokiarchaeota'*)
TCC	total cell counts
THSCG	Terrestrial Hot Spring Crenarchaeotic Group
TMEG	Terrestrial Miscellaneus Euryachaeotic Group
TIC	total inorganic carbon
TOC	total organic carbon

1. Introduction

1.1. The deep biosphere: microorganisms in deep sediments

The term biosphere is not exactly defined. The Russian geologist Vladimir Ivanovich Vernadsky (1863–1945) introduced it in science. "*Vernadsky understood biosphere as the external envelope of the Earth which is inhabited by living things, and comprises both all the living organisms of the planet and the elements of inorganic nature providing the medium for their habitat."* (Piqueras, 1998). In a common sense, a biosphere includes more or less closed habitats and its "inhabitants". "Inhabitants" means any member of all living creatures. In general, terrestrial and marine biospheres can be distinguished.

Balkwill defined the **terrestrial deep biosphere** as the biosphere in terrestrial sediments deeper than 30 – 35 m (Balkwill *et al.*, 1989). The deep terrestrial biosphere can be divided in subtypes. Pedersen distinguished aquifers, ancient salt deposits and continental sedimentary rocks and caves (Pedersen, 2000). In terrestrial subsurface environments (including the shallow terrestrial subsurface) Stevens distinguished sedimentary environments, permafrost, ice sheets and glaciers, and bedrock environments (Stevens, 2002). Stevens gave a more functional definition of the subsurface biosphere, including also the marine deep biosphere: *"The subsurface biosphere is the habitable volume of the Earth's crust, above the isotherm of maximum survivable temperature, but below the root zone, or bioturbated zone of soils and sediments".* (Stevens, 2002).

The **marine deep biosphere** comprises the sediment and the rock that is deeper than 1 meter beneath the seafloor (Jørgensen and Boetius, 2007), (Edwards *et al.*, 2012). "*Almost 95% of the seabed (67% of the Earth's surface) lies in water depths where the light intensity is too low to sustain photoautotrophic production, the temperatures are close to freezing (–1°C to 4°C) and the availability of organic matter controls benthic productivity and biomass.*" (Jørgensen and Boetius, 2007).

Little was known about the marine deep biosphere until 1994 when microorganisms were detected and microbial activity measured up to a sediment depth of 500 mbsf (Parkes *et al.*, 1994). Schippers *et al.* showed the viability of subseafloor populations in 2005 for depths of up to more than 400 m subseafloor sediments (Schippers *et al.*, 2005). Scientific Ocean drilling has also demonstrated that microbial populations are ubiquitously detectable in deep marine subsurface environments. Roussel *et al.* provided evidence for bacterial and archaeal life at a

depth of 1626 m below the seafloor in sediments mainly composed of hemipelagic mudrocks from the Newfoundland Margin (Roussel *et al.*, 2008). For the Canterbury Basin off New Zealand, Ciobanu *et al.* provided evidence for bacterial life up to depths of 1922 mbsf (Ciobanu *et al.*, 2014). Inagaki *et al.* found evidence of microbial life in sediments of the Pacific Ocean of Japan up to 2500 mbsf (Inagaki *et al.*, 2015). Consequently, the question arose how large this habitat is. Whitman *et al.* estimated the total number of prokaryotes and the total amount of their cellular carbon on earth to be 4 - 6 x 10^{30} cells and 350 - 550 Pg (5.5 x 10^{17} g) of carbon, respectively (Whitman *et al.*, 1998). According to Kallmeyer *et al.* who collected a larger data set of cell counts including more open ocean oligotrophic sediments, the total microbial abundance in subseafloor sediments was corrected to a lower number (2.9 x 10^{29} cells) which is similar to the estimates for the total number of prokaryotes in seawater (1.2 x 10^{29}) and in soil (2.6 x 10^{29}) (Kallmeyer *et al.*, 2012). Microbial biomass below the seafloor itself is estimated to be between 4 Gt and 303 Gt (Hinrichs and Inagaki, 2012).

All three domains of life, *Archaea*, *Bacteria* and *Eukarya* are thriving in the deep biosphere as well as spores and viruses exist (Edgcomb *et al.*, 2011), (Engelhardt *et al.*, 2013), (Lomstein *et al.*, 2012), (Schippers *et al.*, 2012). Therefore, conclusions of insights in biomasses and energy fluxes caused by microorganisms in marine and terrestrial sediments are important for several aspects of human life, like storage of waste, ground water flows, natural gasoline usage, methane production and release for example.

Concerning deeply-buried marine sediments, the question which factors are limiting and controlling microbial life was worked out by Parkes *et al.* (Parkes *et al.*, 2000). Hereby, they found the best correlation of overall total cell counts (TCC) with depths but less for porosity and age:

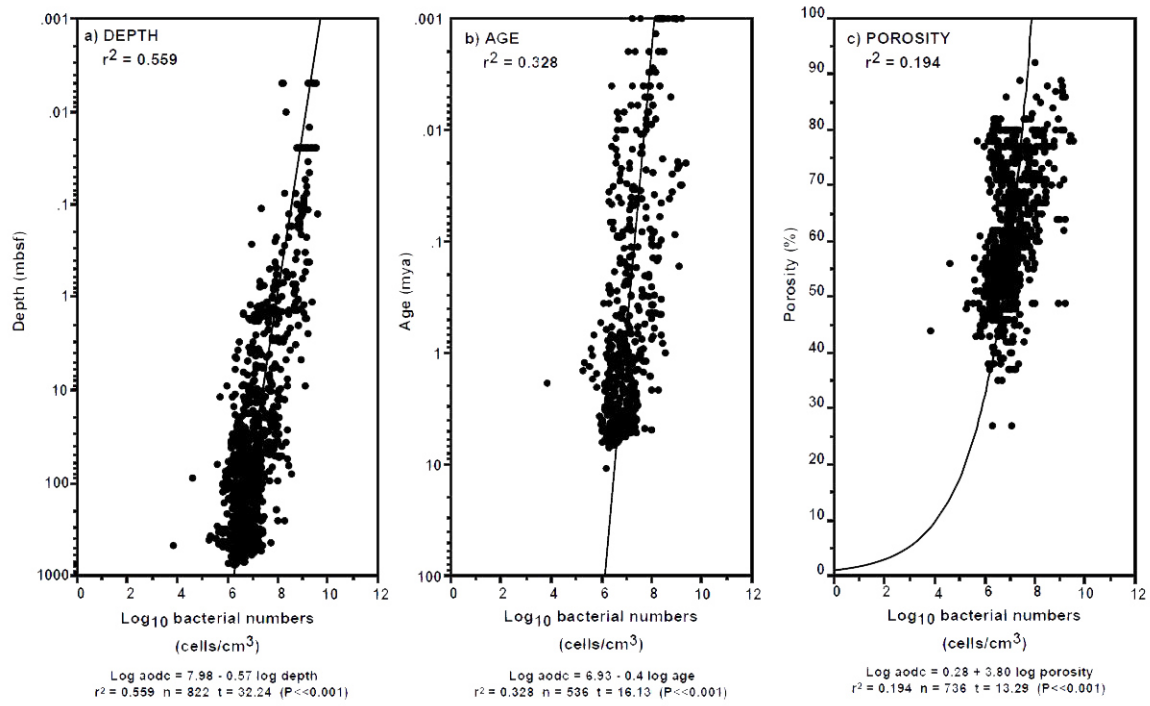


Figure 1. Correlation of subseafloor sediment prokaryotic cell counts with a) depth, b) age, and c) porosity. Data for several ocean drilling sites were compiled. Hydrothermally influenced sediments were not included. Modified from (Parkes *et al.*, 2000)

Kieft *et al.* found similar results for terrestrial sediments; they found a significant positive correlation between total organic carbon (TOC) values with the log of the direct cell counts and a significant positive correlation between the basal respiration rates and the TOC values (Kieft *et al.*, 1995).

These insights and data explain the importance of research in these mainly unexplored ecosystems and raise many questions: What are the energy sources of these from photosynthesis-detached microorganisms? What are electron donors and acceptors? Which kind of microbial loops take place there? How do they survive under extreme conditions such as nutrient limitation, high pressure and high temperature, or in other words, what is limiting microbial life in this environment? For the habitable depths, Heim (Heim, 2011) postulated space, (characterized by rock porosity), the availability of water and in particular temperature as limitation factors. Pedersen focused on temperature and availability of energy (Pedersen, 1993). Ghiorse and Wilson suggested that microbial life might exist up to 4000 m and more if water, pore space and nutrients are available (Ghiorse and Wilson, 1988). Only six years later, in 1994 Szewzyk *et al.* reported about the isolation of a thermophilic bacterium from a depth of 5278 m in a terrestrial sediment (granitic rock in Gravberg, Sweden) (Szewzyk *et al.*, 1994).

One major question concerning how microorganisms can survive in the deep biosphere is what is their energy source? These microorganisms are separated from photosynthesis and therefore need other energy sources. Concerning organic rich and/or reduced sediments, these compounds can serve as energy sources. Another theory about biospheres separated from these sources is that natural hydrogen especially in deeply buried sediments together with natural carbon dioxide could serve as energy source (Heim, 2011). Different ways of abiotic hydrogen production are proposed. Stevens *et al.* investigated hydrogen production by serpentinization. They demonstrated hydrogen production at room temperature by the reaction of water with ultramafic rocks (Stevens and McKinley, 1995), (Stevens, 1997). Another theory supports the radiolysis of water (Pedersen, 2000), (D'Hondt *et al.*, 2009), (Parkes *et al.*, 2014), (Lin *et al.*, 2005), (D'Hondt *et al.*, 2015). There have been several attempts to monitor hydrogen flux. Nevertheless, this question remained largely unacknowledged.

1.2. Biogeochemistry of marine and terrestrial sediments

Understanding the principles, reactions and systems how microorganisms are able to use possible free energy delivering reactions to gain energy is one of the general aims of microbiology. Hereby, sediments represent a stratified system of possible energy releasing substrates. This system is amongst others influenced by deposition of organic material from above, the thermal gradient, the oxygen gradient and the methane gradient. The main principle here is the absence of photoautotrophy as a possible energy providing reaction. Although it is not permissible to display general biogeochemical processes for all sediments, e.g. terrestrial sediments versus marine sediments, the main biogeochemical reactions as related to degradation of organic carbon (= oxidation of organic carbon) can be distinguished:

I. aerobic respiration
II. nitrate reduction
III. ammonification
IV. manganese and iron reduction
V. sulfate reduction
VI. anaerobic methane oxidation at the sulfate methane transition zone
($CH_4 + H_2SO_4 \rightarrow H_2S + H_2CO_3 + H_2O$)
VII. methanogenesis, acetogenesis (Fig. 2)

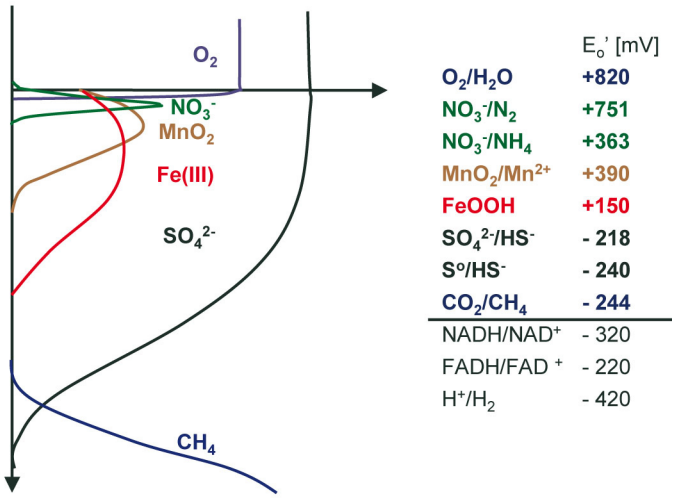


Figure 2. General stratification of concentrations of electron acceptors and methane in sediment pore water and relevant redox pairs. Modified from (Engelen, 2007).

The crossing of the methane and sulfate porewater concentration profiles in this general stratification scheme indicates the sulfate methane transition zone (SMTZ) for which anaerobic methane oxidation has been described. This process occurs in different sediment depths (Reeburgh, 1980), (Iversen and Jørgensen, 1985), (Thomsen *et al.*, 2001), (Treude *et al.*, 2005), (Berelson *et al.*, 2005). In addition, separated SMTZ's in the same core at different depths have been reported for a sediment of the Neuharlingersieler Nacken (53°43'270N and 07°43'718E), German Wadden Sea (Wilms *et al.*, 2007) and IODP Leg site 1229, Peru Margin (D'Hondt *et al.*, 2004).

Thullner *et al.* focused on microbial redox reactions of microorganisms in sediments with special attention to the hydrocarbon degradation following the proposed general cycle (Fig. 3) (Thullner *et al.*, 2007).

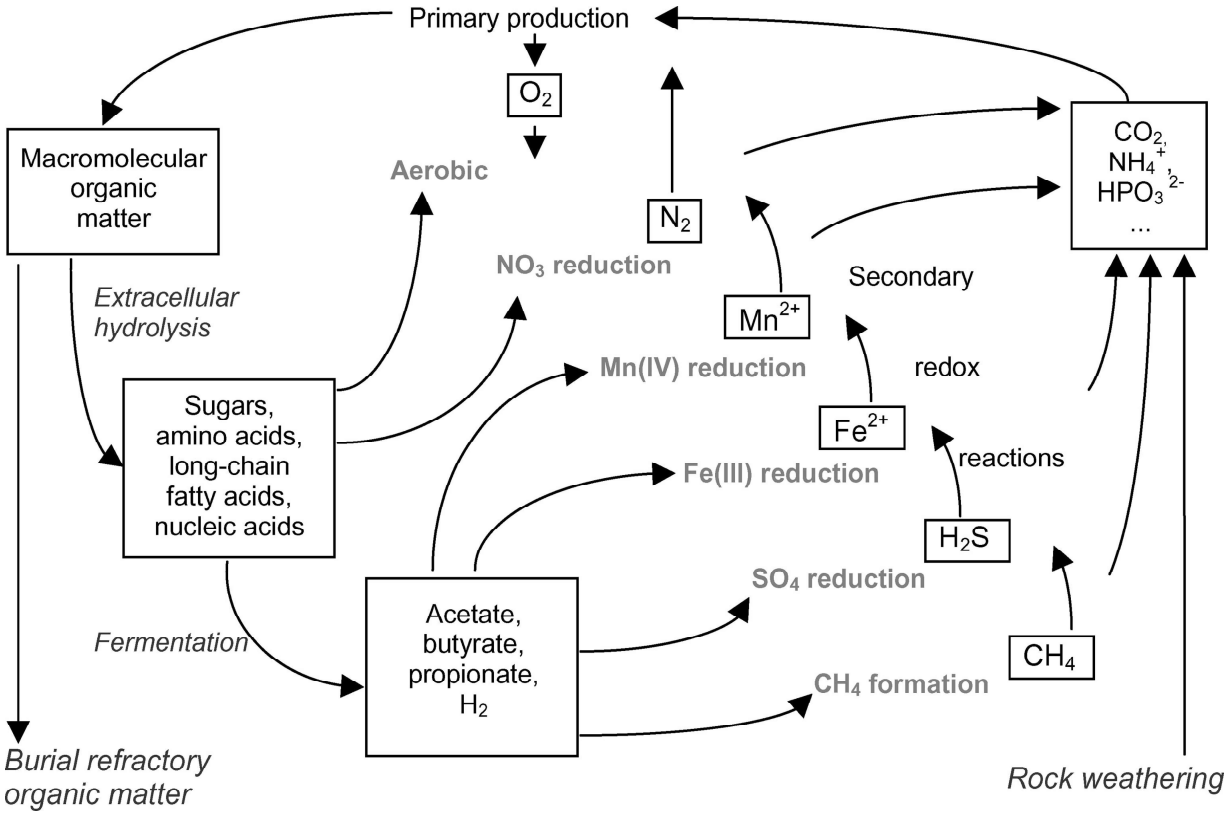


Figure 3. *"Key constituents, reaction pathways and redox species involved in the global organic matter cycle. The degradation of macromolecular organic matter synthesized by primary production causes the successive utilization of external electron acceptors, and the appearance of intermediate electron donor substrates. The reduced chemical species produced by the organic matter degradation pathways may participate in a variety of secondary redox reactions, hence greatly expanding the complexity of biogeochemical reaction networks in subsurface environments. Ultimately the degradation of organic matter regenerates inorganic carbon and nutrients that become again available to primary producers".* Picture from Thullner *et al*. (Thullner *et al.*, 2007), the picture was redrawn by Thullner *et al.*, originating from (Fenchel and Jørgensen, 1977).

Fermentation processes are largely unexplored in the deep biosphere; research so far has more focused on the final step of organic carbon degradation with the various electron-accepting processes. Since more and detailed biogeochemical investigations such as the Ocean Drilling program (ODP) Leg 201 related research in different deeply buried sediments have been carried out, exceptions like reversed zones or co-occurring of zones of this simple stratification model were found and discussed in detail (D'Hondt *et al.*, 2004), (Parkes *et al.*, 2005).

Despite these activities represent only a small part of a) possible reactions and b) known reactions; they are widespreadly found as well in marine, terrestrial and limnic systems (Jannasch and Mottl, 1985), (Nealson and Myers, 1992), (Fenchel and Jørgensen, 1977).

Energy gaining activities (amongst others: **dissimilatory pathways**) can be distinguished from **assimilatory pathways.** Dissimilatory pathways depend on the concurrent energy delivering available redox pairs and consequently the pH and principal physical parameters like temperature and pressure. The pH influences the possible free energy delivering reaction as well as concentrations of substrates and products. Both, dissimilatory and assimilatory pathways (like nitrogen fixation, assimilatory sulfate reduction and carbon fixation) are mediated by specific enzymes encoded by functional genes. Its genetic code is more or less conserved, e.g. the high variable *gyrB* genes that encode the subunit B protein of DNA gyrase (topoisomerase type II), the highly conserved DSR (dissimilatory sulfate reduction, last step) gene *dsrA* or the 16S rRNA gene (Yamamoto and Harayama, 1995), (Yamamoto and Harayama, 1996), (Wagner *et al.*, 1998).

As mentioned above, substrate availability and therefore the diversity of the community is one of the focused items in microbiology. The terminus “oligotrophy” is often used to characterize microorganisms in environments that have different limited kinds of substrate availability. Nevertheless, it is used inconsistently and not a distinct term.

1.2.1. Oligotrophy, extreme environments - what are we talking about?

Schut’s review “Oligotrophy and pelagic marine bacteria: facts and fiction” (Schut Frits, 1997) summarized the research efforts on oligotrophy. The difficulties to define the terminus “oligotrophy” (Schut Frits, 1997) resulted in a list of 16 different definitions (for detailed information see appendix).

They worked out the difficulty to get reliable data of obligatory oligotrophic microorganisms and concluded:

“It is therefore most conceivable that free-living marine bacteria represent cells with a remarkably stable 'low-nutrient-conditioned' phenotype. Often, this is perceived as an obligately oligotrophic state.”

Hütter focused more on photo autotrophy and defined trophy as the intensity of photoautotrophic primary production which reduces carbon of its highest oxidation state (CO_2) to organic compounds (Reitner and Thiel, 2011). It has to be mentioned, that this definition was published for application in limnic systems. According to the OECD definition, the most important factor for trophy is the total amount for phosphate during spring circulation (Lampert and Sommer, 1999) which can be

divided in five subcategories from ultra-oligotrophic to hypereutrophic. Nevertheless, this definition was also related to limnic systems and is not widely used for terrestrial or marine sediments.

Concerning the subseafloor biosphere, Roussel *et al.* (Roussel *et al.*, 2008) confirmed the correlation between the organic matter index and total cell counts. Conclusively, it is not surprising that organic carbon outcompetes other possible energy donating reactions which release less free energy. Durbin and Teske suggested different parameters for marine sediments to define the trophic status (Durbin and Teske, 2012): *"The combination of higher-energy electron acceptor type and slower flux of electron donor substrates likely imposes distinct constraints on life in oligotrophic marine sediments, which cover the majority of the surface of Earth "* (Durbin and Teske, 2012). Hereby, ultra-oligotrophic sediments exhibit no depletion of oxygen or nitrate, oligotrophic sites exhibit nitrate depletion within meters, mesotrophic and eutrophic sites are characterized by high DIC and ammonium pore water concentrations and high sedimentation rates. Schut focused at marine bacteria and defined oligotrophy at least as the inability of bacterial cells to propagate at elevated nutrient concentrations (Schut Frits, 1997). According to this short summary, there are two different ways to approach to the terminus "oligotrophy":

a) by characterizing the environment and therefore to specify the oligotrophic parameter

b) by focusing on the microorganisms and - for example - to include only microorganisms that are able to grow at low nutrient conditions but not at elevated nutrient conditions

Concerning the amount of total organic carbon, it is useful to characterize different categories (Durbin and Teske, 2012) given by the concentrations of organic carbon as shown in Table 1.

Table 1. Characterization of sediments depending on their TOC content according to Durbin and Teske (Durbin and Teske, 2012).

amount of TOC	**characterization**	**examples**
< 1%	organic lean	abyssal plains, e.g. South Pacific Gyre
1% - 2%	between organic lean and organic rich	South China sea sites Gulf of Mexico sites
> 2%	organic rich	Eastern Mediterranean Sea: Amsterdam mud volcano, Kazan mud volcano

The existence of a **deep hot biosphere** was postulated in 1992 by the American astrophysical scientist Thomas Gold (Gold, 1992). He postulated that life under the earth crust is not dependent on photosynthesis as primary energy source but uses chemical sources such as fluids from deeper sections of the earth. Its energy supply comes from chemical sources, e.g. hydrothermal vents and he mentioned "*that microbial life exists in all the locations where microbes can survive"* (Gold, 1992). Gold also favored the *Archaea* to be the inhabitants of this environment (Gold, 1992). *Methanopyrus kandleri* (DSM 6324^{T}) growths between 84 and 110°C (300 kPa pressure) (Kurr *et al.*, 1991); *Methanopyrus kandleri* strain 116 proliferates at temperatures up to 122° C under high pressures (20 MPa) (Takai *et al.*, 2008). An intensive discussion about potential limits of life on earth started (Daniel and Cowan, 2000). Similar to oligotrophic environments, McKay summarized: *"There are two somewhat different approaches to the question of the limits of life. The first approach is to determine the requirements for life. The second approach is to determine the extreme environments in which adapted organisms - often referred to as extremophiles - can survive."* (McKay, 2014).

1.3. *Archaea* and *Bacteria*

In 1676 Antonie van Leuwenhoek observed bacteria among the very little animalcules in sea water, rain water and pepper water (van Leewenhoeck, 1677), (Sapp, 2005). Beside the important work of Robert Koch and Louis Pasteur in prevention, defense and identification of infection ways of diseases caused by *Bacteria*, it took about another ~ 250 years until a representative definition of *Bacteria* (formerly often handled as "germs") differentiating them from *Eukarya* was enunciated: *"Bacteria may be defined as extremely minute, simple, unicellular microörganisms* (sic!)*, which reproduce themselves with exceeding rapidity, usually by transverse division, and grow without the aid of chlorophyl* (sic!)*. They have no morphological nucleus, but contain nuclear material which is generally diffused throughout the cell body in the form of larger or smaller granules."* (Park and Williams, 1914), (Sapp, 2005). Around 50 years later, Woese and Fox introduced the "archaebacteria" as the third of three primary kingdoms (Woese and Fox, 1977). Besides the ongoing discussion about the last universal common ancestor/last eukaryotic common ancestor (LUCA/LECA) (Koonin, 2015), (Evans *et al.*, 2015), (Brochier-Armanet *et al.*, 2008), (Spang *et al.*, 2015), and if two or three primary

kingdoms exist (Williams *et al.*, 2013), the question of abundances, of *Bacteria* versus *Archaea* and their contribution to biogeochemical cycles (e.g. methanogenesis/methane oxidation) has been raised (Lipp *et al.*, 2008), (Hug *et al.*, 2016), (Valentine, 2007), (Gubry-Rangin *et al.*, 2010), (Briggs *et al.*, 2012), (Schippers and Neretin, 2006).

Before this era, most discoveries where made when cultivating techniques where established and pathogenic and not pathogenic microorganisms could be cultivated and their roles for human beings were identified. However, cultivation techniques are often insufficient and need to be supplemented with molecular ecological methods if investigations about unexplored complex ecosystems such as the deep biosphere are carried out. In 1977, Carl Woese and George fox published the article “The primary kingdoms”. This was the beginning of a new era of functional and phylogenetic insights basing initially on the analysis the small subunit gene of the ribosome of eukaryotic and prokaryotic cells (Woese and Fox, 1977). The analysis of the 16S rRNA gene gives enough information to analyze phylogenetic and evolutionary relationships and also to detect and categorize new species/families/classes with a manageable effort. New culture independent approaches not only comprising the 16S rRNA gene (and the eukaryotic 18S rRNA gene) but also other at least in parts conserved genes (e.g. the *dsrA* gene) were widely established. The next milestone in phylogenetic approaches was the development of the single cell genome amplification method by Zhang *et al.* (Zhang *et al.*, 1992). This approach was first intended to be applied to human health questions: "*Whole genome amplification beginning with a single cell, or other samples with very small amounts of DNA, has significant implications for multipoint mapping by sperm or oocyte typing disease diagnosis, forensics, and the analysis of ancient DNA.*" (Zhang *et al.*, 1992).

Since the beginning of phylogenetic analysis and discussion, the place of the *Eukarya* in the phylogenetic tree and therefore the root of the tree as a marker for the latest common ancestor was attended (Spang *et al.*, 2013). With other words, are there two or three primary domains of life and how did they evolve (Williams *et al.*, 2013), (Woese *et al.*, 1990)? By using a large data set based on ribosomal proteins in a multimodal approach including metagenomics analysis and the single-cell amplified genome method (SAG) the analysis of Hug *et al.* sustain the branching of the *Eukarya* with the *Archaea* (Hug *et al.*, 2016) (Fig. 4).

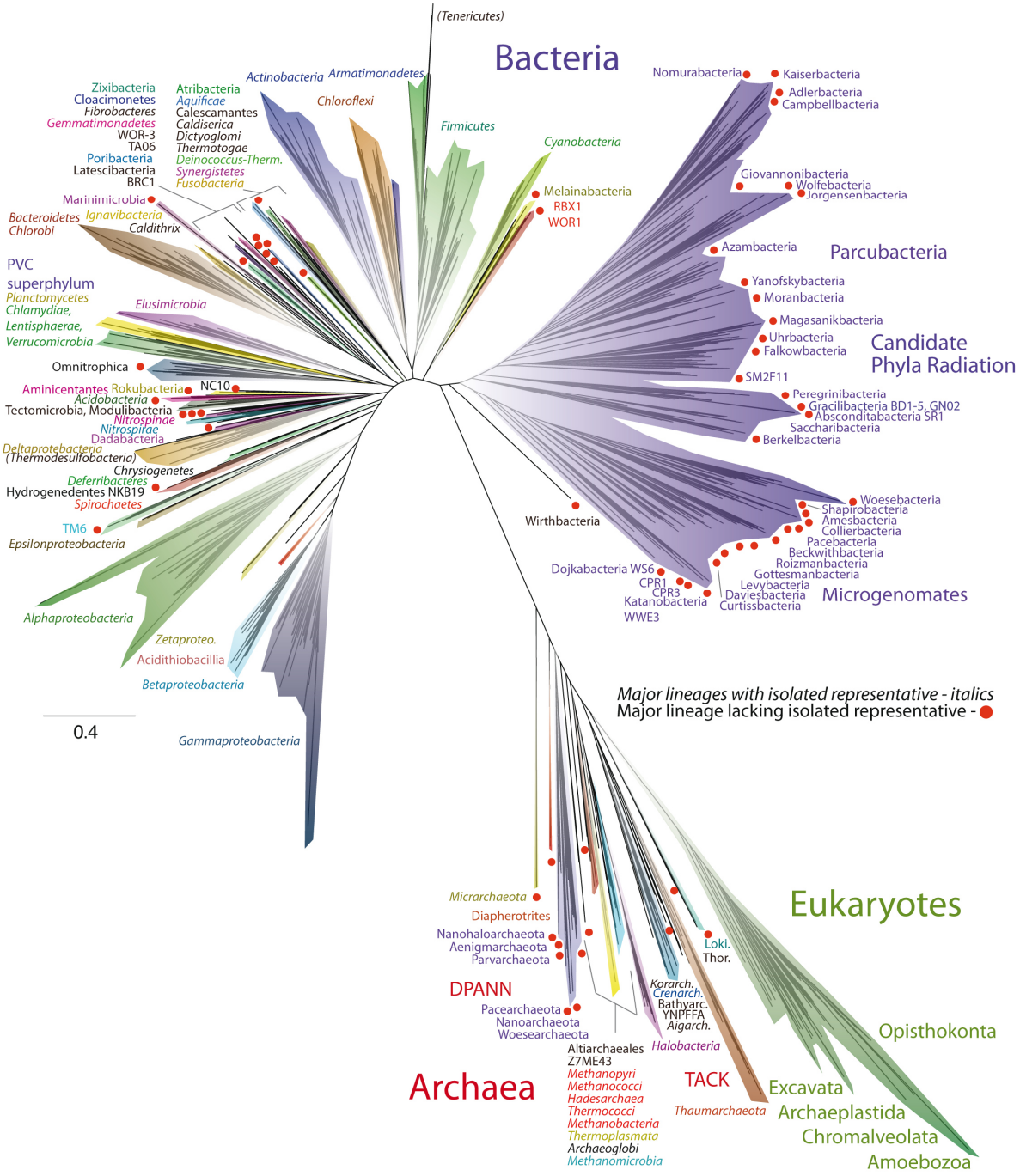


Figure 4. A current view of the tree of life. The tree includes 92 named bacterial phyla, 26 archaeal phyla and all five of the Eukaryotic supergroups. Major lineages are assigned arbitrary colors and named, with well-characterized lineage names, in italics. Lineages lacking an isolated representative are highlighted with non-italicized names and red dots. The names *Tenericutes* and *Thermodesulfobacteria* are bracketed to indicate that these lineages branch within the *Firmicutes* and the *Deltaproteobacteria*, respectively. The CPR phyla are assigned a single color as they are composed entirely of organisms without isolated representatives, and are still in the process of definition at lower taxonomic levels (Hug *et al.*, 2016). Description has been modified from (Hug *et al.*, 2016).

1.3.1. Phylogeny of *Archaea*

The “List of Prokaryotic names with Standing in Nomenclature” (http://www.bacterio.net/) contains a generally accepted taxonomic classification and a depository of scientific descriptions and knowledge of microorganisms. Up to date only two archaeal phyla (the ***Euryarchaeota*** and the ***Crenarchaeota***) are validated. Whole genome analysis allows identifying genes for entire protein/enzyme families. Therefore not only the identification of one metabolic reaction is possible; the prediction of feasible metabolic pathways and bases on the knowledge of the evolutionary conservation of genes, new phylogenetic relationships have been established (Hug *et al.*, 2016), (Baker *et al.*, 2016). For example, the SAG method has revealed two new superphyla concerning the *Archaea*. The **TACK** superphylum includes the ***'Thaumarchaeota'***, *the* ***'Aigarchaeota'***, *the* ***Crenarchaeota*** and the ***'Korarchaeota'*** (Guy and Ettema, 2011); the **DPANN** superphylum includes the ***'Diapherotrites'*** (clone pMC2A384), the ***'Parvarchaeota',*** the ***'Aenigmarchaeota'*** (DSEG), the ***'Nanohaloarchaeota'*** and the ***'Nanoarchaeota'*** (Rinke *et al.*, 2013). It has to be mentioned, that the DPANN superphylum is monophyletic only in *Archaea* restricted trees (Rinke *et al.*, 2013).

These superphyla were expanded recently due to metagenomics sequencing. For example, the ***'Lokiarchaeota'*** (Spang *et al.*, 2015) were described and supposed to belong also to the TACK superphylum. Concerning 16S rRNA gene phylogenetic analysis, the *'Lokiarchaeota'* are part of the **Deep-Sea Archaeal Group/Marine Benthic Group B (DSAG/MBG-B)** (Spang *et al.*, 2015). Phylogenetic analyses of universal proteins of the *'Lokiarchaeota'* revealed that they form a monophyletic group with eukaryotes (Spang *et al.*, 2015), sharing amongst others the ubiquitin protein degradation system with the eukaryotes. They are supposed to represent a descendant of the last common ancestor of the *Eukaryotes* and the *Archaea* (Koonin, 2015), (Spang *et al.*, 2015), (Embley and Williams, 2015). Similarities with the ubiquitin protein modification system genes, an actin skeleton coding signature, genes for eukaryotic signal transduction ways and the eukaryotic endosomal sorting complex (ESCRT) genes amongst others with the eukaryotes support this thesis (Spang *et al.*, 2015).

The DSAG/MBG-B group is found widely in marine sediments (Biddle *et al.*, 2006), (Teske, 2006), (Sørensen *et al.*, 2004) and significantly gas hydrate

associated (Parkes *et al.*, 2014). The DSAG/MBG-B group dominated methane hydrate bearing zones at Peru Margin (site 1230) and Cascadia Margin (site 1244/1245 and site 1251). In contrast, methane free zones contained relatively few clones of DSAG/MBG-B 16S rRNA encoding genes (site 1225 and site 1227) (Inagaki *et al.*, 2006). The group was also present at the sulfate reduction zones and therefore the authors inferred that DSAG/MBG-B group plays an important role in sulfate reduction and methane oxidation (Inagaki *et al.*, 2006). In summary, the DSAG/MBG-B group is active within the SMTZ but is it not limited to this zone (Teske and Sørensen, 2008), (Biddle *et al.*, 2006). These results suggest that the MBG-B Archaea benefit from methane in marine sediments (Teske and Sørensen, 2008), (Sørensen and Teske, 2006) and that the MBG-B assimilate other organic compound than methane, maybe by oxidation of methane without its assimilation (Biddle *et al.*, 2006). The proposed microbial ethanogenesis via acetate reduction (Hinrichs *et al.*, 2006) might be beside fermentation and methane oxidation a third feasible reaction for energy generating of DSAG/MBG-B Archaea (Teske and Sørensen, 2008).

Another recently proposed deeply branching phylum is the ***'Aigarchaeota'*** former known as **HWCG I (Hot Water Crenarchaeotal Group I)**. As an indicator for phylogenetic neighborhood, the *'Aigarchaeota'* share the ubiquitin modification system and a topoisomerase I B encoding gene with the *Eukarya* (Nunoura *et al.*, 2011), verified in its candidate *'Caldiarchaeum subterraneum'*.

The **Marine Group I** (proposed phylum ***'Thaumarchaeota'*** Brochier-Armanet *et al.*, 2008) is another group that is often found in deep marine sediments (Teske and Sørensen, 2008). Similar to the *'Aigarchaeota'*, Brochier-Armanet *et al.* showed that a eukaryotic-like DNA Topoisomerase B encoding gene is present in sequenced genomes of two *Archaea ('Nitrosopumilus maritimus'* and *'Cenarchaeum symbiosum'*) of the *'Thaumarchaeota' (Brochier-Armanet et al., 2008)*. The authors conclude: *"This finding indicates that the last common ancestor of Archaea and Eukarya may have harboured a DNA genome."* (Brochier-Armanet *et al.*, 2008).

The **Miscellaneus Crenarchaeotic Group (MCG, *'Bathyarchaeota'*)** is one major group that is found widespread in marine and terrestrial sediments (Biddle *et al.*, 2006), (Teske, 2006), (Kubo *et al.*, 2012). The MCG group encompasses members of the former named Terrestrial Miscellaneous Crenarchaeotic Group which were first isolated in waters of a South African Gold Mine (Takai *et al.*, 2001). By identifying also members of marine environments, the group was renamed as

Miscellaneous Crenarchaeotic Group (Inagaki *et al.*, 2003). Several attempts to clear up the phylogenetic position and suborders of this group were undertaken. These approaches reflect the high intragroup phylogenetic diversity of the MCG *Archaea* and the attempt to get more insights in phylogenetic and biogeochemical features of these *Archaea*. For further detail about the history of grouping, see appendix.

By performing a metagenomics analysis (including the LSU-SSU rRNA, ribosomal proteins and topoisomerase IB genes), Meng *et al.* supported the thesis that the MCG group forms a sister lineage with the *'Thaumarchaeota'* and the *'Aigarchaeota'* (Lloyd *et al.*, 2013b) and proposed a new phylum *'Bathyarchaeota'* for this group (Meng *et al.*, 2014). The proposal of a new phylum was confirmed by metagenomic analysis (shotgun metagenomic sequencing) of 53 archaeal concatenated conserved single-copy genes (He *et al.*, 2016). The MBG-B and the *'Bathyarchaeota'* were suspected to be responsible for exogenous protein degradation in cold anoxic environments (Lloyd *et al.*, 2013b). This finding was confirmed by Castelle *et al.* for the four-, five-, and six meter assemblies from an aquifer adjacent to the Colorado River, near Rifle, Colorado, USA by identifying peptidases (Castelle *et al.*, 2015): "*This expands the potential role of Archaea in protein remineralization to terrestrial anoxic sediment."*

'Bathyarchaeota' have been found in very different and also extreme environments (Teske and Sørensen, 2008), (Lazar *et al.*, 2015). Although they are typically found in anaerobic, eutrophic marine sediments, *'Bathyarchaeota'* are not restricted to eutrophic sediments (Durbin and Teske, 2012). The working hypothesis that *'Bathyarchaeota'* are heterotrophic anaerobes was expressed (Teske and Sørensen, 2008). Stratification dependent analysis showed the *'Bathyarchaeota'* were exclusively found below the SMTZ (ODP site 1227) and frequently above the SMTZ, whereas the SMTZ was dominated by the MBG-B group (Sørensen and Teske, 2006). For sediments of the White Oak River estuary, an increasing proportion of *'Bathyarchaeota'* was found with increasing reducing conditions and increasing depths (Lazar *et al.*, 2015). Regarding the *'Bathyarchaeota'* subgroups, the MCG-6 subgroup (Kubo *et al.*, 2012) exhibited a controversial pattern with decreasing clone library abundance (Lazar *et al.*, 2015). Interestingly, a recent study of three karstic lakes in Spain identified the MCG-6 subgroup as a "generalist group able to cope with varying reducing conditions" and the subgroups MCG-5a and MCG-5b (Kubo *et al.*, 2012) as "planktonic specialists thriving in euxinic bottom waters" (Fillol *et al.*,

2015). Clone library abundances do not reflect real abundances and therefore have to be handled with caution. For the Pearl River estuary *Jiang et al.* found *'Bathyarchaeota'* to be the most abundant group except the top layer and with a relative minimum at the SMTZ and a stratification depending on the accorded subgroups (Jiang *et al.*, 2011). Nevertheless, *'Bathyarchaeota'* play a major, largely unknown role in terrestrial and marine biogeochemical cycles.

Concerning the *Euryarchaeota*, 16S rRNA gene sequences of the **South African Euryarchaeotic Gold Mine group (SAGMEG)** were first isolated in waters from a South African gold mine (Takai *et al.*, 2001). Biddle *et al.* showed that they are also distributed in marine deep sediments (Nankai Trough, Peru Margin, Sea of Okhotsk) (Biddle *et al.*, 2006). Recently, Baker *et al.* carried out a phylogenomic analysis that places the SAGMEG *Archaea* as a deeply rooting sister clade of the *Thermococci*. They proposed a new phylum ***'Hadesarchaea'*** (former part of the *Euryarchaeota*) (Baker *et al.*, 2016). Although the analyzed bins obviously lack the gene for methyl-CoM reductase (*mcrA*), they contain partially several genes for the carbon monoxide dehydrogenase pathway (reductive acetyl CoA pathway, Wood-Ljungdahl pathway). This pathway is typically used in methanogenic *Archaea* (Baker *et al.*, 2016)). They contain also partially genes for the glycine pathway (Baker *et al.*, 2016), which is supposed to be a common ancestor of carbon fixation (Braakman and Smith, 2012). On the other hand, two of four analyzed bins have the genes of a near full Calvin-Benson-Basham cycle for carbon fixation but lack the genes for the tricarboxylic acid cycle for carbon fixation (Baker *et al.*, 2016). As pointed out by several researchers (Biddle *et al.*, 2006), (Teske and Sørensen, 2008), the *'Hadesarchaea'* are a part of the heterotrophic archaeal community in deep, anaerobic and methanogenic Peru Margin sediments at ODP LEG 201 Site 1227. The stratified abundances of *'Hadesarchaea'* concerning the SMTZ together with the possible capabilities in biogeochemical carbon pathways makes it of special interest to understand their role in methanogenesis/methanotrophy or syntrophy of methanogenic microorganisms. Baker *et al.* concluded, "*They* [the *'Hadesarchaea'*] *share several physiological mechanisms with strict anaerobic Euryarchaeota. Several metabolic characteristics make them successful in the subsurface, including genes involved in CO and H_2 oxidation (or H_2 production), with potential coupling to nitrite reduction to ammonia (DNRA).*" (Baker *et al.*, 2016).

16S rRNA gene sequences belonging to the **Deep-Sea Hydrothermal Vent Euryarchaeotal Group 6 (DHVEG-6)** were firstly isolated from a hydrothermal vent environment (Takai and Horikoshi, 1999). In 2004, 16S rRNA gene sequences from the organic lean Peru Margin ODP Leg 201 site 1231 at 1.8 mbsf were successfully isolated. This group as well as the Terrestrial Miscellaneus Euryarchaeotal Group (TMEG) has representatives in terrestrial sediments (Teske and Sørensen, 2008). According 16S rRNA gene phylogeny, the candidate phylum ***'Parvarchaeota'*** is a subgroup of the DHVEG-6 group. The *'Parvarchaeota'* include the genus *'Parvarchaeum'* (e.g. *Candidatus Parvarchaeum acidiphilum*, ARMAN-4) and some authors also include (depending on the analysis method and the scientific evaluation) the genus *'Micrarchaeum'* (e.g. *Candidatus Micrarchaeum acidiphilum* ARMAN-2), which was detected in the acidic Richmond Mine at Iron Mountain in northern California (Baker *et al.*, 2010), (Comolli *et al.*, 2008), (Rinke *et al.*, 2013), (Baker *et al.*, 2006). The placement of the *'Parvarchaeota'* cannot be reproduced consistently by metagenomic analysis (and 16S rRNA phylogeny); Castelle *et al.* found the genera *'Parvarchaeum'* and *'Micrarchaeum'* of *'Parvarchaeota'* not to be monophyletic as proposed by Rinke *et al.* (Castelle *et al.*, 2015), (Rinke *et al.*, 2013) and supposed the additional phylum ***'Micrarchaeota'***.

Other recently identified subgroups of the DHVEG-6 group are the proposed new phyla ***'Woesearchaeota'*** and ***'Pacearchaeota'*** (Castelle *et al.*, 2015) which form sister lineages to the *'Parvarchaeota'* and are part of the DHVEG-6 group according 16S rRNA gene phylogeny. They were mainly isolated from an aquifer adjacent to the Colorado River, near Rifle, CO, USA. "*The organisms have small genomes, and metabolic predictions indicate that their primary contributions to Earth's biogeochemical cycles involve carbon and hydrogen metabolism, probably associated with symbiotic and/or fermentation-based lifestyles."* (Castelle *et al.*, 2015).

Marine subsurface sediments contain members of the **MBG-D group (Marine Benthic Group D)** (Lloyd *et al.*, 2013b). Similar to other archaeal groups, no isolates have been cultivated yet. This group overlaps with the **Deep Sea Hydrothermal Vent Group 1** (**DHVEG-1** or **DHVE-1**, **Deep-Sea Hydrothermal Vent Euryarchaeota Group 1)** (Takai and Horikoshi, 1999)), (Teske and Sørensen, 2008). In a cultivation experiment with samples from the 0.3 - 0.6 m deep SMTZ of Aarhus Bay, Denmark (Webster *et al.*, 2011) 16S rRNA gene sequences belonging to the MBG-D group

were found continuously during the experiment in stable amounts. SAG analysis containing intra- and extracellular peptidases showed that MBG-D/DHVE-1 *Archaea* similar than *'Bathyarchaeota'* seem to be able to degrade proteins (Lloyd *et al.*, 2013b).

1.3.2. Deep subsurface *Bacteria*

Similarly to the recently efforts in phylogeny of *Archaea*, the phylogeny of the phylum *Bacteria* is under discussion. For example, metagenomic analysis of samples obtained from an aquifer adjacent to the Colorado River near the town Rifle, Colorado, USA, revealed a new group of small, numerous biosynthetic pathways lacking, monophyletic *Bacteria*. For these, the name **Candidate Phyla Radiation (CPR)** which contains for example the ***'Parcubacteria'* (OD1)** (Brown *et al.*, 2015) was proposed. Due to metagenomics analysis, Brown *et al*. concluded that CPR-*Bacteria* are probably obligate fermenters depending on other organisms and exhibiting unusual ribosomes (Brown *et al.*, 2015).

Concerning the deep terrestrial subsurface, most abundant are *Proteobacteria, Actinobacteria, Firmicutes, Chloroflexi,* members of the *Geobacteraceae* family, sulfate reducers, denitrifiers, fermenters, and acetogens (Boivin-Jahns *et al.*, 1996), (Chandler *et al.*, 1997), (Detmers *et al.*, 2001), (Detmers *et al.*, 2004), (Brown and Balkwill, 2008), (Fry *et al.*, 2009). Similarly as described above for the CPR, the bacterial phyla *Actinobacteria, Cyanobacteria, Thermi (Deinococcus-Thermus), Chloroflexi* and *Firmicutes,* were identified to be monophyletic, "terrestrial" *Bacteria* and constitute the proposed superphylum ***'Terrabacteria'*** (Rinke *et al.*, 2013).

Specifically, Balkwill *et al.* isolated in 1989 *Pseudomonas, Acinetobacter* (both *Gammaproteobacteria*) and *Agrobacterium* (*Alphaproteobacteria*) as typical soil *Bacteria* from surface soils and coastal plain subsurface (up to 265 m) with relative high identification security (Balkwill *et al.*, 1989). Similarly, analysis of the Subsurface Microbial Culture Collection (SMCC) containing cultivated microorganisms from three different terrestrial subsurface sites (USA) revealed *Arthrobacter, Bacillus, Streptococcus, Acinetobacter, Comamonas, (Betaproteobacteria), Pseudomonas, Sphingomonas (Alphaproteobacteria)* and *Variovorax (Betaproteobacteria)* as isolates (Balkwill *et al.*, 1997). Gram-positive *Firmicutes* were the most frequently detected phylum followed by smaller contributions of *Proteobacteria, Actinobacteria*

and *Deinococcus-Thermus* found in a borehole from Tau Tona gold mine (Witwatersrand Basin, South Africa) by metagenomics analysis (Magnabosco *et al.*, 2016). Similarly, a previous study was successful in cultivating a novel actinobacterium from a depth of 940 m (sediment-clast brecchia which filled up the crater after the impact), *Tessaracoccus profundi* from Chesapeake Bay. Growth occurred with an optimum between 30 and 40°C as a facultative anaerobe (Finster *et al.*, 2009). Also from the deeper section of the Chesapeake Bay impact structure, Cockell *et al.* enriched several bacterial isolates with close affiliation to *Clostridium* sp., *Bacillus* sp., *Paracoccus* sp., *Halobacterium* sp., *Clostridium* sp. and *Cupriavidus* sp. (Cockell *et al.*, 2012).

Webster *et al.* described in 2004 a novel phylogenetic group, **JS1** (Webster *et al.*, 2004) associated to the phylum *Chloroflexi*. The phylum *Chloroflexi* comprises six classes: the class *Anaerolineae*, the class *Caldinilinea*e, the class *Chloroflexia*, the class *Dehalococoidia*, the class *Ktedonobacteria* and the class *Thermomicrobia* (http://www.bacterio.net/-classifphyla.html#Chloroflexi), (Blazejak and Schippers, 2010). The acronym JS1 originates from the first clones retrieved from the Japan Sea. Webster *et al.* revealed the widespread distribution of members of this group in sediments and suggested due to habitation characterization that members of this group have an anaerobic metabolism. Webster *et al.* were also successful to identify JS1 *Bacteria* related genomic markers in an enrichment culture of the Aarhus Bay sediment slurry originating from the sulfate methane transition zone (SMTZ) (Webster *et al.*, 2011). The analysis with different cultivation independent methods indicated that JS1 bacteria metabolize acetate in the presence of sulfate. The JS1 candidate division is relative closely related to the OP9 group (Webster *et al.*, 2004). Dodsworth *et al.* confirmed this, they proposed for the OP9 candidate division the name ***'Atribacteria'*** (Dodsworth *et al.*, 2013). Nobu *et al.* recently analyzed the relationship of the JS1 candidate division and the OP9 candidate division by using single-cell amplified genome (SAG) sequencing and metagenomics (Nobu *et al.*, 2016). Their analysis supported the monophyly of these two clades and consequently they suggested the new phylum *'Atribacteria'* for both clades. For catabolism, a propionate using metabolic pathway for the JS1 cluster and a sugar using metabolic pathway for the OP9 cluster was presented. 16S rRNA gene clone analysis of Inagaki *et al.* revealed that JS1 *Bacteria* preferentially inhabit strictly anaerobic organic-rich environments associated with methane hydrates (Inagaki *et al.*, 2006). Due to the

fact, that JS1 *Bacteria* were represented throughout the hydrate bearing cores even in sulfate-free deeper zones, Inagaki *et al.* concluded that most likely they were not responsible for sulfate reduction (Inagaki *et al.*, 2006). Accordingly, the *'Atribacteria'* seem to be anaerobic heterotrophic microorganisms specialized to habitats which have low concentrations or no external electron acceptors and are rich in organic compounds (Nobu *et al.*, 2016). Due to their unculturability there is still little known about these widespread microorganisms; nevertheless, Blazejak *et al.* developed a qPCR primer to enumerate JS1 and related *Bacteria* of the classes *Anaerolineae* and *Caldinilinae* of the phylum *Choroflexi* (Blazejak and Schippers, 2010).

1.3.2.1. Iron and manganese reduction: the bacterial family *Geobacteraceae*

Several *Bacteria* and *Archaea* are able to reduce iron(III) to iron(II) e.g. *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilus, Archaeoglobus fulgidus* and *Pyroccocus furiosus* (Lovley, 2006). Nevertheless, the ability to gain energy by ATP generation is restricted to a smaller but diverse group of microorganisms (Fig. 5).

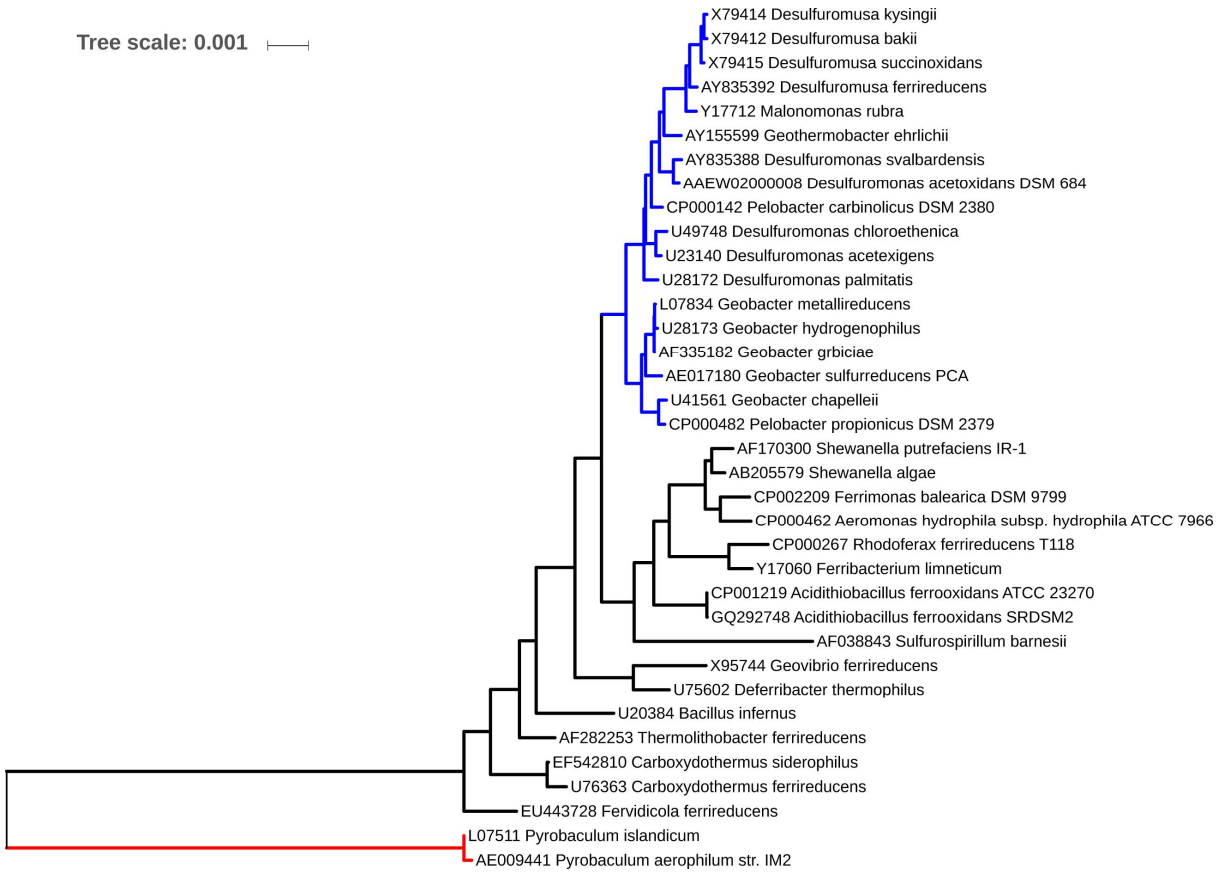


Figure 5. Dissimilatory iron reducers. A phylogenetic tree based on 16S rRNA gene sequences of microorganisms known to conserve energy to support growth from iron(III) reduction. *Archaea*: red clade, *Deltaproteobacteria*: blue clade. Microorganisms as depicted in a phylogenetic tree by Lovley *et al*. (Lovley, 2006) were identified in arb (www.arb.home de) and recently described microorganisms were added. For further tree construction see appendix.

Most iron(III) reducing bacteria are also able to reduce manganese(IV) and some additionally reduce uranium(VI). Interestingly, several acidophilic iron oxidizers like *Acidithiobacillus ferrooxidans* are also able to reduce ferric iron by a dissimilatory pathway (Bridge, T. A. M. and Johnson, 1998), for review: (Johnson *et al.*, 2012). The neutrophilic iron(III) reducing bacteria belong to different families: to the family *Geobacteraceae* (*Deltaproteobacteria*, e.g. *Geobacter metallireducens*), the family *Desulfurobacteraceae (Deltaproteobacteria*), *Deferribacteraceae* (class *Deferribacteres,* e.g. *Geovibrio ferrireducens* and *Deferribacter thermophilus*), *Ferribacter limneticum* (*Betaproteobacteria*), the family *Aeromonadaceae*, the family *Ferrimonadaceae*, the family *Shewallenaceae* (all *Gammaproteobacteria*) for example (Slobodkina *et al.*, 2015), (Lovley, 2006). Concerning the *Archaea*, the genus *Pyrobaculum* (*Crenarchaeota*) contains several members who are able to reduce iron(III) like *Pyrobaculum aerophilum, Pyrobaculum arsenaticum,*

Pyrobaculum islandicum, Pyrobaculum calidifontis and the recently isolated strain *Pyrobaculum ferrireducens* (Slobodkina *et al.*, 2015) (Fig.5). The use of nitrate, sulfur, thiosulfate, sulfite, selenite, selenate and arsenate as electron acceptor varies among these strains (Slobodkina *et al.*, 2015). Responsible for the most active part of iron(III) reduction in natural habitats seems to be the family *Geobacteraceae* (Holmes *et al.*, 2002). Consequently, Holmes *et al.* developed a qPCR assay to quantify *Geobacteraceae* (Holmes *et al.*, 2002).

1.3.2.2. Sulfate reduction: Enumerating sulfate reducers via qPCR quantification of *dsrA* and *aprA* encoding genes

Sulfate reduction is an important biogeochemical process and is found in terrestrial (e.g. the Romashkinskoe oil field, Nazina *et al.*, 1995) as well as in marine sediments (D'Hondt *et al.*, 2002), (Leloup *et al.*, 2007) }, (Schippers and Neretin, 2006), (Schippers *et al.*, 2010), (Blazejak and Schippers, 2011), (Schippers *et al.*, 2012), (Jørgensen, 1982). Oxidation of organic matter (or methane or hydrogen) by reducing sulfate is a main diagenetic process (Kasten and Jørgensen, 2000), (Froelich *et al.*, 1979). The most relevant reactions of the biogeochemical sulfur cycle on earth are shown in Fig. 6.

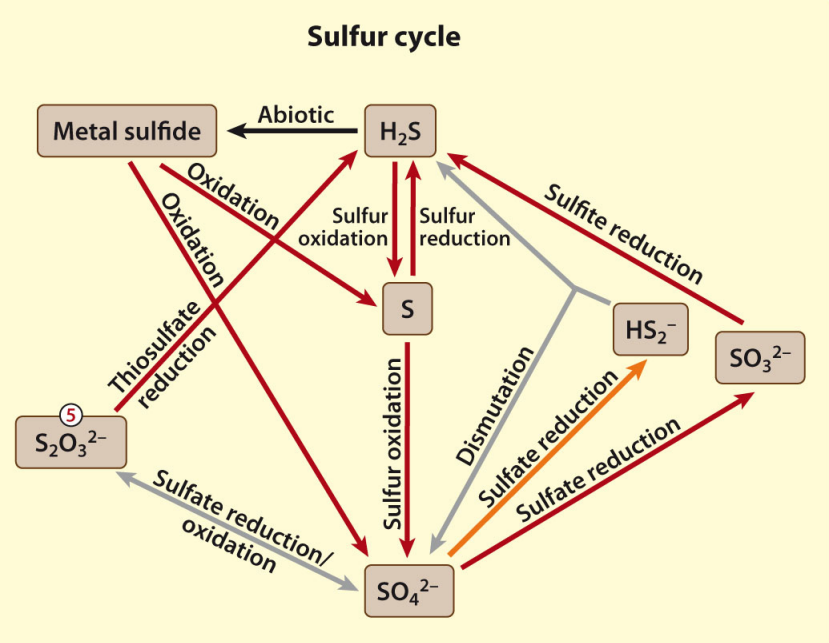


Figure 6. The sulfur cycle. Red arrows indicate metabolic reactions known from *Archaea* and *Bacteria*, gray arrows indicate metabolic reactions only known from *Bacteria* and orange arrows indicate metabolic reactions only known from *Archaea*. 5: $S_2O_3^{2-}$ is produced in several different ways including abiotic processes. The scheme and description has been slightly modified from Offre and Spang (Offre *et al.*, 2013).

Sulfate reducing *Bacteria* can be divided in two groups, those who oxidize organic compounds (proteins, lipids, polysaccharides) incompletely to acetate and those that oxidize them completely to carbon dioxide (Muyzer and Stams, 2008). Interestingly, some sulfate reducing *Bacteria* respire oxygen and are able to form ATP by oxygenic respiration (Dilling and Cypionka, 1990). Sulfate reduction coupled to methane oxidation occurs at the SMTZ and is an anaerobic process that normally occurs at a distinct zone below the zone of iron and manganese reduction. It is under discussion, whether this energetically unfavorable process is mediated by a consortium of *Bacteria* and *Archaea* or methanotrophic *Archaea* alone (Muyzer and Stams, 2008), (Milucka *et al.*, 2012), (Offre *et al.*, 2013), (Boetius *et al.*, 2000). Different mechanisms have been proposed for the reaction carried out by a consortium of *Bacteria* and *Archaea,* (Wagner, 2015): Firstly, electron transfer through diffusible metabolites (e.g. H_2); secondly, direct electron transfer through archaeal-bacterial connections; thirdly, methane oxidation and sulfate reduction to zero-valent sulfur by the *Archaea* and after sulfur release formation of disulfide in presence of sulfide, this disulfide is used by the *Deltaproteobacteria* and finally converted to sulfide and sulfate (Wegener *et al.*, 2015), (McGlynn *et al.*, 2015), (Milucka *et al.*, 2012), (Hoehler *et al.*, 1994), (Meyerdierks *et al.*, 2010), (Moran *et al.*, 2008).

The known dissimilatory sulfate reducers belong to *Deltaproteobacteria*, *Firmicutes* or *Archaea* (*Archaeoglobus fulgidus*) (Wagner *et al.*, 1998), (Widdel and Pfennig, 1977). In dissimilative sulfate reducers, adenosine 5'- phosphosulfate reductase catalyzes the two-electron reduction of adenosine 5'-phosphosulfate (APS) to sulfite and adenosine-5'-monophosphat (AMP). Dissimilatory sulfite reductase catalyzes the 6-electron reduction of sulfite to hydrogen sulfide.

Wagner *et al.* investigated the phylogeny of the *dsrA* gene (encoding gene of the α-subunit of dissimilatory sulfite reductase), (Wagner *et al.*, 1998). They found a great accordance between the phylogeny of the DSR gene and 16S rRNA gene phylogeny. Therefore they concluded that the "early ancestors of *Bacteria* and *Archaea* already possessed a key enzyme of sulfate and sulfite respiration" (Wagner *et al.*, 1998, 2975). For both genes, *dsrA* and *aprA*, qPCR assays were developed (Kondo *et al.*, 2004), (Schippers and Blazejak, 2011).

1.3.2.3. Carbon fixation via RuBisCo: *cbbl* gene copy numbers

The Calvin-Benson-Bassham cycle or ribulose-1,5-bisphosphate carboxylase/oxygenase cycle is one of six known carbon fixation cycles (Berg *et al.*, 2010) and seems to be a younger evolutionary occurrence (Berg *et al.*, 2010). Interestingly, a recent research focused on the phylogenetic evolution of the known carbon fixation pathways and their integrated analysis postulated an ancestral carbon-fixation pathway by a serine-glycine bypass that *"is different from any modern form, but better suited to the capabilities of the earliest primitive cells.*" (Braakman and Smith, 2012).

The enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo) catalyzes the first step, the addition of one molecule CO_2 to one molecule 1,5-ribulosebisphosphate. The unstable intermediate dissociates in two molecules 3-phosphoglycerate, which are further reduced or regenerated to 1,5-ribulosebisphosphate to complete the cycle and provide triosephosphates.

Four different forms of RuBisCo or RuBisCo related proteins were postulated (Tabita, 1999) and found (Selesi *et al.*, 2005), (Ashida *et al.*, 2003). The most common enzyme is the form I (Selesi *et al.*, 2007), which is found in photosynthetic organisms such as higher plants, algae and autotrophic *Proteobacteria* (Ashida *et al.*, 2003). The encoding gene *cbbl* exist in two forms, the green-like and the red-like form (Watson and Tabita, 1997). Analysis of terrestrial soil samples revealed a high diversity of the encoding gene of the red-like form of the *cbbl* gene (Selesi *et al.*, 2005). Phylogenetic analysis of *cbbl*-positive bacterial isolates based on 16S rRNA gene sequences revealed bacteria belonging to the gram-positive genera *Bacillus, Streptomyces* and *Arthrobacter* (Selesi *et al.*, 2005). Consequently, a qPCR assay to quantify the *cbbl* gene (red-like form) was developed (Selesi *et al.*, 2007).

1.4. Exploration of the deep biosphere via scientific drilling

This work analyzes samples of several ICDP/ODP/IODP expeditions, thus the general aims of theses scientific programs are illustrated.

The **Ocean Drilling Programm (ODP)** started in 1983 as a successor of the Deep Sea Drilling Project, a scientific American deep ocean coring and downhole logging program (Pedersen, 2000). *"Research based on the samples strongly supported the hypotheses of seafloor spreading - the relationship of crustal age to*

the record of Earth's magnetic reversals - and plate tectonics." (Consortium for Ocean Leadership, Inc.).

The **Integrated Ocean Drilling Program (IODP)**, (2003 - 2013), (http://www.iodp.org/history), followed it. In 2013, the successor **International Ocean Discovery Program (IODP)**, (2013 - 2023) started. IODP is an international marine research collaboration to explore subseafloor environments. Aim of IODP is to understand earth history and dynamics, to recover data from subseafloor and rocks in order to understand geological and biological features (Fig. 7). Twenty-six nations are working together within IODP (www.iodp.org).

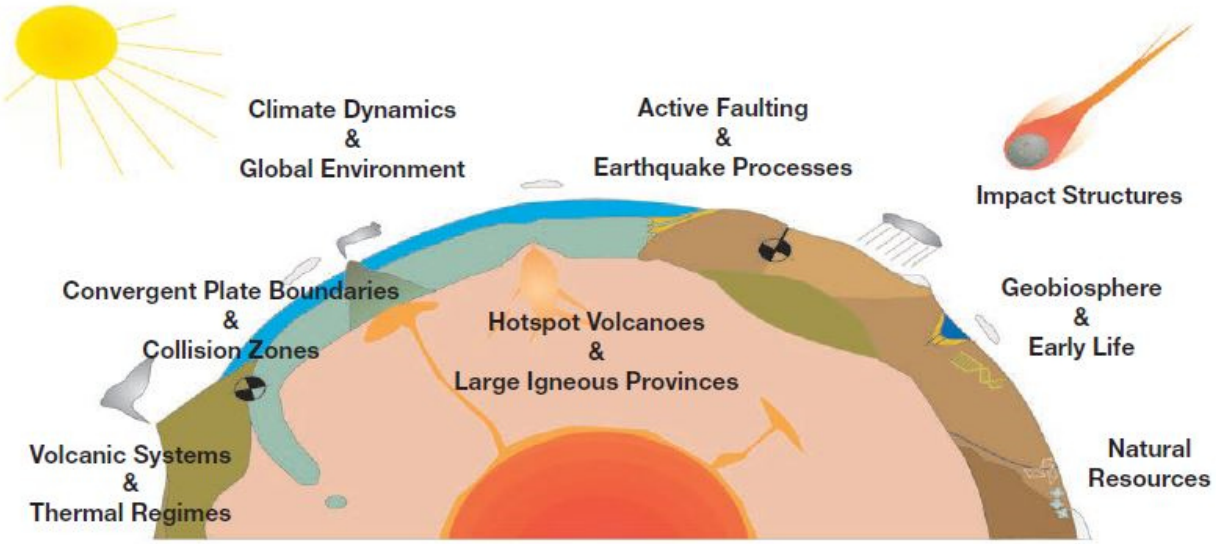


Figure 7. Investigations on earth history and dynamics. (ICDP Office 2015, GFZ German Research Centre for Geoscience).

The **International Continental Drilling Program (ICDP)** was a logic successor of the German Continental Deep Drilling Program KTB and started in 1996 as an international collaboration. Similar to IODP, the main aims are to understand the terrestrial subsurface with all its implications like earthquakes, volcanic activities, and deep fluids. Though the terrestrial biosphere is a dynamic system, the main principles are to probe, collect, monitor and analyze the subterrean earth (http://www.icdp-online.org/profile/).

1.5. The sediment sampling sites

Distinctive features of the sediments and a short summary of special questions concerning the microbiology are described in this chapter. An overview about the sampling sites is given in Table 2.

Table 2: Overview about the sediment sampling sites of this thesis.

Sampling area	CBIS	North Pond	New Jersey	Okinawa Trough
Expedition	ICDP - Chesapeake Bay impact structure	RV Maria S. Merian Cruise MSM11/1	IODP Expedition 313 New Jersey shallow shelf	IODP Expedition 331 Deep Hot Biosphere
geographic locaction	Chesapeake Bay area	mid Atlantic ridge flank	New Jersey shallow shelf	Hydrothermal field of Okinawa Trough
terrestrial or marine	terrestrial	marine	marine	marine
overlying water	-	4000 - 4500 m	~ 35 m	~ 1100 m
drilling depths	~ 140 m for this study (1766 m in total)	~ 9 mbsf (different cores)	~ 50 mbsf for this study (~ 740 mbsf in total)	~ 130 mbsf
sites	Eyreville hole C	GEOB 13501, 13502, 13504, 13507, 13510, 13512	Hole M0027A	site C0014 site C0017
special characteristics	post impact sediments	very organic lean	alternating freshwater and saltwater layers	hydrothermal influenced area
mean of TOC	total: 0.18%, uppermost soil (7m): 0.077%	0.15%	total: 1.17% upper 50 mbsf: 0.47%	site C0017: 0.3% site C0014: total: 0.13% upper 40 mbsf: 0.18%

As explained above, the applicability of a general definition of oligotrophy to characterize sediments correctly is difficult and a clear evident definition to characterize marine and terrestrial sediments by the composition of possible energy donating substrates and the released products (e.g. DOC, nitrate) cannot be performed at all. No previous definition of oligotrophy or another general characterization which fits to all the investigated sediments was applicable and accurate for this study. Nevertheless, the investigated sediments have relative low carbon contents.

1.5.1 The Chesapeake Bay impact structure (CBIS)

In 2006, the ICDP drilled in the Chesapeake Bay impact structure (CBIS), Virginia, USA, which was formed during the late Eocene meteoric impact approximately 35.5 million years ago. The total crater of this structure has a diameter of 85 km with a central 35 - 40 km wide inner crater (Poag *et al.*, 2004), (Fig.8).

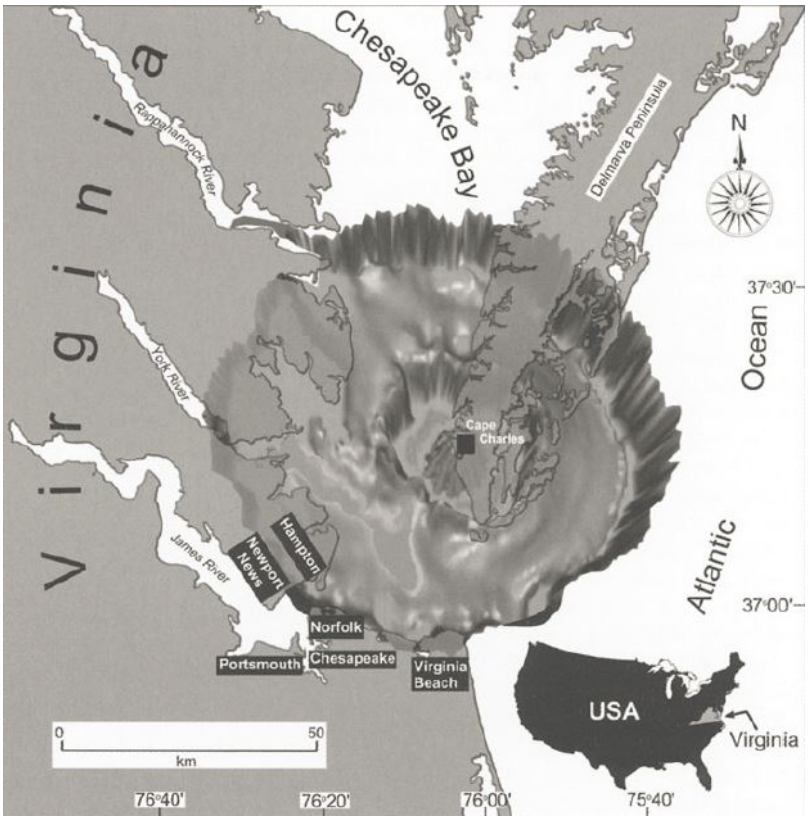


Figure 8: Computer-generated 3-D perspective of the Chesapeake Bay impact crater, showing its location beneath the lower part of the Chesapeake Bay, its surrounding peninsulas, and inner part of adjacent Atlantic Continental Shelf. (Poag *et al.*, 2004) (Image and description modified from Poag *et al.* 2004).

The impact eruption formed a crater that was filled with breccia and post impact sediments. In the CBIS Project post impact terrestrial sediments were drilled up to 1766 m depths at a site within the central crater. The upper 140 m of sediment composed of post-impact sandy and clayish sediments (Miocene to Pleistocene) were cored in Eyrevillehole C during April and May 2006 and analyzed in this thesis. Contamination tests using microscopic counting of fluorescent microsphere beads were done.

1.5.2. New Jersey - IODP Expedition 313

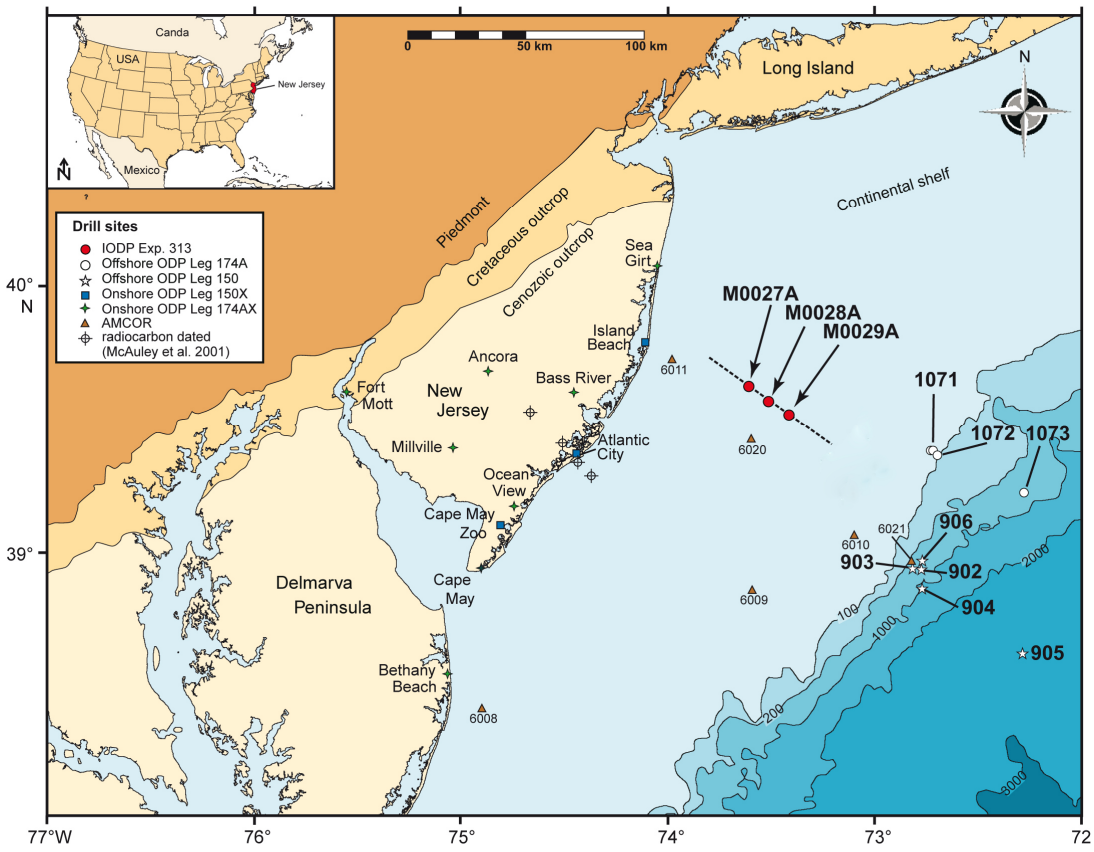


Figure 9: IODP Expedition 313 Site Map with drilling holes of previous expeditions modified from from van Geldern *et al*. (van Geldern *et al.*, 2013).

The New Jersey shallow shelf marine sediments were drilled in summer 2009 during IODP expedition 313 (Fig. 9). The overlying seawater was about 35 m deep. The sediments are generally characterized by terrigenous input of organic material. The more or less sandy sediment is also interstratified by various types of sediment, e.g. clay layers. Three different layers can be distinguished: 1) saltwater layers, 2) freshwater layers and 3) a deeply buried brine layer (Fig. 10). At the hereby investigated Hole M0027A the brine started at 415 mbsf (van Geldern *et al.*, 2013). Contamination tests were performed by microscopic counting of fluorescent microsphere beads.

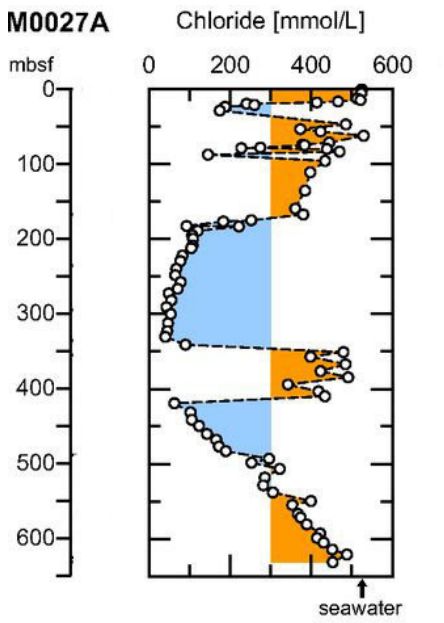


Figure 10. Profile of porewater chloride concentration at site M0027A on the New Jersey shallow shelf. Alternating saltwater and freshwater layers and a deeply buried brine are indicated by the chloride profile. Blue: freshwater, yellow: saltwater. Brine is starting below 415 mbsf. Modified from van Geldern *et al.* (van Geldern *et al.*, 2013).

1.5.3. North Pond - RV Maria S. Merian Cruise MSM11/1

The very organic lean sediments of the North Pond basin are 7 million year old and located on the western flank of the Mid-Atlantic Ridge at 23°N. They were drilled during the IODP Expedition 336 (Fig. 11). Before, gravity core sampling was done on the site survey cruise with the German R/V Maria S. Merian in 2009 (MSM 11/1) and samples for this thesis were taken. The depth of the overlaying water at the sampling sites was between 4040 and 4480 m (Villinger, 2009).

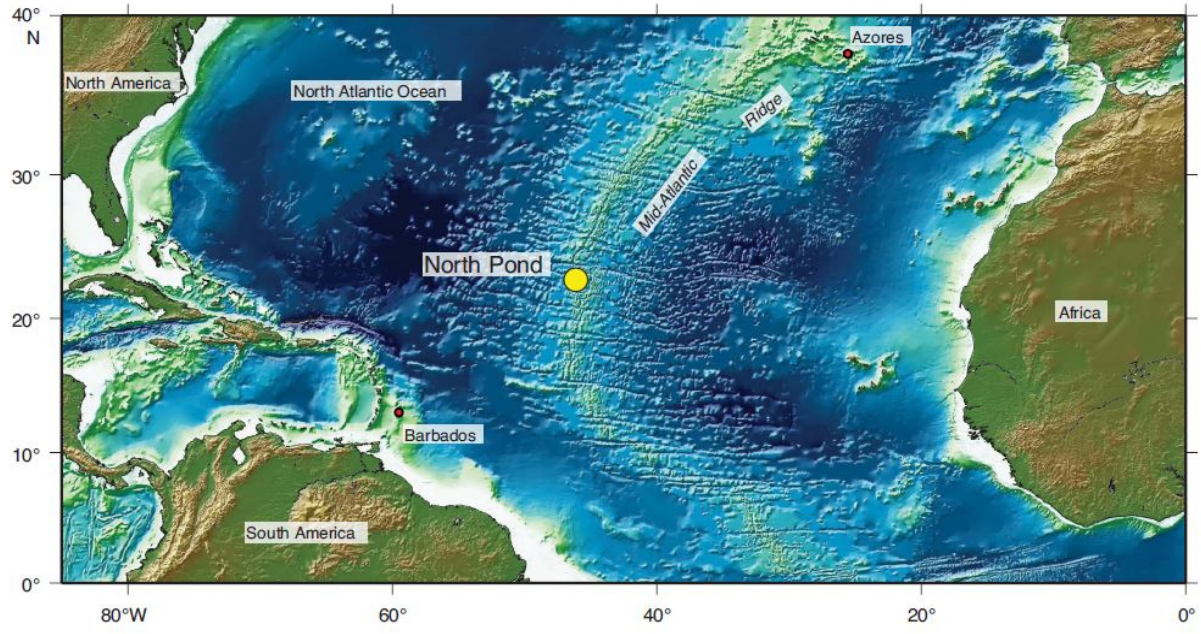


Figure 11. Location of the sediment basin North Pond on the western flank of the Mid-Atlantic Ridge at 23°N (Edwards, K. J. *et al.*, 2010).

1.5.4. Okinawa Trough – IODP Expedition 331

IODP Expedition 331 with the Japanese drilling vessel Chikyu and Anja Breuker onboard took place in the autumn of 2010. The drilling sites were located nearby the Iheya North Knoll field in the Okinawa trough. Due to previous expeditions (Nakagawa *et al.*, 2005), the existence of a deep hot biosphere was supposed and one of the major aims of this study was to prove this hypothesis. Five sites were drilled and sampled for different scientific disciplines including microbiology done by Japanese scientist and partly within this thesis (Fig. 12).

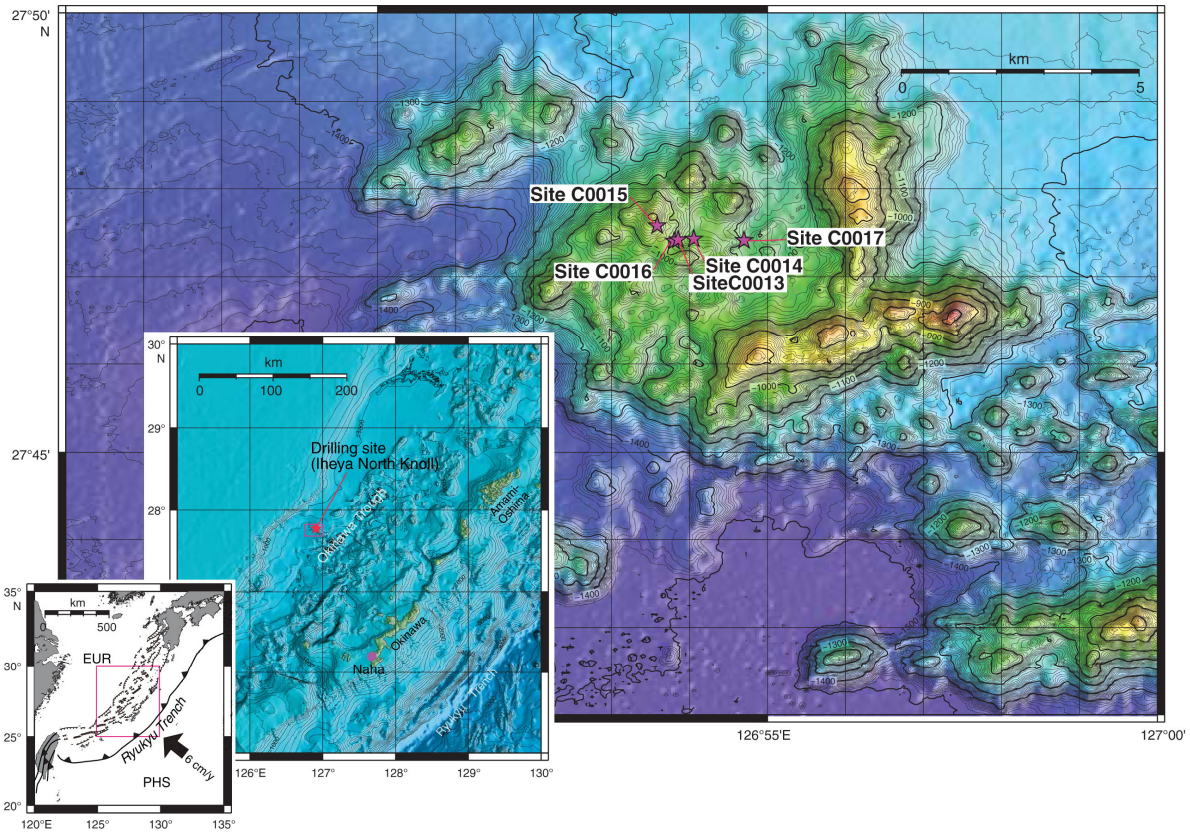


Figure 12. IODP Expedition 331 site map with five drilling locations in the Iheya North Knoll field in the Okinawa trough (Takai, K., Mottl, M.J., Nielsen, S.H., and the Expedition 331 Scientists, 2011).

Additionally, a comparative analysis concerning potential contamination of sediments with seawater organisms during drilling was carried out using microsphere beads and perfluorocarbon tracers (PFT) supplied to the sediments simultaneously (Yanagawa *et al.*, 2013).

1.6. Aims of the study

The microbial communities (deep biosphere) at different sediment depths at the various sites introduced above were analyzed via total cell counting, real-time PCR (qPCR), CARD-FISH and 16S rRNA gene sequencing. By comparing the results with those of other laboratories for some sites in joint papers, with geochemical analyses and also with published data for other deep biosphere sites, the following research questions were addressed:

Do either *Bacteria* or *Archaea* dominate and which distinct prokaryotic groups are most abundant in the various sediments?

Besides sediment depths which other physiochemical factors in marine and terrestrial sediments determine the abundance of prokaryotes?

Is there a relation of geological and biogeochemical stratification with special groups of microorganisms?

Can molecular methods be sufficiently improved to investigate oligotrophic sediments with low abundances of microorganisms?

2. General discussion

This thesis revealed a distinct microbial composition of the deep biosphere in each of four different sediments. These were all separated from photosynthesis but differed in their characteristics and can be characterized as follows: terrestrial, marine hydrothermally influenced, marine mesotrophic-oligotrophic and marine deep oligotrophic.

2.1. Abundances of microorganisms estimated by different methods

The abundances of *Bacteria* versus *Archaea* varied strongly. Using an improved DNA extraction protocol, for the first time a dominance of *Archaea* was found for the cold oligotrophic sediment of a marine basin at the mid-Atlantic ridge (North Pond). A similar abundance of the two domains occurred in the relative organic lean sediments of the shallow shelf off New Jersey, and *Bacteria* dominated in marine sediments influenced by hydrothermal fluids in the Okinawa Trough off Japan as well as in the terrestrial sediments in the Chesapeake Bay area, VA, USA. Total cell counts (TCC) decreased with sediment depth and with decreasing organic carbon content in agreement with previous studies, and overall fitted well to the qPCR data.

As summarized by Schippers (Schippers, 2016) a quantification of particular prokaryotic groups (i.e. *Bacteria* and *Archaea*) in deep subsurface sediments has been done by qPCR in several studies (Inagaki *et al.*, 2003), (Schippers *et al.*, 2005), (Inagaki *et al.*, 2006), (Schippers and Neretin, 2006), (Wilms *et al.*, 2007), (Engelen *et al.*, 2008), (Nunoura *et al.*, 2009), (Webster *et al.*, 2009), (Schippers *et al.*, 2010), (Schippers *et al.*, 2012), (Breuker *et al.*, 2013), (Breuker and Schippers, 2012), (Ciobanu *et al.*, 2014). Eukaryotic 18S rRNA genes were orders of magnitude less abundant than prokaryotic 16S rRNA genes (Schippers and Neretin, 2006), (Schippers *et al.*, 2010), (Schippers *et al.*, 2012), (Ciobanu *et al.*, 2014). Published qPCR data on the abundance of *Bacteria* and *Archaea* of several sediment studies show that the ratio of *Archaea* versus *Bacteria* seems to be variable depending on the type of sediment (Breuker *et al.*, 2013), (Breuker and Schippers, 2012) and/or the qPCR protocols applied in different laboratories (Lloyd *et al.*, 2013a). Using qPCR, an almost equal abundance of *Bacteria* and *Archaea* has been found for the Porcupine Seabight (IODP Exp. 307; Webster *et al.*, 2009), the northeast Pacific ridge-flank (IODP Exp. 301; Engelen *et al.*, 2008), Sumatra forearc basins (Schippers *et al.*, 2010), and sediments of the Black Sea and the Benguela upwelling system off the

Atlantic coast of Namibia (Schippers *et al.*, 2012). By contrast, Bacteria dominated other sediments such as the Sea of Okhotsk (Inagaki *et al.*, 2003), the Gulf of Mexico (IODP Exp. 308), (Nunoura *et al.*, 2009), the Peru continental margin and the equatorial Pacific sediments (ODP Leg 201), (Schippers *et al.*, 2005) as well as gas-hydrate bearing sediments from the Cascadia margin (ODP Leg 204), (Inagaki *et al.*, 2006) as well as very deep sediments of the Canterbury basin (IODP Exp. 317), (Ciobanu *et al.*, 2014).

The results of the published literature in general and also those in this thesis support the thesis that *Bacteria* dominate in general in subsurface sediments and are in accordance with conclusions from metagenomics analyses carried out by Hug *et al.*: *"Domain Bacteria includes more major lineages of organisms than the other Domains* (sic!)*. We do not attribute the smaller scope of the Archaea relative to Bacteria to sampling bias because metagenomics and single-cell genomics methods detect members of both domains equally well. Consistent with this view, Archaea are less prominent and less diverse in many ecosystems (for example, seawater, hydrothermal vents, the terrestrial subsurface and human-associated microbiomes).*" (Hug *et al.*, 2016).

2.1.1. Comparison of TCC, qPCR and CARD-FISH data for the CBIS sediments

The ribosomal RNA targeting method CARD-FISH was applied to the terrestrial CBIS sediments. For the uppermost 7 m sediment depth, CARD-FISH data for the domains *Bacteria* and *Archaea* were obtained (Fig. 13); in the deeper section, they were below the detection limit. The cell numbers were low (mean of CARD-FISH data: 1.38 $x10^6$ cells/g dw, standard deviation: 7.68 $x10^5$ cells/g dw, median: 1.23 x 10^6 cells/g dw), whereas the TCC were much higher (mean: 3.84 $x10^8$ cells/g dw, standard deviation: 1.37 $x10^9$ cells/g dw, median: 1.00 x 10^7 cells/g dw). The mean relative proportion of CARD-FISH counts to the TCC was 9.72 % (median 10.87 %, standard deviation: 6.72 %).

In summary, the proportion of living cells was about one-tenth of the TCC. Previous studies on this topic revealed inconsistent results regarding different sediments and conditions. Comparison of total cell counts (AODC) and CARD-FISH data was performed by Schippers *et al.* for sediments in the open-ocean of the Equatorial Pacific and for the Peru ocean-margin (ODP expedition Leg 201) (Schippers *et al.*, 2005). A different proportion of the AODC counts was detected by

CARD-FISH analysis: about one-third for the organic-poorer open-ocean and up to one-tenth for the organic-richer ocean-margin sediments. Schippers *et al.* suggested the better availability of different electron acceptors at the open ocean sites as an explanation for this difference (Schippers *et al.*, 2005).

In cultivation experiments of a mixed culture of iron oxidizing microorganisms (*Acidithiobacillus ferrooxidans, Acidithiobacillus thiooxidans* and *Leptospirillum ferrooxidans*) cultivated on mine tailings concentrate the CARD-FISH data were nearly as much as high as the TCC during the incubation experiment (Schippers *et al.*, 2008). Cultivation on tailings concentrate provides optimal conditions for the cells, so this should promote growths and enhance the proportion of living microorganisms; however this laboratory experiment does not represent environmental conditions. Nevertheless, a high proportion of CARD-FISH cell number data as indicator for living microorganisms (mean of ~ 12 %) was found by Kock *et al.* in a study of an uncovered, pyrrhotite-containing mine tailings dam (Kock and Schippers, 2006). Selection of microorganisms and enhanced growth in the pyrrhotite enhanced environment compared with CBIS sediment explain the data.

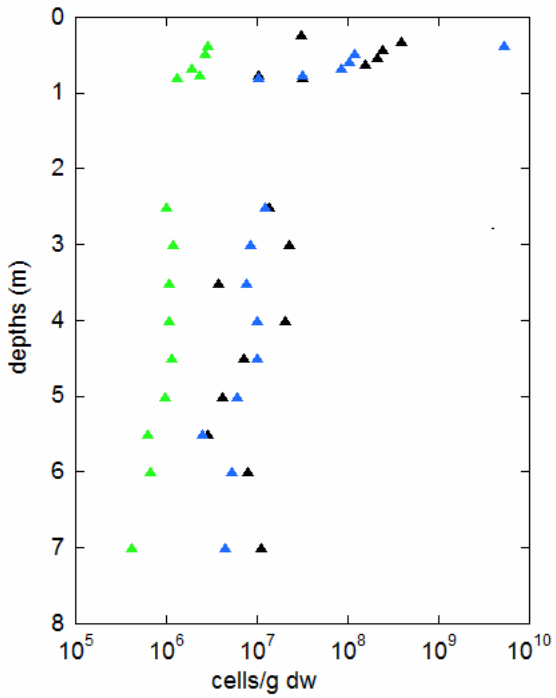


Figure 13. Depth dependent comparison of cell numbers determined by three different methods for the CBIS sediments. Black triangles: TCC, blue triangles: sum of qPCR cell numbers for *Bacteria* and *Archaea*, green triangles: sum of CARD-FISH cell numbers for *Bacteria* and *Archaea*.

More attention was given to the question of the abundances of *Archaea* and *Bacteria* in sediments highlighting methodological influences on the different proportion of FISH or CARD-FISH counts to TCC by Lloyd *et al.* (Lloyd *et al.*, 2013a). They carried out a meta-analysis including data from 65 published studies and found

the relative proportion of FISH/CARD-FISH numbers to TCC to be in average ~ 40% for marine sediments, whereas the proportion of FISH/CARD-FISH numbers to TCC in seawater was clearly higher. Two reasons might explain this result: Firstly, seawater can be “concentrated” to overcome a low detection limit. Secondly, a high proportion of cells in sediments may be dead (Lloyd *et al.*, 2013a). They also mentioned that below 10 mbsf the relative proportion of *Archaea* to all microorganisms increased with depths. Previous reported depths dependent decreasing distribution of all microorganisms as depicted by Parkes *et al.* (Parkes *et al.*, 1994) was confirmed only for the uppermost 10 mbsf for all microorganisms, below 10 mbsf; Lloyd *et al.* found stable numbers for *Bacteria* calculated by qPCR and CARD-FISH or FISH and concluded: *"Unexpectedly, the trend of decreasing bacteria with depth in marine sediments ended at 10 mbsf; below this depth, bacterial cell density was not correlated with sediment depth. This may indicate the presence of a more stable deep subsurface population that is better equipped for subsurface living."*

Concerning the terrestrial CBIS sediment, the proportion of CARD-FISH counts (and also the qPCR data) of *Archaea* (Fig. 14) were relatively low compared to the meta-analysis of Lloyd *et al.* (Lloyd *et al.*, 2013a). An increasing proportion for *Archaea* to the sums of *Archaea* and *Bacteria* as found by Lloyd *et al.* for the marine sediments was not observed. This might be either indicate that the terrestrial CBIS sediment hosts enough organic carbon and/or electron acceptors to favor bacterial life. Due to the very low archaeal qPCR percentages below 60 m (mean 0,36%) and only one maximum at 25 m (100%) it seems to be likely that *Bacteria* outcompete *Archaea* in this terrestrial environment (data not shown).

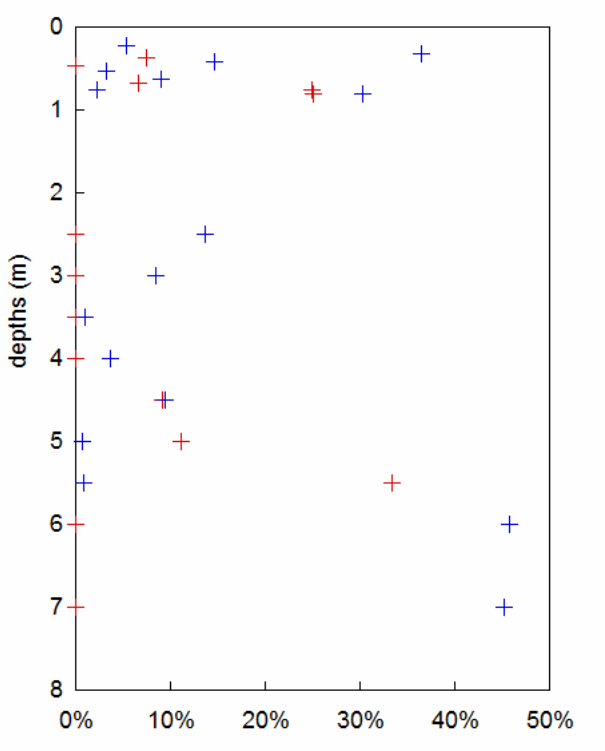


Figure 14. CBIS: Percentage of *Archaea* to prokaryotic abundance. Blue: percentage of archaeal qPCR data. Red: percentage of archaeal CARD-FISH data.

Nevertheless, the CBIS data do not support the explanation of Schippers *et al.* mentioned above. If organic-lean sediments harbor a higher proportion of living bacteria, one would expect higher CARD-FISH counts for CBIS which was not the case. It has to be mentioned that no comparable CARD-FISH data for a terrestrial sediment site with higher organic input are available to verify general differences between marine and terrestrial sediments.

A recent study by Boungiorno *et al.* (Bastin, 1926), (in review) attended methodological problems for achieving reliable CARD-FISH data for sediments. They investigated the proposed possible positive effects of proteinase K for a better detection of *Archaea* by CARD-FISH suggested by Lloyd *et al.* (Lloyd *et al.*, 2013a). Deeply-buried sediments of the Baltic Sea (IODP expedition 347) were analyzed in parallel in two different laboratories. In both laboratories, the proposed use of proteinase K did not increase the yield of archaeal CARD-FISH data; (Buongiorno *et al.*). It seems unlikely that their results should not apply to the CBIS sediment and so the use of proteinase K would likely not yield to higher archaeal CARD-FISH counts. The most probable explanation for the low CARD-FISH counts of the Chesapeake sediment is either a loss of cells during the CARD-FISH protocol washing steps and/or low ribosomal contents and low activity of cells as pointed out by Buongiorno *et al.* and Schippers *et al.* (Schippers *et al.*, 2005), (Buongiorno *et al.*) also for marine

sediments. Due to the fact that comparisons for different cell degradation processes for marine versus terrestrial sediments have not carried out, it might also be that dead cells are better conserved in the terrestrial sediment. In summary, the studies by Lloyd *et al.* and Buongiorno *et al*. indicate that FISH and CARD-FISH are not reliable methods for an absolute quantification of living cells; however they are suitable methods for a relative comparison of cell abundances in different samples of the same site. Consequently for the CBIS sediment, a correlation between the CARD-FISH counts data and TCC data could be found (Fig. 15).

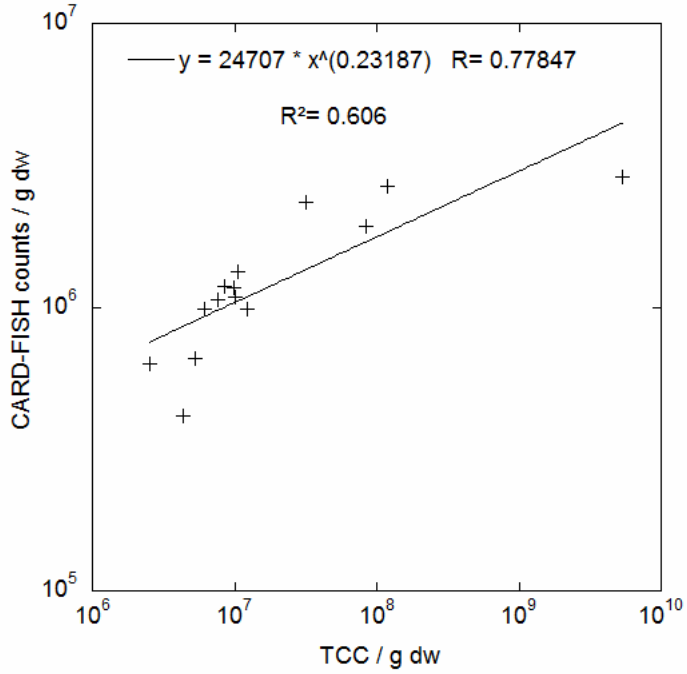


Figure 15. Correlation between CARD-FISH data and TCC for the uppermost 7 m at the CBIS sediment.

For the CBIS sediments the qPCR data of the sum of *Archaea* and *Bacteria* were closer to the TCC than the CARD-FISH data; however a close match of qPCR data and TCC was not found (Fig. 13). This finding is in agreement with Lloyd *et al.* who concluded of their meta-analysis study: *"In sediments, the sum of bacterial and archaeal 16S rRNA gene qPCR counts was not closely related to cell counts, even after accounting for variations in copy numbers per genome."* (Lloyd *et al.*, 2013a). To estimate if total organic carbon has an influence on the TCC or the qPCR data, a correlation analysis between TOC/TCC and TOC/sum of qPCR data was carried out using the CBIS data and the New Jersey data (data not shown). For both analyses, there was no correlation. This result seems to be in contrast to the findings of Parkes

et al. and Kieft *et al.* (Parkes *et al.*, 2000), (Kieft *et al.*, 1995) who determined a correlation between TOC and cell counts as well as for marine and terrestrial sediments. An explanation might be that such a correlation is more difficult to find for relative organic lean sediments and the influence of other geochemical features than TOC might be higher for the CBIS and New Jersey sediments.

2.1.2. Diversity of *Bacteria* in terrestrial sediments

For the CBIS sediment, full length 16S rRNA bacterial gene sequences were retrieved from 50.05 m, 74.71 m, 101 m, 108.86 m and 125.18 m depths. The identified bacteria belonged to *Alphaproteobacteria, Gammaproteobacteria, Betaproteobacteria* and *Actinobacteria*, some single 16S RNA gene sequences to other groups (Fig.16). These groups are typically found also in upper soils, as e.g. confirmed by Roesch et *al*., who conducted a pyrosequencing analysis of four upper soils in Brazil, Florida, Illinois and Canada. Hereby, the *Betaproteobacteria* were the dominant class except for a Brazilian soil and the second most abundant class were the *Bacteroidetes* (Roesch *et al.*, 2007).

Similarly, these groups were identified in deep terrestrial sediments before (Magnabosco *et al.*, 2016), (Boivin-Jahns *et al.*, 1996), (Balkwill *et al.*, 1989). A comparison of the 16S rRNA gene sequences of this study with the previously described 16S rRNA sequences from the deeper section at the CBIS sediment (Cockell *et al.*, 2012) revealed no accordance indicating autochthonous microbial communities or selective enrichment of very low abundant *Bacteria* of the deeper section. Primer biases should play a minor role for the relatively well described *Bacteria*. Interestingly, although the qPCR analysis revealed significant copy numbers of the *'Atribacteria'* (OP9/JS1) and the *Chloroflexi* classes *Anaerolineae* and *Caldinilinae,* neither 16S rRNA gene sequences of the '*Atribacteria'* nor *Chloroflexi Anaerolineae* and *Caldinilinae* were found by 16S rRNA gene analysis.

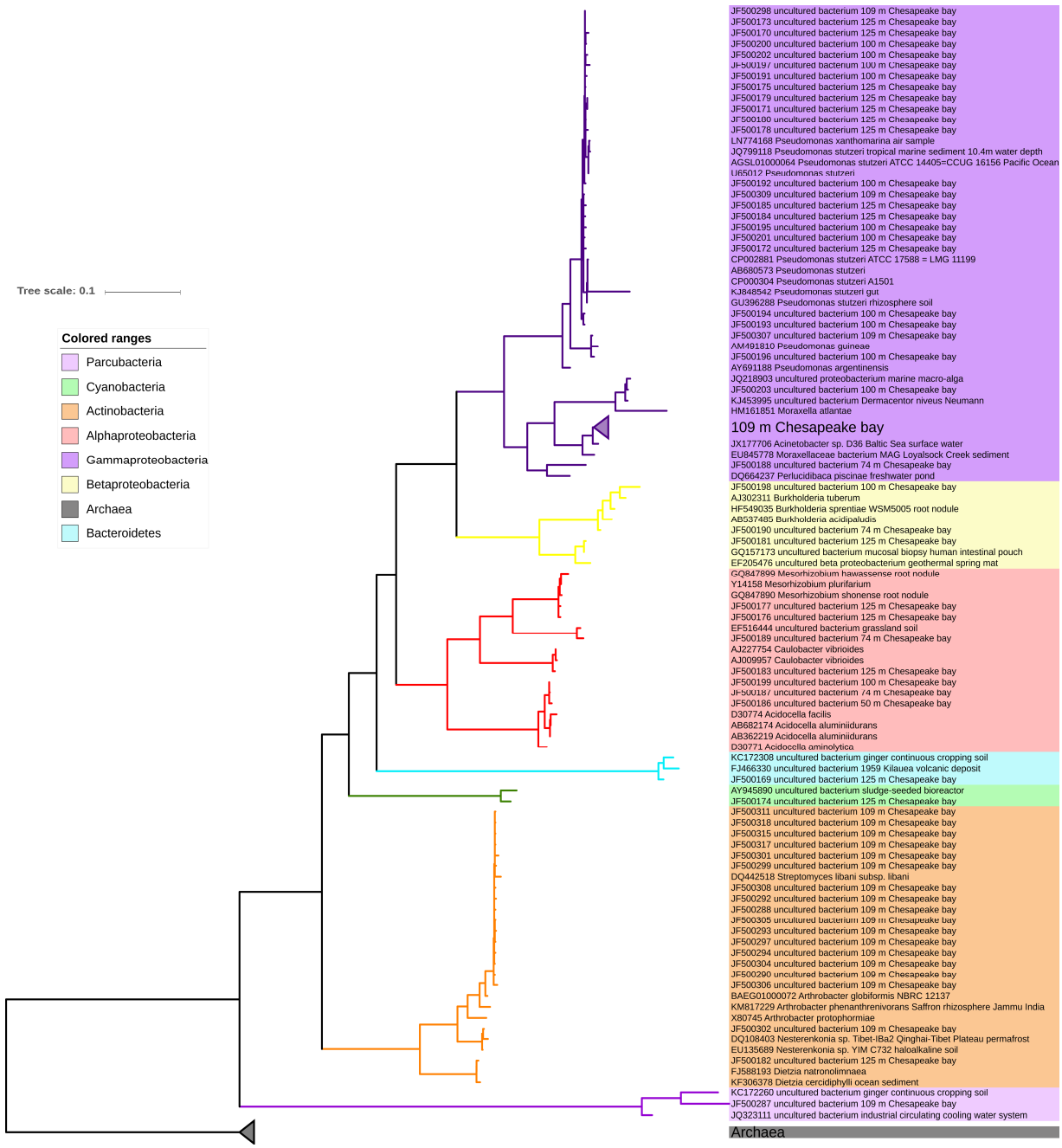


Figure 16. Phylogenetic tree representing the identified bacterial 16S rRNA gene sequences and major groups. For tree construction see appendix.

In particular, a 16S rRNA gene sequences belonging to the *'Parcubacteria'* was retrieved from 109 m depths (Fig. 16). The ***'Parcubacteria'*** (OD1) belong to the Candidate Phylum Radiation (CPR), for which Wrighton *et al*. found evidence to carry out widespread fermentation-based metabolism. They also detected several pathways for anoxic carbon, hydrogen and sulfur cycling in these organisms which share features previously documented only for Archaea (Wrighton *et al.*, 2012). The genomes were collected from an anoxic aquifer five, seven and 10 days after starting

with addition of acetate (Wrighton *et al.*, 2012). Reduced metabolic capabilities (no electron chain) were reported (Rinke *et al.*, 2013), (Wrighton *et al.*, 2012). Nelson and Stegen confirmed the reduced genome of *'Parcubacteria'* which lacks specific encoding sequences. These encoding gene sequences are typically absent in symbionts and Nelson and Stegen concluded that *'Parcubacteria'* might have a symbiotic lifestyle (Nelson and Stegen, 2015). Similarly, Hug *et al.* confirmed the small genome size and metabolic limitations in CPR *Bacteria* containing incomplete tricarboxylic acid cycles and lacking electron transport chain complexes. Conclusively the terrestrial deep biosphere may comprise typical soil *Bacteria* but also specific autochthonous organisms and probably even those with a symbiotic lifestyle.

2.1.3. Manganese and iron reduction: The family *Geobacteraceae*

In this study *Geobacteraceae* were detected at the uppermost layers of the sediments off New Jersey, the Okinawa Trough and CBIS supporting the importance of this family in sediments (Holmes *et al.*, 2002), (Holmes *et al.*, 2007) *Geobacteraceae* were found with 1 $x10^5$ copy numbers/g dw at the uppermost soil layer (1 m) at CBIS. At the two marine sediments, *Geobacteraceae* were detected in lower copy numbers (mean: 6.7 $x10^3$ copies/ml up to 13 mbsf for the New Jersey, mean: 2.4 x 10^4 copies per g wet weight for the Okinawa Trough at site C0017 up to 30 mbsf). Abundances were in the same order of magnitude than previously reported data; for example in the porcupine Seabight sediments, abundances of *Geobacteraceae* varied between 0 and 10^4 copies/ml sediment (Webster *et al.*, 2009). Frerichs et al. found for upper soils of a volcanic CO_2 vent system near the Laacher See, Eifel, Germany higher abundances of *Geobacteraceae* (reference site mean 4.97 x 10^6 copies/g wet weight, vent site mean 1.6 x 10^5 copies/g wet weight); the proportion of *Geobacteraceae* to the abundances of total *Bacteria* were by mean 0.56% at the reference site and 0.12% at the CO_2 vent site (Frerichs *et al.*, 2013). For the CBIS sediment studied here, these values ranged from 1.35% - 22.63% (data not shown). *Geobacteraceae* were almost exclusively detected in the layers, where reactive iron was detected in higher concentrations (Table 4).

Table 4. Abundances of *Geobacteraceae,* total iron and reactive iron at the CBIS sediment.

depths (m)	Fe in %	reactive Fe in %	proportion of reactive Fe to total Fe	*Geobacteraceae:* copy numbers/g dw
0.23	3.94	0.07	0.02	3.35×10^4
0.33	2.10	0.56	0.27	$1{,}89 \times 10^5$
0.43	2.41	1.49	0.62	$1{,}93 \times 10^6$
0.53	2.35	0.74	0.32	$1{,}15 \times 10^5$
0.63	2.25	1.07	0.48	$7{,}82 \times 10^5$
0.75	1.85	0.68	0.37	3.73×10^4

A correlation between the concentration of reactive iron and *Geobacteraceae* 16S rRNA gene copy numbers was found for the CBIS sediment (Fig. 17).

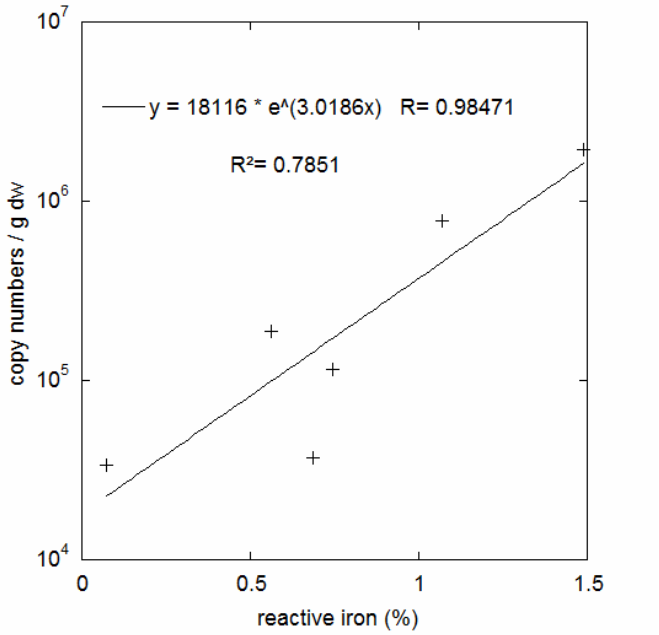


Figure 17. Correlation between the concentration of reactive iron and *Geobacteraceae* 16S rRNA gene copy numbers including the data of the uppermost 7 m of CBIS sediments (no *Geobacteraceae* were identified by qPCR below this depths).

These data stress, although many microorganisms are able to reduce iron, the ecological important role of iron(III) reducing *Geobacteraceae* depending on the concentrations of reactive iron in the sediment.

Concerning the New Jersey shallow shelf sediment and the recharge site C00017 at the Okinawa trough, no correlation could be verified between amounts of *Geobacteraceae* and iron or manganese (reactive iron and manganese for site C00017 and Fe^{2+} and Mn^{2+} interstitial pore water at the New Jersey shallow shelf sediment). This might be due to not corresponding depths of iron and manganese data with those of qPCR data for New Jersey. Concerning the Okinawa trough, for

site C0017 only a few data points for *Geobacteraceae* ranging from 10^3 -10^4 copies/g sediment were obtained.

2.1.4. Analysis concerning the *'Atribacteria'* (OP9/JS1) and the *Chloroflexi* classes *Anaerolineae* and *Caldinilinae*

Concerning the CBIS, the New Jersey and the Okinawa Trough sediment, qPCR data were obtained for all sites. For the CBIS and New Jersey sediments, members of the '*Atribacteria*' JS-1 candidate division and the classes *Anaerolineae* and *Caldinilineae* were found over the complete sampling depths with maximal copy numbers of up to 1.32 x 10^8 copies/g wet weight at the CBIS sediment at 0.33 m depths (mean 1.35 x10^6 copies g wet weight). In the New Jersey sediment, the qPCR copy numbers were slightly lower (mean 3.76 x10^5 copies/g wet weight) and at the Okinawa Trough sediment, mean copy numbers were of 3.93 x10^6 copies/g wet weight were detected for site C0017 up to 30 mbsf. The *'Atribacteria'* (OP9/JS1) and the *Chloroflexi* classes *Anaerolineae* and *Caldinilinae* are not restricted to special environments and have abundantly detected in other marine sediments as well (Blazejak and Schippers, 2010). Nobu et al. (Nobu *et al.*, 2016) resumed that the *'Atribacteria'* are likely heterotrophic anaerobes that lack respiratory capacities. A SAG- and pyrosequencing analysis of samples of the deep marine sediment at IODP Site U1357 in the Adélie Basin offshore Antarctica confirms this; the results of Carr *et al.* showed evidence for sugar and amino-sugar metabolism in the atribacterial genomes which would produce fermentation products as acetate, ethanol and CO_2 providing substrates for methanogens (and other) (Carr *et al.*, 2015).

2.1.5. Sulfate reduction: Enumerating sulfate reducers via qPCR quantification of *dsrA* and *aprA* encoding genes

In marine sediments, sulfate reduction is a major biogeochemical process because a constant downward flux of seawater maintains sulfate as an electron donator (Jørgensen, 1982). The situation in soils is more complex due to changing water saturation and therefore different redox potentials and pH favoring more or less sulfate reduction depending on the availability of sulfate (Connell and Patrick, 1968). As in marine sediments, Lovley and Phillips showed for terrestrial sediments that the presence of ferric iron as a terminal electron acceptor outcompetes sulfate reduction (and methane production) (Lovley and Phillips, 1987).

The functional genes of sulfate reduction *dsrA* and *aprA* were at all three investigated sites detectable (not done for the oxic North Pond sediments). Concerning the terrestrial CBIS sediment, sulfate reduction seems to play a minor role because *aprA* encoding genes occurred in low copy numbers (mean 4 x10^5 copies/g dwt) at distinct layers at about 0,5 m and 80 m depths. In the New Jersey sediment, *dsrA* and *aprA* encoding genes were in the order of narrow 10^5 (mean 1.2 x 10^5 copies of the *dsrA* gene per g sediment) with the highest copy numbers at ~ 20 mbsf. Gene copy numbers of *dsrA* were generally higher than the gene copy numbers of *aprA* (mean 1.3 x 10^4 copies/g sediment up to 20 mbsf). Occurring only in a few samples, the highest copy numbers were found at ~20 mbsf similar to the *dsrA* copy numbers. The highest gene copy numbers occurred at 20 mbsf where gradients of porewater sulfate concentrations and alkalinity and a TOC maximum at the boundary between freshwater and saltwater were found. Nevertheless, it is not possible to discriminate clearly between freshwater intrusions and/or microbial sulfate reduction as mentioned by van Geldern *et al.* (van Geldern *et al.*, 2013). If active sulfate reducers cause the peak of *dsrA* and *aprA* gene copy numbers, the electron donors for this process should be diffusing organic compounds from the underlying freshwater intrusion. The lack of the gene *mcrA* of methanogens and the absence of methane in the uppermost 50 mbsf (van Geldern *et al.*, 2013) do not indicate sulfate reduction coupled to methane oxidation.

For the Okinawa Trough sediments, gene copy numbers were in the order of magnitude of 10^5 per g sediment for *dsrA* and 10^4 for *aprA*. The copy numbers were generally lower for the *aprA* gene. The gene copy numbers of *dsrA* and *aprA* decreased with sediment depths, following the general trend of 16S rRNA qPCR data. Sulfate reduction is supported by the detection of *Deltaproteobacteria* up to 74.9 mbsf. Lower copy numbers of *aprA* and *dsrA* were reported from the Porcupine sea bight sediments with ~10^3 copies/cm^3 sediments as well as for the thereby investigated carbonate mound site and the marine sediment reference site. Up to nearly 10^8 copies/g of *aprA* and *dsrA* were reported for two sediments at the Peru Margin Ocean and also for one sediment of the Black Sea (station 20). The *aprA* and *dsrA* gene copy numbers did not vary from each other (Schippers and Blazejak, 2011). In forearc sediment basins off Sumatra, *dsrA* copy numbers were between 10^3 - 10^7 genes/ml (Schippers *et al.*, 2010). Hereby, a high number of *dsrA* genes corresponded to the calculated sulfate reduction rates. The overall sulfate reduction

rates were similar to those in other deeply buried sediments but considerably lower than those in other near-surface sediments. Schippers *et al.* concluded that this might be due to the overall low sulfate reduction rates in the sediments off Sumatra (Schippers *et al.*, 2010). A comparative analysis of sediments of the Black sea with sediments of the Benguela upwelling area off Namibia revealed *dsrA* and *aprA* copy numbers at all sites with higher copy numbers of the *aprA* gene at the Benguela upwelling sites (Schippers *et al.*, 2012). The observed differences between *dsrA* and *aprA* gene copy numbers may be explained by primers specificity for the *dsrA* and *aprA* gene with primer mismatches for the *dsrA* gene.

Another open question to interpret the *dsrA* and *aprA* copy numbers is, whether the amplified *dsrA* and *aprA* genes result from different or identical microorganisms (Schippers and Blazejak, 2011). If the *dsrA* and *aprA* result from the same phylogenetic species, the qPCR copy numbers should represent the relation of copy numbers of the *aprA* gene and the *dsrA* gene in these species. Evidence for the amplification of *dsrA* and *aprA* genes resulting from the same microorganisms was great for the analysis of the Black Sea sediment at 2.7 mbsf due to the fact, that both amplified genes could be allocated to the same two species with high similarity (Schippers and Blazejak, 2011). However, the observed different distribution of *dsrA* and *aprA* gene copy numbers in this study (higher gene copy numbers for *dsrA* at the marine sediments, no *dsrA* gene copy numbers at the terrestrial sediment) remains to be clarified. The observed results underline sulfate reduction as a general microbial process.

2.1.6. Carbon fixation: *cbbl* gene copy numbers

For the CBIS sediment, the large subunit encoding gene *cbbl* was detected in high copy numbers throughout nearly the whole sediment depths with the highest copy numbers at the upper two meters (up to nearly 10^7 copies/g dwt), the percentage of *cbbl* copy numbers was around one order of magnitude lower than that of *Bacteria*. In contrast, the *cbbl* gene copy numbers were found for the New Jersey sediment to be low (~8 x 10^3 copies/g sediment); the overall copy numbers were around two orders of magnitude lower than those of *Bacteria*. Previous analysis of *cbbl* copy numbers were performed for top soil samples from a long-term field experiment and the *cbbl* gene copy numbers reached up to 3.4 x 10^7 per g sediment (Selesi *et al.*, 2007). These copy numbers were therefore in the same order of

magnitude as the gene copy numbers for the CBIS sediment in the uppermost soil. Analysis of top arable soils revealed high *cbbl* gene copy numbers (Yousuf *et al.*, 2012), (Yousuf *et al.*, 2014). The percentage of *cbbl* copy numbers to bacterial copy numbers for the CBIS sediment were similar to one of the analyzed rhizosphere soils and the agriculture soil (Yousuf *et al.*, 2012) and higher than in the other rhizosphere soil, the two saline soils and in the paddy soil (Table 5).

Table 5. Percentages of *cbbl* copy numbers to total bacterial 16S rRNA gene copy numbers for different sediments and soils, respectively.

this study	**this study**	**Yousuf *et al.* 2012**[1]	**Yousuf *et al.* 2014**[2]	**Yousuf *et al.* 2014**[2]	**Yousuf *et al.* 2014**[2]	**Yousuf *et al.* 2014**[2]	**Xiao *et al.* 2014**[3]
CBIS sediment	New Jersey sediment	rhizosphere soil, agricultural field, India	rhizosphere soil, Gujarat, India	agricultural soil, Gujarat, India	low saline soil, Gujarat, India	high saline soil, Gujarat, India	paddy soil, Taoyuan, China
17.76%	0.43%	17.27%	4.62%	11.51%	0.83%	1.92%	0.57%

[1]: (Yousuf *et al.*, 2012), [2]: (Yousuf *et al.*, 2014), [3]: (Xiao *et al.*, 2014).

In nearly the same low copy numbers than in the New Jersey sediment, the *cbbl* gene was previously also detected in marine organic rich sediments of the Black Sea with copy numbers ~10^5 copies/ml sediment and in sediments of the Benguela upwelling system off the Atlantic coast (Namibia) with 10^3-10^4 copies/ml sediment (Schippers *et al.*, 2012). Quantification of the *cbbl* gene in groundwater samples from two superimposed limestone aquifers located in the Hainich region in northwest Thuringia revealed abundances of *cbbl* genes ranging from 1.14 x 10^3 to 1.75 x 10^6 genes/liter over a two year period. Hereby, up to 17% of the microbial population had the genetic potential to fix CO_2 via the Calvin cycle (Herrmann *et al.*, 2015). It has to be mentioned, that the hereby used primers were developed to detect the green like form of RuBisCo (Herrmann *et al.*, 2015) which occurs in *Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Cyanobacteria, Prochlorales*, Eukaryotes-Viridiplantae *(Streptophyta, Chlorophyta),* Euglenozoa and the Sargasso Sea metagenome whereas the red like form occurs in *Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Chloroflexi*, Eukaryotes-Stramenopiles, *Rhodophyta and Haptophyceae* (Tabita *et al.*, 2007).

In summary, in the CBIS sediment high *cbbl* copy numbers with a relatively high proportion to the total *Bacteria* were detected indicating that autotrophy beside

heterotrophy contributes to carbon cycling. This conclusion is supported by a *cbbl* gene diversity and RuBisCo activity study of different soils incubated in light and dark which revealed that the RuBisCo enzyme activity of these soils was closely positive related to the synthesis rate of soil organic carbon and the bacterial *cbbl* gene abundance (Yuan *et al.*, 2012).

2.2. Phylogenetic analysis of *Archaea*

A main overview of previously identified major taxonomic isolates and recently identified new phyla with relationship to the hereby identified 16S rRNA gene sequences and the phylogenetic relationship is given in Figure 18.

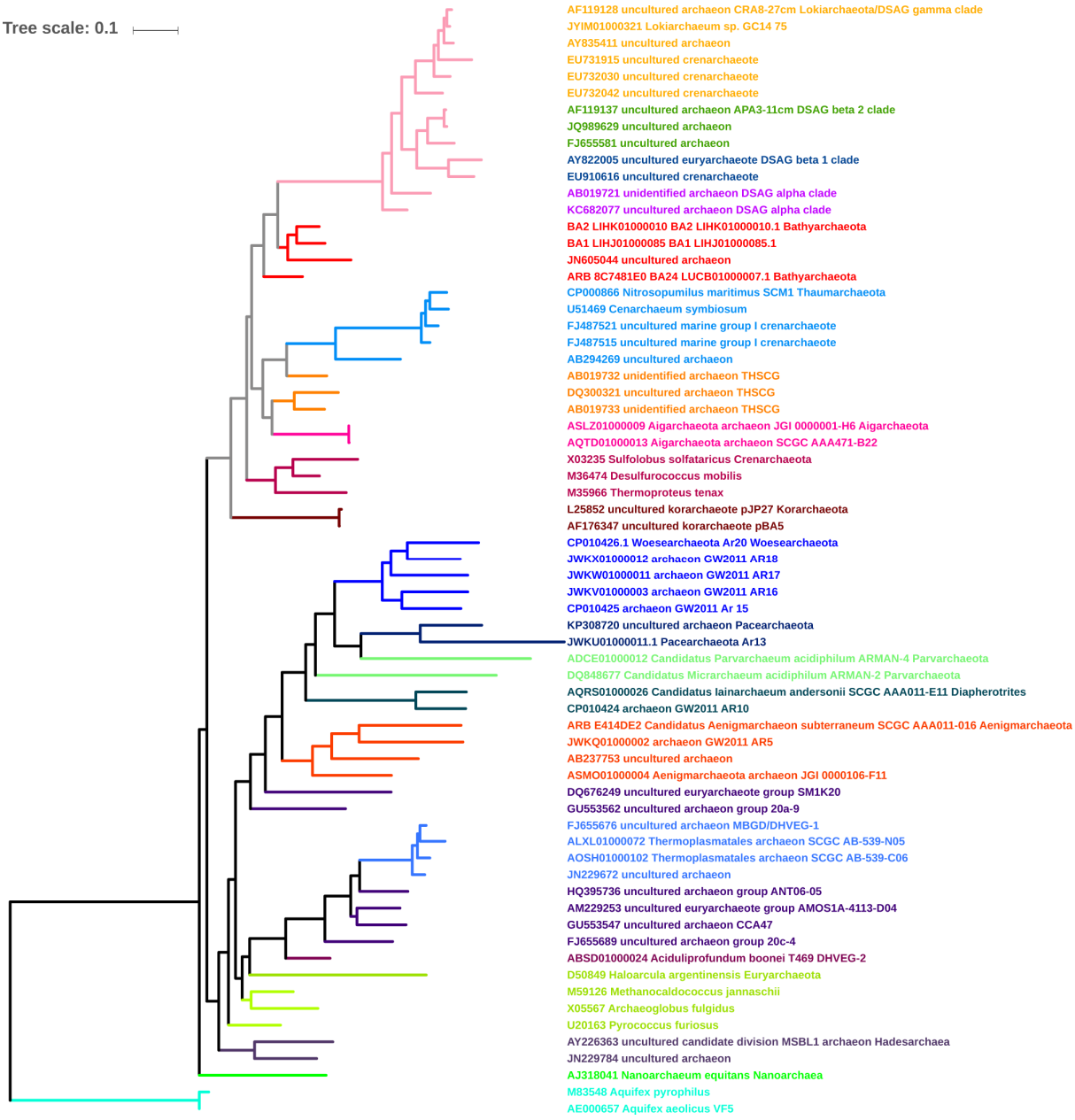


Figure 18. Phylogenetic tree representing major archaeal taxonomic lineages according Baker *et al.* (Baker *et al.*, 2003), representative 16S rRNA gene sequences of recently proposed new phyla (*'Aigarchaeota', 'Bathyarchaeota', 'Lokiarchaeota', 'Woesearchaota', 'Parvarchaeota', 'Diapherotrites', 'Thaumarchaeota', 'Hadesarchaea', 'Pacearchaeota'*) and representatives of small groups to which the hereby identified 16S rRNA gene sequences could be allocated. Grey branches lead to the TACK superphylum. Light pink branches lead to the DSAG group. For tree reconstruction see appendix, the tree was rooted with *Aquifex.*

For the CBIS sediment 16S rRNA gene sequences could be affiliated to the phyla *'Bathyarchaeota', 'Pacearchaeota', 'Hadesarchaea'* and one 16S rRNA sequence to the *'Aenigmarchaeota'* (sequence not published, clone 1572_10, 125m depths), (Fig. 19a, Table 6).

For the New Jersey sediment, representatives of *'Thaumarchaeota', 'Aigarchaeota', 'Lokiarchaeota'*, *'Bathyarchaeota', 'Aenigmarchaeota'*, *'Hadesarchaea'* the THSCG group, the MBG-D/DHVEG-1 and different small euryarchaeotic groups (SM1K20, 20a-9, 20c-4, CCA47, AMOS1A-4113-D04) were identified (Fig. 19b, Table 6).

'Lokiarchaeota' could be detected in the New Jersey sediment at 8.94 msbf. *'Lokiarchaeota'* belong to the γ-clade of the DSAG group (Spang *et al.*, 2015). A comparison of 16S rRNA gene sequences for the sediment off New Jersey with the β1-, the β2- and α-clade as described by Spang *et al.* revealed allocation with the γ-clade and therefore to the *'Lokiarchaeota'* (Fig. 19b).

Table 6. Overview and depths dependent phylogenetic community composition of the CBIS and the New Jersey sediments. The TACK superphylum is highlighted in blue.

Phylogenetic classification	CBIS sediment	Sediment off New Jersey
'Bathyarchaeota'	all depths	all depths
'Lokiarchaota'		8.94 mbsf
'Aigarchaeota'		8.94 mbsf, 10.23 mbsf, 12.99 mbsf
'Thaumarchaeota'		12.99 mbsf
polyphyletic THSCG group		8.94 mbsf, 10.23 mbsf
'Hadesarchaea'	109 m, 125 m	8.94 mbsf, 10.23 mbsf, 12.99 mbsf
'Pacearchaeota'	125 m	
'Aenigmarchaeota'	125 m (clone 1572_10arch, not published)	8.94 mbsf, 10.23 mbsf, 12.99 mbsf
small euryarchaeotic groups: SM1K20, 20a-9, 20c-4, CCA47, AMOS1A-4113-D04		8.94 mbsf, 10.23 mbsf, 12.99 mbsf
MBG-D/DHVEG-1		8.94 mbsf, 10.23 mbsf, 12.99 mbsf

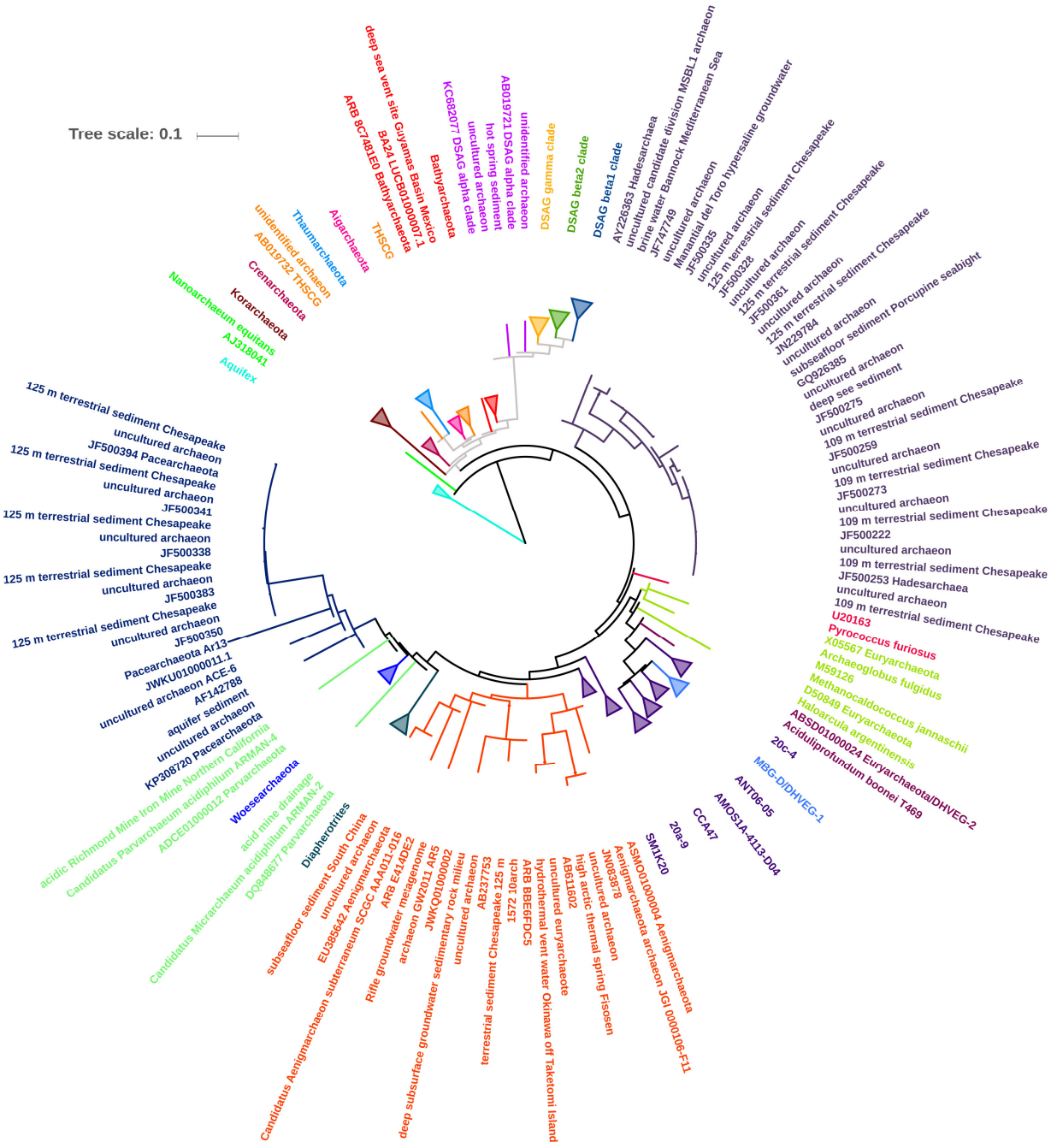


Figure 19a. Tree of main phylogenetic groups with the hereby identified 16S rRNA gene sequences of the CBIS sediment excluding bathyarchaeotal 16S rRNA gene sequences. For tree construction see appendix.

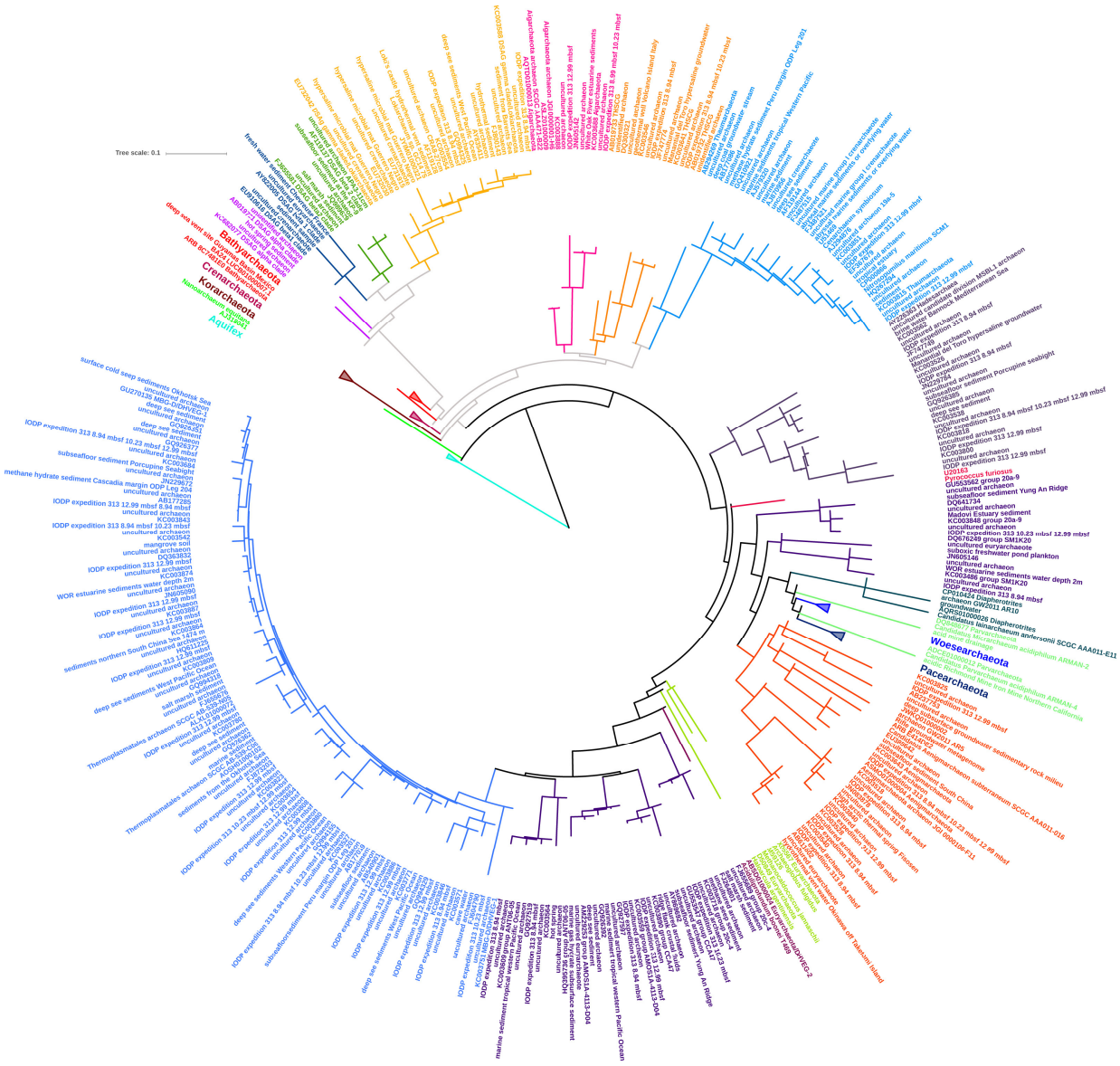


Figure 19b. Tree of main phylogenetic groups with the hereby identified 16S rRNA gene sequences of the New Jersey sediment excluding bathyarchaeotal 16S rRNA gene sequences. For tree construction see appendix.

Jørgensen *et al.* observed a significant correlation between the relative abundance of DSAG 16S rRNA genes and the content of organic carbon and a significant co-variation with iron oxide and dissolved iron and manganese with the abundances of DSAG copy numbers in sediment near Loki's castle vent field in the rift valley of the Arctic mid ocean ridge. They concluded, that DSAG *Archaea* are directly or indirectly linked to iron and manganese cycling (Jørgensen *et al.*, 2013). In this study, *'Lokiarchaeota'* were only retrieved from a depths of 8.94 mbsf, a depth with a total organic carbon content of 0.12 w% and extrapolated manganese and iron porewater concentration of 7.96 µM and 16.11 µM respectively (data not shown). Compared to the Loki's sediment, manganese and iron concentration in the sandy

part of the New Jersey sediments are relative low. On the other hand, at depths with higher iron concentrations in the pore water and higher organic content, e.g. 12.99 mbsf, no *'Lokiarchaeota'* were detected. Nevertheless, '*Lokiarchaeota'* might be involved in in manganese and iron cycling at the New Jersey sediment as suggested by Jorgensen *et al.* because concentrations do not reflect the fluxes and therefore a direct comparison may fail even if *'Lokiarchaeota'* are active.

'Aigarchaeota' represented by the first described genome sequence of Candidatus *'Caldiarchaeum subterraneum'* (Nunoura *et al.*, 2011) were identified at 8.94 mbsf, 10.23 mbsf and 12.99 mbsf in the New Jersey sediment. Besides sharing the ubiquitin modification system and a topoisomerase I B encoding gene with the *Eukarya* (Nunoura *et al.*, 2011) and their deeply branching rooting concerning phylogeny, the predicted gene set suggests the potential of chemolithotrophic growth in *'Aigarchaeota'* using hydrogen or carbon monoxide as electron donor and oxygen, nitrate or nitrite as electron acceptor (Nunoura *et al.*, 2011). A characteristic marker of *'Aigarchaeota'* seems to be the presence of a heme copper (terminal) oxidase complex, which might be an indicator of the predicted use of oxygen as terminal electron acceptor although other electron acceptors (nitrate, nitrite, sulfate) are discussed (Hedlund *et al.*, 2014), (Beam *et al.*, 2016). As a possible carbon fixation pathways in *'Aigarchaeota'* an incomplete dicarboxylate/4-hydroxybutyrate pathway missing the 4-hydroxybutyryl-CoA dehydratase was described for *'Caldiarchaeum subterraneum'* (Nunoura *et al.*, 2011), (Hedlund *et al.*, 2014). Recently, metabolic reconstruction of genomic and metatranscriptomic data from Candidatus *'Calditenuis aerorheumensis'* suggested an aerobic, chemoorganoheterotrophic lifestyle with autotrophic potential (Beam *et al.*, 2016). Similar as for Candidatus *'Caldiarcharchaeum subterraneum'*, an incomplete 3-hydroxypropionate/4-hydroxybutyrate cycle was detected. Samples of this analysis were retrieved from an alkaline siliceous geothermal spring located in the White Creek Area of the Lower Geyser Basin Region of YNP, WY, USA. The hereby described *'Aigarchaeota'* are distributed in geothermal environments with temperatures ~ 68 - 87 °C (and moderately acidic to alkaline pH values, pH: ~5 - 9) (Beam *et al.*, 2016). Although the temperature of the New Jersey sediment was not measured during sampling, such high temperatures up to 13 mbsf could be excluded and therefore the aigarchaeotal New Jersey 16S rRNA gene sequences are not typical. In fact, a check of all aigarchaeotal 16SrRNA gene sequences available at the silva arb website (www.arb-

silva.de) revealed that a minority of ~25% sequences of all aigarchaeotal 16S rRNA gene sequences are not from geothermal isolation sources.

16S RNA gene sequences belonging to the THSCG group were detected at 8.94 mbsf and 10.23 mbsf at the New Jersey sediment. The THSCG group is a polyphyletic group with relationships to the *'Aigarchaeota'* and *'Bathyarchaeota'*.

'Thaumarchaeota' were identified in the New Jersey sediment at 12.99 mbsf but not in upper layers. This group is dominant in prokaryotic picoplankton in seawater below 3000 m depths comprising there a major portion of all *Archaea* (Karner *et al.*, 2001). In marine sediments above two meter below seafloor, they are the most abundant archaeal group (Parkes *et al.*, 2014). The first cultured representative is the candidate *'Nitrosopumilus maritimus'*, a chemolithoautotrophic microorganism that oxidizes ammonia to nitrite (Könneke *et al.*, 2005). Indications for nitrification by *'Thaumarchaeota'* were also provided by Tully and Heidelberg for sediments of the South Pacific Gyre by metagenomics analysis (Tully and Heidelberg, 2016). Teske and Sorensen concluded: *"The habitat preference of MG-I Archaea for the surface layers of oxidized, organic poor marine sediments is consistent with an aerobic metabolism and an ability to take up inorganic dissolved carbon and to fix carbon autotrophically*." (Teske and Sørensen, 2008).

'Bathyarchaeota' were detected at all depths in the CBIS sediment and in the New Jersey sediment. Phylogenetic analysis of archaeal 16S rRNA gene sequences confirmed that *'Bathyarchaeota'* are a widespread group; they constituted 90% of all clones of the clone library for the terrestrial CBIS sediment and 76 % of all clones of the clone library of the marine New Jersey sediment (data not shown). A meta-analysis of 11 studies by Fry *et al.* found that 33% of all archaeal clones belonged to the *'Bathyarchaeota'* (Fry *et al.*, 2008). Although clone library abundances do not represent real abundances, these much higher abundances may be explained by high percentages of primer mismatches for the hereby used primer ARC958R (more than 80%) of all analyzed clones in DHVE-6 group *(Euryarchaeota*), the DSAG/MBG-B group (*'Lokiarchaeota'*) and the MG-1 (*'Thaumarchaeota'*) (Teske and Sørensen, 2008) as shown in Table 7. This would lead to an overestimation of *'Bathyarchaeota'*; nevertheless 16S rRNA gene sequences of the groups with a high proportion of mismatches could be detected in the CBIS sediment as well as in the New Jersey sediments.

Table 7: Evaluation of the mismatches of primer ARC958r which was used in this study according the data of Teske *et al.* (Teske and Sørensen, 2008).

phylogenetic group	sequences with mismatches	sequences analyzed	percentage of sequences with mismatches
SAGMEG: *'Hadesarchaea'*	1	10	10%
DSAG/MBG-B: including the *'Lokiarchaeota'*	6	7	86%
MCG: *'Bathyarchaeota'*	0	1	0%
DHVE-6 (including the *'Pacearchaeota'*)	14	14	100%
MG-1: *'Thaumarchaeota'*	17	18	94%
MBG-D: *'Aenigmarchaeota'*	2	4	50%
other Euryarchaeota	10	24	42%
other Crenarchaeota	10	16	6%

At 125 m depths of the CBIS sediments and all depths of the New Jersey sediments bathyarchaeotal 16S rRNA gene sequences could be allocated to MCG-8/MCG-G subgroup (Fig. 21 and 22) for which Meng *et al.* found indications that members of this group may have the ability to utilize aromatic compounds (hereby, protocatechuate was used in a feeding experiment) (Meng *et al.*, 2014).

SAG analysis showed the possible coexistence of the Embden-Meyerhoff-Pathway for glycolysis beside parts of the CO_2 fixating acetyl-CoA pathway (Wood-Ljungdahl-Pathway) in *'Bathyarchaeota'* (Evans *et al.*, 2015). Acetyl-CoA is hereby a central metabolite interlinked between pyruvate from glycolysis and methyltetrahydromethanopterin (in one of the analyzed genomes, BA1, associated with MCG-3, Fig. 21, Fig. 22). He *et al.* classified BA1 as a member of MCG-3 (He *et al.*, 2016). Methyltetrahydromethanopterin itself can be produced from CO_2 fixation on the one hand and on the other hand from methyl compound oxidation whereas the enzyme for energy conservation via methanogenesis (methyl-tetrahydromethanopterin: coenzyme M methyltransferase complex, MTR) is absent (Evans *et al.*, 2015). Both analyzed genomes harbor the machinery for the CoM-S-S-CoB cycling with *mcrABG* (amongst others) homologs (Evans *et al.*, 2015). Interestingly, the analyzed genomes lack ATP synthase encoding genes. This raises the question, for which reaction the methane cycling machinery is used. It also

restricts the microorganisms to substrate level phosphorylation. In conclusion, Evans *et al.* showed for one of the analyzed bathyarchaeotal genomes (BA1) that parts of the Wood-Ljungdhal pathway may contribute for CO_2 fixation and that glycolysis (via Embden-Meyerhoff pathway) and non hydrogenotrophic methanogenesis may contribute to energy generation (Evans *et al.*, 2015). Linked over parts of the methanogenic machinery, methyl compounds may contribute to carbon assimilation (Evans *et al.*, 2015). Similarly, parts of the Wood-Ljungdhal pathway and the Emden-Meyerhoff pathway were found in other bathyarchaeotal genomes (He *et al.*, 2016), (Lazar *et al.*, 2016). For the second analyzed bathyarchaeotal genome (BA2, Fig. 21 Fig. 22), Evans *et al.* predicted the use of fatty acids for energy metabolism via acetyl-CoA/acetate and also possible the incorporation of acetate as a carbon source via acetylCoA into biomass due to the identified metabolism genes (Evans *et al.*, 2015). Concerning 16S rRNA gene analysis, BA2 is associated with subgroup MCG-8/MCG-C or, depending on strictness of phylogenetic interpretation, a member of subgroup MCG-8/MCG-C for which 16S rRNA gene sequences from 125 m depths in the CBIS sediment and all depths in the New Jersey sediment could be allocated (Fig. 21, Fig. 22, He *et al.*, 2016). Concerning methanogenesis, a surprising result of the study of Evans *et al.* is that there is substantial *mcrA* gene diversity outside of the phylum *Euryarchaeota*. Evans *et al.* showed that the commonly used *mcrA* primers have mismatches to the previously identified euryarchaeotal *mcrA* genes and therefore amplification of the *mcrA* with the available PCR primers gene may fail. In conclusion, *"The congruent topologies of these gene trees support the hypothesis that the MCR complex has coevolved as a functional unit and that methane metabolism was present in the last common ancestor of Euryarchaeota and Bathyarchaeota.'* (Evans *et al.*, 2015). Nevertheless, not all analyzed bathyarchaeotal genomes harbor *mcrA* genes (Evans *et al.*, 2015). He *et al.* found the presence of the Wood-Ljungdahl pathway for carbon fixation beside parts of the tricarboxylic acid cycle and the acetogenesis pathway but not the *mcr* gene (key enzyme) for methanogenesis in bathyarchaeotal genomes (He *et al.*, 2016). For the role of *'Bathyarchaeota'* they proposed acetogenesis as an important archaeal pathway in marine sediments (He *et al.*, 2016) (Fig. 20).

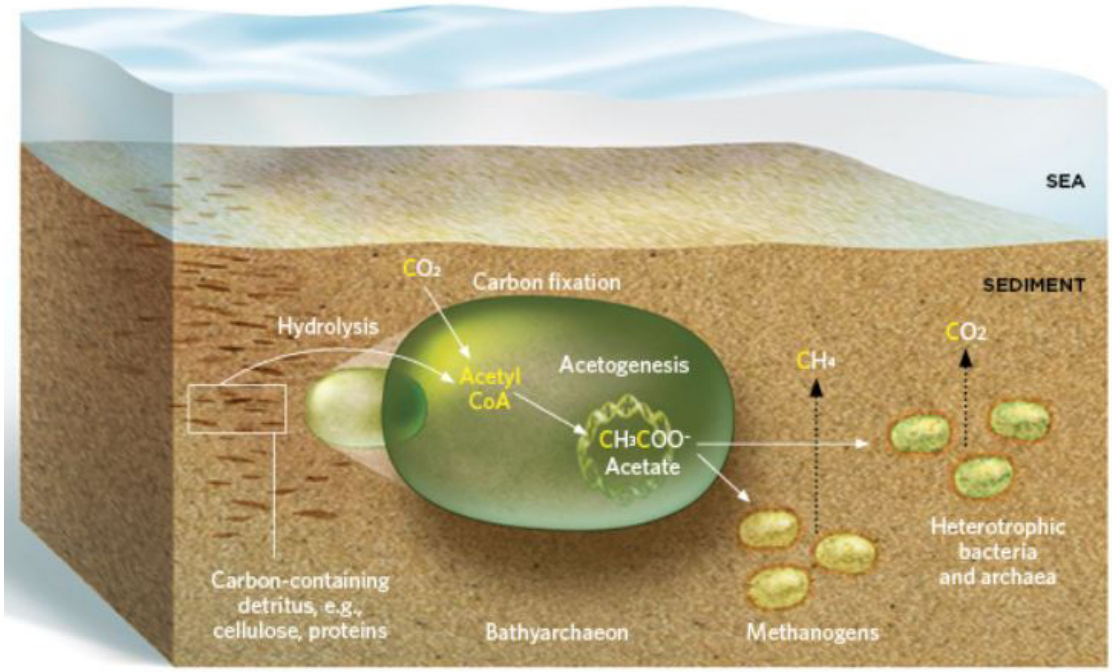


Figure 20. Proposed bathyarchaeotal contribution at marine carbon cycling. Picture from "The Scientist", (http://www.the-scientist.com/?articles.view/articleNo/46336/title/A-New-Role-for-Marine-Archaea/).

Due to low abundances of the *mcrA* and *dsrA* gene, Lever *et al.* compiled that methanogenesis and sulfate reduction in the deep subsurface biosphere only support a small fraction of the total biomass in the deep biosphere unless the microorganisms involved in these processes are too genetically different to be detected with conventionally methods (Lever, 2013). Hence, the recently detected dissimilarity of *mcrA* genes in *'Bathyarchaeota'* and *Euryarchaeota* (Evans *et al.*, 2015) underlines the importance of methane cycling in deeply buried sediments and argues for the latter hypothesis implicating an important role of the detected bathyarchaeotal 16S rRNA sequences in the CBIS and the New Jersey sediments.

'Pacearchaeota' were detected in the CBIS sediment at 125 m depths. *'Pacearchaeota'* (and *'Woesearchaeota')* were detected in high abundances in surface waters of high-altitude Pyrenean lakes. Hereby, the relative abundances of *'Pacearchaeota'* (and *'Woesearchaeota')* correlated significantly and positively with the phylogenetic diversity of bacterial communities supposing interactions between theses archaeal groups and *Bacteria* (Ortiz-Alvarez and Casamayor, 2016) in the high alpine freshwater lakes. Although the total diversity of *Bacteria* at the CBIS sediment was relatively low, it can be asserted that there was no loss in diversity of

Bacteria with depths and interactions between the bacterial community and *'Pacearchaeota'* would be reasonable.

'Hadesarchaea' were detected at both sampling sites and all depths except at 16.71 mbsf in the New Jersey sediment.

'Aenigmarchaeota' were identified up to 12.99 mbsf in the New Jersey sediment and with one clone at 125 m depths in the CBIS sediment.

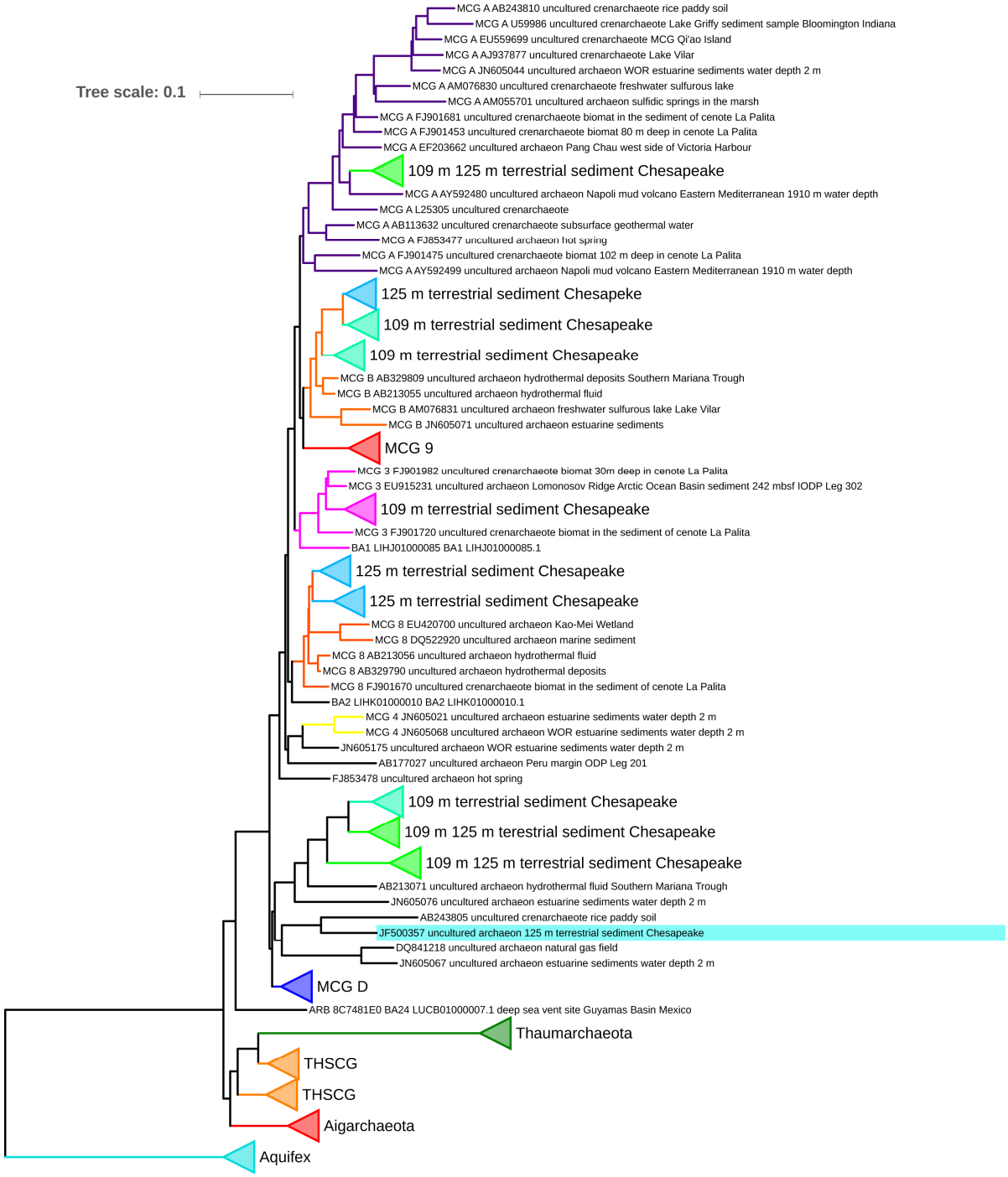
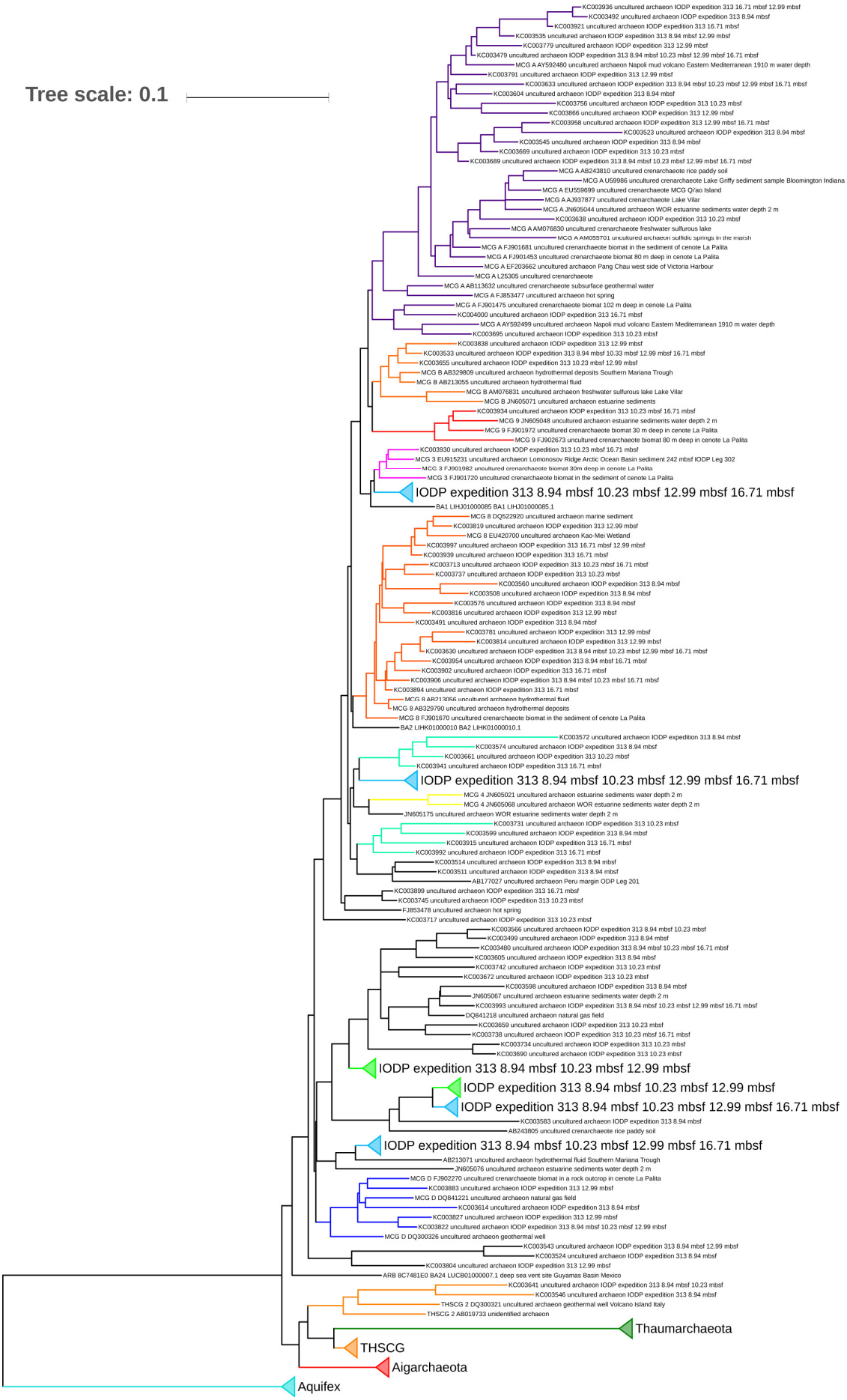


Figure 21 (this side) and 22 (next side): Diversity of the *'Bathyarchaeota'*. Colored clades: described phylogenetic groups according to Meng *et al.* and Kubo *et al.*, proposed related phyla. For tree construction see appendix.


Tree scale: 0.1
KC003936 uncultured archaeon IODP expedition 313 16.71 mbsf 12.99 mbsf
KC003492 uncultured archaeon IODP expedition 313 8.94 mbsf
KC003921 uncultured archaeon IODP expedition 313 16.71 mbsf
KC003535 uncultured archaeon IODP expedition 313 8.94 mbsf 12.99 mbsf
KC003779 uncultured archaeon IODP expedition 313 12.99 mbsf
KC003479 uncultured archaeon IODP expedition 313 8.94 mbsf 10.23 mbsf 12.99 mbsf 16.71 mbsf
MCG A AY592480 uncultured archaeon Napoli mud volcano Eastern Mediterranean 1910 m water depth
KC003791 uncultured archaeon IODP expedition 313 12.99 mbsf
KC003633 uncultured archaeon IODP expedition 313 8.94 mbsf 10.23 mbsf 12.99 mbsf 16.71 mbsf
KC003604 uncultured archaeon IODP expedition 313 8.94 mbsf
KC003756 uncultured archaeon IODP expedition 313 10.23 mbsf
KC003866 uncultured archaeon IODP expedition 313 12.99 mbsf
KC003958 uncultured archaeon IODP expedition 313 12.99 mbsf 16.71 mbsf
KC003523 uncultured archaeon IODP expedition 313 8.94 mbsf
KC003545 uncultured archaeon IODP expedition 313 8.94 mbsf
KC003669 uncultured archaeon IODP expedition 313 10.23 mbsf
KC003689 uncultured archaeon IODP expedition 313 8.94 mbsf 10.23 mbsf 12.99 mbsf 16.71 mbsf
MCG A AB243810 uncultured crenarchaeote rice paddy soil
MCG A U59986 uncultured crenarchaeote Lake Griffy sediment sample Bloomington Indiana
MCG A EU559699 uncultured crenarchaeote MCG Qi'ao Island
MCG A AJ937877 uncultured crenarchaeote Lake Vilar
MCG A JN605044 uncultured archaeon WOR estuarine sediments water depth 2 m
KC003638 uncultured archaeon IODP expedition 313 10.23 mbsf
MCG A AM076830 uncultured crenarchaeote freshwater sulfurous lake
MCG A AM055701 uncultured archaeon sulfidic springs in the marsh
MCG A FJ901681 uncultured crenarchaeote biomat in the sediment of cenote La Palita
MCG A FJ901453 uncultured crenarchaeote biomat 80 m deep in cenote La Palita
MCG A EF203662 uncultured archaeon Pang Chau west side of Victoria Harbour
MCG A L25305 uncultured crenarchaeote
MCG A AB113632 uncultured crenarchaeote subsurface geothermal water
MCG A FJ853477 uncultured archaeon hot spring
MCG A FJ901475 uncultured crenarchaeote biomat 102 m deep in cenote La Palita
KC004000 uncultured archaeon IODP expedition 313 16.71 mbsf
MCG A AY592499 uncultured archaeon Napoli mud volcano Eastern Mediterranean 1910 m water depth
KC003695 uncultured archaeon IODP expedition 313 10.23 mbsf
KC003838 uncultured archaeon IODP expedition 313 12.99 mbsf
KC003533 uncultured archaeon IODP expedition 313 8.94 mbsf 10.33 mbsf 12.99 mbsf 16.71 mbsf
KC003655 uncultured archaeon IODP expedition 313 10.23 mbsf 12.99 mbsf
MCG B AB329809 uncultured archaeon hydrothermal deposits Southern Mariana Trough
MCG B AB213055 uncultured archaeon hydrothermal fluid
MCG B AM076831 uncultured archaeon freshwater sulfurous lake Lake Vilar
MCG B JN605071 uncultured archaeon estuarine sediments
KC003934 uncultured archaeon IODP expedition 313 10.23 mbsf 16.71 mbsf
MCG 9 JN605048 uncultured archaeon estuarine sediments water depth 2 m
MCG 9 FJ901972 uncultured crenarchaeote biomat 30 m deep in cenote La Palita
MCG 9 FJ902673 uncultured crenarchaeote biomat 80 m deep in cenote La Palita
KC003930 uncultured archaeon IODP expedition 313 10.23 mbsf 16.71 mbsf
MCG 3 EU915231 uncultured archaeon Lomonosov Ridge Arctic Ocean Basin sediment 242 mbsf IODP Leg 302
MCG 3 FJ901082 uncultured crenarchaeote biomat 30m deep in cenote La Palita
MCG 3 FJ901720 uncultured crenarchaeote biomat in the sediment of cenote La Palita
IODP expedition 313 8.94 mbsf 10.23 mbsf 12.99 mbsf 16.71 mbsf
BA1 LIHJ01000085 BA1 LIHJ01000085.1
MCG 8 DQ522920 uncultured archaeon marine sediment
KC003819 uncultured archaeon IODP expedition 313 12.99 mbsf
MCG 8 EU420700 uncultured archaeon Kao-Mei Wetland
KC003997 uncultured archaeon IODP expedition 313 16.71 mbsf 12.99 mbsf
KC003939 uncultured archaeon IODP expedition 313 16.71 mbsf
KC003713 uncultured archaeon IODP expedition 313 10.23 mbsf 16.71 mbsf
KC003737 uncultured archaeon IODP expedition 313 10.23 mbsf
KC003560 uncultured archaeon IODP expedition 313 8.94 mbsf
KC003508 uncultured archaeon IODP expedition 313 8.94 mbsf
KC003576 uncultured archaeon IODP expedition 313 8.94 mbsf
KC003816 uncultured archaeon IODP expedition 313 12.99 mbsf
KC003491 uncultured archaeon IODP expedition 313 8.94 mbsf
KC003781 uncultured archaeon IODP expedition 313 12.99 mbsf
KC003814 uncultured archaeon IODP expedition 313 12.99 mbsf
KC003630 uncultured archaeon IODP expedition 313 8.94 mbsf 10.23 mbsf 12.99 mbsf 16.71 mbsf
KC003954 uncultured archaeon IODP expedition 313 8.94 mbsf 16.71 mbsf
KC003902 uncultured archaeon IODP expedition 313 16.71 mbsf
KC003906 uncultured archaeon IODP expedition 313 8.94 mbsf 10.23 mbsf 16.71 mbsf
KC003894 uncultured archaeon IODP expedition 313 16.71 mbsf
MCG 8 AB213056 uncultured archaeon hydrothermal fluid
MCG 8 AB329790 uncultured archaeon hydrothermal deposits
MCG 8 FJ901670 uncultured crenarchaeote biomat in the sediment of cenote La Palita
BA2 LIHK01000010 BA2 LIHK01000010.1
KC003572 uncultured archaeon IODP expedition 313 8.94 mbsf
KC003574 uncultured archaeon IODP expedition 313 8.94 mbsf
KC003661 uncultured archaeon IODP expedition 313 10.23 mbsf
KC003941 uncultured archaeon IODP expedition 313 16.71 mbsf
IODP expedition 313 8.94 mbsf 10.23 mbsf 12.99 mbsf 16.71 mbsf
MCG 4 JN605021 uncultured archaeon estuarine sediments water depth 2 m
MCG 4 JN605068 uncultured archaeon WOR estuarine sediments water depth 2 m
JN605175 uncultured archaeon WOR estuarine sediments water depth 2 m
KC003731 uncultured archaeon IODP expedition 313 10.23 mbsf
KC003599 uncultured archaeon IODP expedition 313 8.94 mbsf
KC003915 uncultured archaeon IODP expedition 313 16.71 mbsf
KC003992 uncultured archaeon IODP expedition 313 16.71 mbsf
KC003514 uncultured archaeon IODP expedition 313 8.94 mbsf
KC003511 uncultured archaeon IODP expedition 313 8.94 mbsf
AB177027 uncultured archaeon Peru margin ODP Leg 201
KC003899 uncultured archaeon IODP expedition 313 16.71 mbsf
KC003745 uncultured archaeon IODP expedition 313 10.23 mbsf
FJ853478 uncultured archaeon hot spring
KC003717 uncultured archaeon IODP expedition 313 10.23 mbsf
KC003566 uncultured archaeon IODP expedition 313 8.94 mbsf 10.23 mbsf
KC003499 uncultured archaeon IODP expedition 313 8.94 mbsf
KC003480 uncultured archaeon IODP expedition 313 8.94 mbsf 10.23 mbsf 16.71 mbsf
KC003605 uncultured archaeon IODP expedition 313 8.94 mbsf
KC003742 uncultured archaeon IODP expedition 313 10.23 mbsf
KC003672 uncultured archaeon IODP expedition 313 10.23 mbsf
KC003598 uncultured archaeon IODP expedition 313 8.94 mbsf
JN605067 uncultured archaeon estuarine sediments water depth 2 m
KC003993 uncultured archaeon IODP expedition 313 8.94 mbsf 10.23 mbsf 12.99 mbsf 16.71 mbsf
DQ841218 uncultured archaeon natural gas field
KC003659 uncultured archaeon IODP expedition 313 10.23 mbsf
KC003738 uncultured archaeon IODP expedition 313 10.23 mbsf 16.71 mbsf
KC003734 uncultured archaeon IODP expedition 313 10.23 mbsf
KC003690 uncultured archaeon IODP expedition 313 10.23 mbsf
IODP expedition 313 8.94 mbsf 10.23 mbsf 12.99 mbsf
IODP expedition 313 8.94 mbsf 10.23 mbsf 12.99 mbsf
IODP expedition 313 8.94 mbsf 10.23 mbsf 12.99 mbsf 16.71 mbsf
KC003583 uncultured archaeon IODP expedition 313 8.94 mbsf
AB243805 uncultured crenarchaeote rice paddy soil
IODP expedition 313 8.94 mbsf 10.23 mbsf 12.99 mbsf 16.71 mbsf
AB213071 uncultured archaeon hydrothermal fluid Southern Mariana Trough
JN605076 uncultured archaeon estuarine sediments water depth 2 m
MCG D FJ902270 uncultured crenarchaeote biomat in a rock outcrop in cenote La Palita
KC003883 uncultured archaeon IODP expedition 313 12.99 mbsf
MCG D DQ841221 uncultured archaeon natural gas field
KC003614 uncultured archaeon IODP expedition 313 8.94 mbsf
KC003827 uncultured archaeon IODP expedition 313 12.99 mbsf
KC003822 uncultured archaeon IODP expedition 313 8.94 mbsf 10.23 mbsf 12.99 mbsf
MCG D DQ300326 uncultured archaeon geothermal well
KC003543 uncultured archaeon IODP expedition 313 8.94 mbsf 12.99 mbsf
KC003524 uncultured archaeon IODP expedition 313 8.94 mbsf
KC003804 uncultured archaeon IODP expedition 313 12.99 mbsf
ARB 8C7481E0 BA24 LUCB01000007.1 deep sea vent site Guyamas Basin Mexico
KC003641 uncultured archaeon IODP expedition 313 8.94 mbsf 10.23 mbsf
KC003546 uncultured archaeon IODP expedition 313 8.94 mbsf
THSCG 2 DQ300321 uncultured archaeon geothermal well Volcano Island Italy
THSCG 2 AB019733 unidentified archaeon
Thaumarchaeota
THSCG
Aigarchaeota
Aquifex

3. Short summary of publications and author's contribution

Chapter 4.1. The deep biosphere in terrestrial sediments of the Chesapeake Bay impact structure, Virginia, USA

For the first time, quantitative data of *Bacteria* and *Archaea* as well as phylogenetic and functional microbial groups in organic lean terrestrial sediment were obtained. Iron(II) and manganese(IV) reducing *Bacteria* of the family *Geobacteriaceae* were almost exclusively found in the uppermost meter, where reactive iron was detected in higher amounts. By 16S rRNA gene sequencing typical soil bacteria were found. Concerning *Archaea*, new members of the proposed phyla *'Hadesarchaea', 'Aenigmarchaeota'*, *'Pacearchaeota'* and *'Bathyarchaeota'* were detected.

Authors: Anja Breuker, Gerrit Köweker, Anna Blazejak and Axel Schippers

Author's contribution:

Anja Breuker conducted and evaluated the cloning experiments, evaluated and partially conducted the qPCR experiments and evaluated the total cell counts. Gerrit Köwecker partially conducted the qPCR experiments and the total cell counting. Anna Blazejak obtained the samples, amended and reviewed the manuscript. Anja Breuker and Axel Schippers designed the experiments and wrote the manuscript.

(Frontiers in Microbiology, 2, 2011)

Chapter 4.2. Microbial community analysis of deeply buried marine sediments of the New Jersey shallow shelf (IODP Expedition 313)

Analysis of the organic lean sediments of the shallow shelf at New Jersey revealed similar abundances for *Bacteria* and *Archaea* by qPCR and relatively low total cell counts below 10^7 cells per ml sediment. In the lowermost part up to 50 mbsf *Bacteria* dominated. The bacterial candidate division JS1 and the classes *Anaerolineae* and *Caldinilineae* of the phylum *Chloroflexi* supplied a high proportion of *Bacteria*. Similarly, high *dsrA* gene copy numbers were found for sulfate reducers. Geobacteriaceae (Fe(III) and Mn(IV) reducers) in the uppermost 15 mbsf correlated with the concentration of manganese and iron in the pore water. Diversity analysis by 16S rRNA gene sequencing via clone libraries revealed a high proportion of the *'Bathyarchaeota' (*formerly MCG, Miscellaneus Crenarchaeotic Group) with a high intragroup diversity. 16S rRNA gene sequences of the *Euryarchaeota*, the DHVEG-6/MBG-D group and the proposed phyla *'Thaumarchaeota'*, *'Aenigmarchaeota',*

'Aigarchaeota', 'Lokiarchaeota' and *'Hadesarchaea'* with typical subsurface groups were identified. Representatives of small groups were also detected. A detailed depth distribution analysis with correlations to geochemical features was carried out.
Authors: Anja Breuker, Susanne Stadler and Axel Schippers
Author's contribution:
Susanne Stadler obtained the samples. Anja Breuker did the TCC - calculations and conducted the qPCR experiments with the help of the laboratory technicians. Anja Breuker conducted and evaluated the cloning and sequencing experiments. Anja Breuker interpreted the data set with the help of Susanne Stadler and Axel Schippers. Susanne Stadler reviewed and amended the manuscript. Anja Breuker and Axel Schippers designed the experiments and wrote the manuscript.
(FEMS Microbiology Ecology, 85, 578–592, 2013)

Chapter 4.3. Data Report: Total cell counts and qPCR abundance of *Archaea* and Bacteria in shallow subsurface marine sediments of North Pond: Gravity cores collected on site survey cruise prior to IODP Expedition 336 Higher abundances of *Archaea* than *Bacteria* in the very oligotrophic sediment of the North Pond basin were found. This result supports the thesis, that *Archaea* are specialized to extreme environments (Valentine, 2007). The methods for qPCR and TCC especially for *Archaea* in this carbonate rich sediment were improved.
Authors: Anja Breuker and Axel Schippers
Author's contribution:
Axel Schippers obtained the samples. Anja Breuker prepared the TCC and the qPCR experiments with the help of the laboratory technicians. Anja Breuker interpreted the data set. Anja Breuker and Axel Schippers designed the experiments and wrote the manuscript.
(In: Edwards, K. J., Bach, W., Klaus, A., and the Expedition 336 Scientists, Proceedings of the Integrated Ocean Drilling Program, Volume 336, 2013)

Chapter 4.4. Microbial community stratification controlled by the subseafloor fluid flow and geothermal gradient at the Iheya North hydrothermal field in the mid-Okinawa trough (Integrated Ocean Drilling Program Expedition 331).

The multiphasic approach investigation of the proposed recharge site of the Iheya North Knoll hydrothermal field in the Mid-Okinawa Trough raised insights in a

stratification controlled microbial community up to 151 mbsf. The sediments were composed of hemipelagic muds and volcaniclastic deposits. Members of the *Chloroflexi* and Deep Sea Archaeal Group mainly colonized the low-temperature hemipelagic sediments. In contrast, the interbedded coarse-grained pumiceous gravels were dominated by 16S rRNA gene sequences of Marine Group I/ *'Thaumarchaeota'*. In summary, the pore water chemistry together with the microbial community analysis supports strongly a potential recharging flow of oxygenated seawater. Below this layer, the detection of an uncultivated lineage of the Hot Water Crenarchaeotic Group I (*'Aigarchaeota'*) may be associated with hydrothermal fluids.
Authors: Katsunori Yanagawa, Anja Breuker, Axel Schippers, Manabu Nishizawa, Akira Ijiri, Miho Hirai, Yoshihiro Takaki, Michinari Sunamura, Tetsuro Urabe, Takuro Nunoura, and Ken Takai
Author's contribution:
Anja Breuker obtained the samples. Anja Breuker conducted and evaluated the qPCR experiments with the help of laboratory technicians. Anja Breuker and Katsunori Yanagawa conducted the TCC. Katsunori Yanagawa designed the study and wrote the manuscript with the help of all coauthors.
(Applied and Environmental Microbiology, 80, 6126–6135, 2014)

Chapter 4.5. Defining boundaries for the distribution of microbial communities beneath the sediment-buried, hydrothermally active seafloor.

Investigations of the microbial community beneath an active hydrothermal vent at the Iheya North hydrothermal field in the Mid-Okinawa Trough detected microbial cells, metabolic activities and molecular signatures in the shallow sediments down to 15.8 m bsf. Profiles of methane and sulfate concentrations and the isotopic compositions of methane suggested the laterally flowing hydrothermal fluids inputs of phase-separated hydrothermal fluids and infiltrated seawater and influenced the in situ microbial anaerobic methane oxidation. 16S rRNA gene phylotypes found in the deepest habitable zone were related to those of thermophiles, although sequences typical of known hyperthermophilic microbes were absent from the entire core. The results shed new light on the distribution and composition of the boundary microbial community close to the high-temperature limit for habitability in the subseafloor environment of a hydrothermal field.
Authors: Katsunori Yanagawa, Akira Ijiri, Anja Breuker, Sanae Sakai, Youko Miyoshi,

Shinsuke Kawagucci, Takuroh Noguchig, Miho Hiraih, Axel Schippers, Jun-ichiro Ishibashi, Yoshihiro Takaki, Michinari Sunamura, Tetsuro Urabe, Takuro Nunoura and Ken Takai.

Author's contribution:

Anja Breuker obtained the samples. Anja Breuker conducted and evaluated the qPCR experiments with the help of a laboratory technician. Anja Breuker and Katsunori Yanagawa conducted the TCC experiments. Katsunori Yanagawa designed the study and wrote the manuscript with the help of all coauthors.

(The ISME Journal, 11, 529–542, 2017.)

Publications not included in this study

Yanagawa, K., T. Nunoura, S. McAllister, M. Hirai, A. Breuker, L. Brandt, C. House, C. L. Moyer, J.-L., K. Aoike, M. Sunamura, T. Urabe, M. Mottl and K. Takai. 2013. The first microbiological contamination assessment by deep-sea drilling and coring by the D/V Chikyu at the Iheya North hydrothermal field in the Mid-Okinawa Trough (IODP Expedition 331). *Frontiers in Microbiology* 4: 327.

Ciobanu, M.-C., G. Burgaud, A. Dufresne, A. Breuker, V. Re´dou, S. BenMaamar, F. Gaboyer, O. Vandenabeele-Trambouze, J. S. Lipp, J., A. Schippers, P. Vandenkoornhuyse, G. Barbier, M. Jebbar, A. Godfroy, and K. Alain. 2014. Microorganisms persist at record depths in the subseafloor of the Canterbury Basin. *The ISME Journal.* 7: 1370–1380.

Schippers, A., A. Breuker and M. Blöthe. 2016. Data report: microbial activity determined by microcalorimetry and cultivation of bacteria from hydrothermally influenced subsurface marine sediments in the mid-Okinawa Trough (IODP Expedition 331). *In: Takai, K., Mottl, M.J., Nielsen, S.H., and the Expedition 331 Scientists, Proceedings of the Integrated Ocean Drilling Program, 331: Tokyo (Integrated Ocean Drilling Program Management International, Inc.)*

4. Manuscripts

4.1. The deep biosphere in terrestrial sediments in the Chesapeake Bay area, Virginia, USA

Anja Breuker[1,2], Gerrit Köweker[1], Anna Blazejak[1, §] and Axel Schippers[1, 2, *]

[1]Geomicrobiology, Federal Institute for Geosciences and Natural Resources (BGR), Stilleweg 2, 30655 Hannover, Germany

[2]Faculty of Natural Sciences, Leibniz Universität Hannover,
Appelstraße 11A, 30167 Hannover, Germany

*Correspondence:

Prof. Dr. Axel Schippers
Bundesanstalt für Geowissenschaften und Rohstoffe
Stilleweg 2
30655 Hannover
Germany
axel.schippers@bgr.de

[§]Current address: Max Planck Institute for Marine Microbiology, Bremen, Germany

Running title: Deep biosphere in terrestrial sediments

Keywords: CARD-FISH / Chesapeake Bay / *Crenarchaeota* / deep biosphere / *Euryarchaeota* / real-time PCR / sediments / subsurface

Abstract

For the first time quantitative data on the abundance of *Bacteria, Archaea* and *Eukarya* in deep terrestrial sediments are provided using multiple methods (total cell counting, quantitative real-time PCR (Q-PCR) and catalyzed reporter deposition – fluorescence in situ hybridization (CARD-FISH)). The oligotrophic (organic carbon content of ~ 0.2 %) deep terrestrial sediments in the Chesapeake Bay area at Eyreville, Virginia, USA, were drilled and sampled up to a depth of 140 m in 2006. The possibility of contamination during drilling was checked using fluorescent microspheres. Total cell counts decreased from 10^9 to 10^6 cells per g dry weight (dw) within the uppermost 20 m, and did not further decrease with depth below. Within the top 7 m, a significant proportion of the total cell counts could be detected with CARD-FISH. The CARD-FISH numbers for *Bacteria* were about an order of magnitude higher than those for *Archaea*. The dominance of *Bacteria* over *Archaea* was confirmed by Q-PCR. The down core quantitative distribution of prokaryotic and eukaryotic small subunit ribosomal RNA genes as well as functional genes involved in different biogeochemical processes was revealed by Q-PCR for the uppermost 10 m and for 80-140 m depth. *Eukarya* and the Fe(III)- and Mn(IV)-reducing bacterial group *Geobacteriaceae* were almost exclusively found in the uppermost meter (arable soil), where reactive iron was detected in higher amounts. The bacterial candidate division JS-1 and the classes *Anaerolineae* and *Caldilineae* of the phylum *Chloroflexi*, highly abundant in marine sediments, were found up to the maximum sampling depth in high copy numbers at this terrestrial site as well. A similar high abundance of the functional gene *cbbL* encoding for the large subunit of RubisCO suggests that autotrophic microorganisms could be relevant in addition to heterotrophs. The functional gene *aprA* of sulfate reducing bacteria was found within distinct layers up to ca. 100 m depth in low copy numbers. The gene *mcrA* of methanogens was not detectable. Cloning and sequencing data of 16S rRNA genes revealed sequences of typical soil *Bacteria*. The closest relatives of the archaeal sequences were *Archaea* recovered from terrestrial and marine environments. Phylogenetic analysis of the *Crenarchaeota* and *Euryarchaeota* revealed new members of the uncultured SAGMEG, DHVEG6, and MCG clusters.

1. Introduction

The Earth's deep biosphere includes a variety of subsurface habitats, such as mines and deep aquifer systems in the continental realm, and sediments and igneous rock in the marine realm. It has been estimated that nearly half of total biomass on Earth resides in the deep biosphere (Whitman *et al.*., 1998). However, the existing data used to generate this global census are highly skewed and in reality reflect habitat accessibility. Deeply-buried marine sediments are among the best studied deep biosphere habitats. They are populated by a huge number of prokaryotes mainly belonging to uncultivated phylogenetic lineages (Parkes *et al.*., 2000; Teske, 2006; Teske and Sørensen, 2008; Biddle *et al.*., 2008; Fry *et al.*., 2008). The abundance of particular phylogenetic and physiological prokaryotic groups, i.e. *Archaea* and *Bacteria*, methanogens or sulfate reducers, in deeply-buried marine sediments has been quantified based on 16S rRNA and functional gene analysis by quantitative, real-time PCR (Q-PCR), FISH and CARD-FISH (catalyzed reporter deposition – fluorescence in situ hybridization; Schippers *et al.*., 2005; Biddle *et al.*., 2006; Inagaki *et al.*., 2006; Schippers and Neretin, 2006; Engelen *et al.*., 2008; Nunoura *et al.*., 2009; Webster *et al.*., 2009).

The terrestrial deep subsurface biosphere has been studied so far only by total cell counting, cultivation techniques as well as by molecular 16S rRNA gene diversity analyses. The hard rock terrestrial deep biosphere in e.g. granite, basalt or metabasalt has been mainly explored by groundwater analyses rather than by deep rock drilling (Stevens and McKinley, 1995; Pedersen, 1993; 1997; Fredrickson *et al.*., 1997; Chapelle *et al.*., 2002; Moser *et al.*., 2003; Lin *et al.*., 2006; Hallbeck and Pedersen, 2008; Sahl *et al.*., 2008; Borgonie *et al.*., 2011; Itävaara *et al.*., 2011).

Deep subsurface terrestrial sediments defined as deeper than 30-35 m (Balkwill *et al.*., 1989) have just begun to be studied by molecular techniques. Cell numbers determined by total cell counting or cultivation indicate that a correlation of cell numbers with depth as described for marine sediments (Parkes *et al.*., 1994; 2000) does not exist. Fry *et al.*. (2009) did not find a decrease in cell numbers with depth in a terrestrial drill core of 148 m length including an interbedded coal deposit in New Zealand. Hoos and Schweisfurth (1982) also did not find a decreasing number of colony forming units (CFU) with depth after analyzing cultivable aerobic and anaerobic bacteria up to a sediment depth of 90 m in Lower Saxony, Germany. The

lack of decreasing cell numbers with sediment depth is also supported by AODC and CFU numbers in coastal plain and fluvial sediment cores from South Carolina (Savannah River Site) and Washington State (Hanford Site), USA, sampled up to 265 m depth (Balkwill *et al.*., 1989; Sinclair and Ghiorse, 1989; Fredrickson *et al.*., 1991; Kieft *et al.*., 1995) and Creataceaous sedimentary rock in New Mexico, USA at 190 m depth (Takai *et al.*., 2003).

Analysis of the microbial diversity by 16S rRNA gene sequencing revealed the dominance of the following prokaryotic groups in deep terrestrial sediments. Most abundant among the *Bacteria* were *Proteobacteria, Actinobacteria, Firmicutes, Chloroflexi*, members of the *Geobacteraceae* family, sulfate-reducers, denitrifiers, fermenters, and acetogens. The most frequently occurring *Archaea* were the Miscellaneous Crenarchaeotic Group, *Methanosarcinales* and *Methanobacteriales* (Boivin-Jahns *et al.*., 1996; Chandler *et al.*., 1997; Detmers *et al.*., 2001; 2004; Inagaki *et al.*., 2005; Takai *et al.*., 2003; Kovacik *et al.*., 2006; Brown and Balkwill, 2009; Fry *et al.*., 2009).

Organic carbon seems to be most important for the long term survival of microorganisms in the terrestrial deep biosphere because a correlation was found between total organic carbon (TOC) and direct counts, basal respiration as well as aerobic glucose mineralization (e.g. Kieft *et al.*., 1995).

The aim of this study was a comprehensive microbial community analysis of deep terrestrial sediments in order to provide missing quantitative data on the abundance of prokaryotes in the terrestrial deep biosphere. As terrestrial study site, deep sediments up to a depth of 140 m in the Chesapeake Bay area at Eyreville, Virginia, USA, were chosen. Total cells stained with SYBR Green were counted with three different methods and the microbial diversity was explored by 16S rRNA gene cloning and sequencing. In addition, Q-PCR and CARD-FISH were applied for the first time to study the deep biosphere in terrestrial sediments. With these quantitative methods, 16S rRNA and functional genes of phylogenetic and physiological prokaryotic groups relevant in deeply-buried marine sediments were analyzed.

2. Materials and Methods

2.1. Site and sediment description

The Chesapeake Bay impact structure (CBIS), Virginia, USA, was formed during the late Eocene meteoric impact approximately 35.5 million years (Ma) ago. It has been explored by an international team of scientists in a project of the International Continental Scientific Drilling Program (ICDP) and the U.S. Geological Survey (USGS, Gohn *et al.*., 2006; 2008; 2009). A cross-section figure showing main features of the CBIS and the drill site location is shown elsewhere (Gohn *et al.*., 2008). The CBIS project acquired continuously cored sections from three holes drilled to a composite depth of 1766 meters at a site within the central zone of the structure at the Eyreville drill site near Cape Charles, Virginia, USA. The drill bit penetrated a 1322-meter-thick section of impact-related rocks and sediments and an overlying 444-meter-thick section of post-impact sandy and clayish sediments. The latter consist of upper Eocene to Pliocene (~5.3 to ~1.8 Ma) continental-shelf sediments and Pleistocene (~1.8 to ~0.01 Ma) non-marine sediments. The upper 140 meters (Miocene to Pleistocene) studied here were cored in Eyreville hole C during April and May 2006. In this depth interval, the porosity is between 36 to 54 %, and the pore water chemistry indicates freshwater conditions, however the NaCl concentration is ~ 0.2 % at 100 m depth and increases to ~ 1 % at the bottom of the core (Gohn *et al.*., 2006; 2008; 2010). In this study only post-impact sediments up to 140 m depth not influenced by the meteoric impact were analyzed.

2.2. Sediment sampling

In this study, cores from Eyreville hole C were sampled for terrestrial microbial community analysis. 50 sediment samples were taken from the surface (arable soil) down to a depth of up to 140 m. To avoid contamination, samples for microbiological analysis were only taken from the centre of each sediment core (63.5 mm diameter) using sterilized cut 5 mL syringes or sterilized spatulas. Depth intervals for sampling were selected based on the quality of the cores with a higher depth resolution near the surface and a lower one at greater depth. As a contamination control, fluorescent microspheres were applied during coring for every second core and samples were

taken from the periphery as well as the centre of the cores for microscopic inspection as previously described (Kallmeyer *et al.*., 2006; Gohn *et al.*., 2009). Four samples could be identified as potentially contaminated and were not further analyzed.
For CARD-FISH and counting total cells with fluorescence microscopy, sediment samples were fixed in 4% formaldehyde-PBS (phosphate buffered saline) as described by Llobet-Brossa *et al.*. (1998) and finally stored at -20°C in PBS-ethanol (1:1). For DNA based molecular as well as geochemical analysis, a parallel set of samples was directly frozen at - 20°C. All samples were transported to BGR frozen with dry ice as air-freight, and afterwards stored at - 20°C until analysis.

2.3. Geochemical analysis

The elemental composition of the solid material was determined by XRF analysis (Philips PW 2400). Total organic carbon (TOC) and the total amount of carbon (TC) and sulfur (TS) were measured with the instrument LECO CS 200 (LECO Corporation). TC and TS were measured after acid removal of carbonates. Reactive iron was extracted with buffered citrate-dithionite as described by Canfield (1989), and measured by ICP-OES (Jobin Yvon Emission 166 Ultrace HR 1000).

2.4. Total cell counts and CARD-FISH

Total cell numbers were determined in formaldehyde fixed samples by staining with SYBR Green II following three different protocols. Cells were counted in the sediment matrix as described by Weinbauer *et al.*. (1998) and were detached from sediment particles before counting as described by Kallmeyer *et al.*. (2008) and Lunau *et al.*. (2005). The latter protocol was modified by replacing the ultrasonic bath with an ultrasonic probe. Catalyzed reporter deposition – fluorescence in situ hybridization (CARD-FISH) was carried out as described (Pernthaler *et al.*., 2002, Schippers *et al.*., 2005) and filters were hybridised for *Archaea* and *Bacteria* using probes ARCH915 or EUB338 I-III as a mixture. As a negative hybridization control the probe NON338 was applied. For contamination control fluorescent beads of bacterial size were used and counted.

2.5. Quantitative, real-time PCR (Q-PCR) analysis

The quantitative composition of the microbial community was analyzed by Q-PCR after DNA extraction. High-molecular-weight DNA was extracted from 0.5 g of a frozen sediment sample following a modified FastDNA Spin Kit for Soil (Bio101) protocol (Webster *et al.*., 2003). Sterilized quartz sand treated in a muffle furnace for organic carbon removal was used as negative control in the extraction procedure. Extracted DNA was amplified by Q-PCR using the device ABI Prism 7000 (Applied Biosystems) and master mixes from the companies Applied Biosystems, Eurogentec or Invitrogen. Each DNA extract was measured in triplicate. After each Q-PCR, melting curves were measured for SYBR Green I assays. The copy numbers of the 16S rRNA gene were quantified for *Archaea* (Takai and Horikoshi, 2000), *Bacteria* (Nadkarni *et al.*., 2002), the JS-1- and *Chloroflexi*-related bacteria (Blazejak and Schippers, 2010), and the Fe(III)- and Mn(IV)-reducing family *Geobacteraceae* (Holmes *et al.*., 2002). The 18S rRNA gene of *Eukarya* was determined as previously described (Schippers and Neretin, 2006). Functional genes were quantified as described: *mcrA* for methyl coenzyme M reductase subunit A (Wilms *et al.*., 2007), *aprA* for adenosine 5´-phosphosulfate reductase subunit A (Blazejak and Schippers, submitted), and *cbbL* for the large subunit of the enzyme ribulose-1.5-bisphosphate carboxylase/oxygenase (RubisCO, form I 'red-like'; Selesi *et al.*., 2007).

2.6. Cloning and sequencing

High-molecular-weight DNA was extracted from 0.5 g of a frozen sediment sample as described above. Four depths (75 - 108 m) were analysed for bacterial 16S rRNA gene sequences and two depths (108 - 125 m) were analysed for archaeal 16S rRNA gene sequences. PCR reactions were carried out with the 1.1 or 2 Master Mix® (Thermo Scientific). PCR for *Bacteria* was carried out with the universal primers GM3f (AGA GTT TGA TCM TGG C) and GM4r (TAC CTT GTT ACG ACT T) (Muyzer, 1995). The following thermocycling conditions were used: one cycle at 95 or 96°C for 5 min; 26-30 cycles at 95° or 96°C for 1 min, 42°C for 1 min, and 72°C for 3 min; and one cycle at 72°C for 7 min. PCR for *Archaea* was carried out with the primers 109f (ACK GCT CAG TAA CAC GT) (Grosskopf *et al.*., 1998) and 912r (CTC CCC CGC CAA TTC CTT TA) (Lueders and Friedrich, 2000). These thermocycling conditions

were used: one cycle at 95 °C for 5 min; 26-30 cycles at 95°C for 1 min, 52°C for 1 min, and 72°C for 3 min; and one cycle at 72°C for 6 min. Cloning in *Escherichia coli* was carried out with the pGEM-t-Easy Vector Systems (Promega®) Kit following the instruction manual. For screening of 16S rRNA genes, 96 clones per sample were randomly picked. For template DNA, a small amount of cells from each clone colony was picked with a sterile toothpick and suspended in 20 µl of sterile water. One or two microliter of this template DNA, after preheating to 95°C for 2 min, were amplified by PCR as described above by using a 25-50 µl (total volume) mixture. PCR products of the correct size (~1,500 bp resp. 850 bp) were purified with the QIAquick PCR Purification Kit (Quiagen®) or directly send for sequencing. Sequencing reactions were carried out by Seqlab Laboratories, Göttingen, Germany. Sequences were edited with BioEdit (www.mbio.ncsu.edu/BioEdit/bioedit.html). A negative DNA extraction control without sediment was treated in parallel. For *Bacteria*, PCR products were also obtained for this negative control (presumably contaminants). The negative control of the PCR reaction itself was negative. In conclusion presumably contamination happened during the DNA extraction procedure. Fifty clones resulting from the negative control were analyzed using BLAST. Partial sequences from sediment samples which exhibited more than 98% similarity to the assumed contaminants were not included in the analysis of bacterial 16S rRNA gene sequences. For *Archaea*, the negative DNA extraction control did not result in a PCR product. All archaeal sequences were aligned by using the SINA Webaligner (www.arb-silva.de/aligner) or the integrated Aligner of the ARB software (www.arb-home.de; Ludwig *et al.*., 2004) and were manually adjusted. Closest relatives of all bacterial and archaeal 16S rDNA sequences found with BLAST (www.ncbi.nlm.nih.gov) were also included in the phylogenetic analysis. For tree construction, sequences were grouped together in a clone family if they exhibited 99 % sequence identity using similarity matrix in the ARB software.

Rarefaction curves were calculated with the mothur software (www.mothur.org; Schloss *et al.*., 2009).

2.7. Phylogenetic analysis

Chimera check was done with the Greengenes Bellerophon program (greengenes.lbl.gov/cgi-bin/nph-index.cgi). The closest sequence relatives of the 16S rRNA gene sequences based on BLAST searches were imported into ARB and aligned using the integrated aligner and manually adjusted. The 16S rRNA gene sequence data were analyzed with the ARB software package (www.arb-home.de). Phylogenetic trees were calculated by performing distance matrix methods (Neighbour Joining with 1000 bootstrap values, both with the Jukes-Cantor correction (Jukes and Cantor, 1969)), maximum parsimony analysis and Maximum-Likelihood analysis. For tree reconstruction only sequences with more than 800 bp were used. Phylogenetic trees were calculated via ARB using the Maximum-Likelihood method. The closest sequence relatives and representatives of the major taxonomic groups were included (Baker *et al.*., 2003). Similarity analysis and clone grouping was done with the ARB similarity matrix with Jukes Cantor correction (Jukes and Cantor, 1969). The phylogenetic groups were arranged according to Teske and Sorensen (2008) and Spang *et al.*. (2010).

3. Results

In this study, we analyzed the microbial ecology and bulk geochemistry of 50 samples from the post-impact Chesapeake Bay impact structure (CBIS) terrestrial sediment from land surface (arable soil) up to a depth of 140 m.

3.1. Geochemical results

Data for the geochemical solid phase analysis of 48 sediment samples are summarized in Table 1. Total organic carbon (TOC) as substrate for heterotrophic microorganisms remained low in the complete sediment depth range with a mean value of ~ 0.2 % and a maximum value of 0.9 % (w/w) characterizing the sediments as oligotrophic. Reactive iron, relevant for Fe(III)-reducing microorganisms, was more than twice as high in the uppermost meter than the mean for the total 140 m (data not shown).

3.2. Microbiological results

3.2.1. Total cell counts and CARD-FISH

Total cells stained with SYBR Green were counted following three different protocols. Depth profiles of total cell counts are shown in Fig. 1. For all three protocols, the maximal cell counts were detected near the surface. The total cell counts indicate a logarithmic decline with depth within the upper 20 m, and show no significant depth correlation between 20 and 140 m. The method comparison shows that the highest cell counts for all depths were obtained with the protocol without detaching cells from sediment particles after Weinbauer *et al.* (1998). Maximum cell counts of more than 10^9 cells / g at the surface declined to about 10^6 cells / g at 20 m depth. Below 20 m, counts were highly variable and not correlated with depth. In comparison, the overall counts obtained with protocols in which the cells were detached from sediment particles before counting gave about half an order of magnitude (Kallmeyer *et al.*., 2008) and about one order of magnitude (Lunau *et al.*., 2005, modified) lower cell counts.

A comparison of the highest total cell counts after Weinbauer *et al.*. (1998) with numbers of living *Bacteria* and *Archaea* obtained by CARD-FISH is given in Fig. 2 for the top 7 m sediment depth. A significant proportion of the total cell counts could be detected with CARD-FISH. Interestingly, the CARD FISH numbers for *Bacteria* were about an order of magnitude higher than those for the *Archaea*. At some depth *Archaea* were not detectable at all. Below 7 m sediment depth the CARD-FISH cell signals were below the detection limit of 10^5 cells / g.

3.2.2. Quantitative microbial community analysis by Q-PCR

Results of Q-PCR analysis for the uppermost 10 m depth and for 80-140 m depth are shown in Fig. 3. The Q-PCR data on bacterial 16S rRNA gene copy numbers matched well with the total cell counts after Weinbauer *et al.*. (1998). *Archaea* were found in lower copy numbers than the *Bacteria* in the top ten meters. At 80-140 m depth, *Archaea* were detected only at a few depths, and always in lower copy numbers using Q-PCR. Thus, the dominance of *Bacteria* over *Archaea* in the CBIS post-impact sediment was confirmed by Q-PCR and CARD-FISH.

In addition to the prokaryotic domains *Bacteria* and *Archaea*, *Eukarya* and specific prokaryotic groups were quantified via Q-PCR by 16S rRNA or functional gene quantification. *Eukarya* and the Fe(III)- and Mn(IV)-reducing bacterial group *Geobacteriaceae* were found in the uppermost meter (arable soil) only (besides at 4 m). In the uppermost meter, reactive iron was detected in higher amounts as a potential electron acceptor for the *Geobacteriaceae*. The bacterial candidate division JS-1 and the classes *Anaerolineae* and *Caldilineae* of the phylum *Chloroflexi*, highly abundant in marine sediments (Blazejak and Schippers, 2010), were found in high copy numbers up to the maximum sampling depth of 140 m. A similar high abundance was found for the functional gene *cbbL* coding for the large subunit of the form I 'red-like' ribulose 1.5-bisphosphate carboxylase/oxygenase (RubisCO) occurring in autotrophic *Proteobacteria* that fix CO_2 via the Calvin-Benson-Bassham (CBB) cycle (Selesi *et al.*., 2007; Badger and Bek, 2008). The functional gene *aprA* coding for adenosine 5´-phosphosulfate reductase occurring in sulfate reducing bacteria was found within distinct layers up to ca. 100 m depth. The gene *mcrA* for methyl coenzyme M reductase of methanogenic *Archaea* could not be detected.

3.2.3. Microbial diversity

In order to reveal the prokaryotic diversity in the CBIS sediment, a phylogenetic analysis of 16S rRNA gene sequences from four depths for *Bacteria* and two depths for *Archaea* was performed. The results for the *Bacteria* are shown in Table 2, those for the *Archaea* in Fig. 4 - 7. Overall, the bacterial diversity seems to be very low. This finding may partly be a result of the limited number of reported bacterial clone data. Many bacterial clones had to be excluded since their 16S RNA gene sequences exhibited more than 98 % similarity (checked with BLAST) to the 16S rRNA gene sequences obtained from the negative DNA extraction control with no sediment (contaminants). Sequences of the remaining bacterial 16S rRNA genes revealed typical soil bacteria (Table 2).

The analysis of 16S rRNA gene sequences of *Archaea* from two depths, 109 m and 125 m, resulted in 13 and 103 clones which could be allocated to the phyla *Euryarchaeota* or *Crenarchaeota*, respectively. The rarefaction curves of the 16S rRNA gene sequences indicate a good coverage of the archaeal diversity as can be seen in the flattening of the two curves (Fig. 4). The composition of the archaeal

community shows a similar ratio of euryarcheotic and crenarcheotic contingents (109 m: 5 euryarchaeotal sequences, 58 crenarchaeotal sequences, 125 m: 8 euryarchaeotal sequences, 57 crenarchaeotal sequences) for the two analyzed sediment depths (Fig. 5).

The phylogenetic analysis with different methods (ARB neighbor joining with 1000 bootstraps, maximum parsimony method, data not shown) gave similar results as the maximum likelihood analysis. Phylogenetic trees for the two archaeal phyla, *Euryarchaeota* and *Crenarcheaota,* are shown in Figs. 6 and 7. Two euryarcheotic clone groups, E1 and E2, could be allocated to the South African Gold Mine Group (SAGMEG). Group E1 clustered together with a clone received from deeply-buried sediments of the Peru margin (AB177011). Group E2 represents a novel phylogenetic subgroup of archaeal sequences with less than 98 % similarity to its closest sequence, AY093454. A third clone group, E3 could be allocated to the Deep Sea Hydrothermal Vent Euryarchaeotal Group 6 (DHVE6). The 16S rRNA gene sequence similarity to its closest sequence EU750878 is less than 89 %. Both SAGMEG and DHVE6 contain 16S rRNA gene sequences of terrestrial as well as of marine origin (Teske and Sørensen, 2008). The three clone groups could be allocated to the two different depths. Group E1 includes only sequences from 109 m depth while group E2 and E3 include only sequences from 125 m depth.

All 16S rRNA gene sequences from the phylum *Crenarcheaota* belong to the Miscellaneous Crenarcheotic Group (MCG). Sequences received could be grouped into eight different clone groups. Five groups (C1, C2, C3, C6, C7) represent new phylogenetic clusters with less than 99 % similarity to their closest related sequences in GenBank. Some clone groups were found in one sample only (e.g. C7, 109 m; C4, 125 m), while others occurred in both samples. Interestingly, the clone groups C3a (125 m) and C3b (109 m) are related to each other and belong together to a new cluster, but the similarity between C3a and C3b is 96.7 % and thus below the species level.

4. Discussion

4.1. Abundance of total and living cells

The microbial community in terrestrial sediments up to a depth of 140 m in the Chesapeake Bay area, Virginia, USA, was thoroughly analyzed by SYBR Green total cell counting, Q-PCR and CARD-FISH, and 16S rRNA gene cloning. The organic carbon content is low (mean ~ 0.2 %) in these oligotrophic deep terrestrial sediments, thus little substrate is available for sustaining a thriving heterotrophic microbial community. Nevertheless total cell counts after Weinbauer *et al.*. (1998) and Q-PCR data exhibited an average of about 10^6 cells / g between 20 m and 140 m depth without a decrease with depth. In comparison, the overall counts obtained with protocols that detached cells from sediment particles before counting resulted in one (Lunau *et al.*., 2005) to half (Kallmeyer *et al.*., 2008) an order of magnitude lower cell counts. This difference between the protocols can be explained by a loss of cells during the detachment procedure and/or counting of unspecific signals without detaching cells from sediment particles. A comparison of the total cell counts with the 16S rRNA gene copy numbers of *Bacteria* obtained by Q-PCR gives the best match with the highest cell counts (Weinbauer *et al.*., 1998). Thus, cell loss during the cell detachment procedures seemed to be more relevant than an overestimation by counting unspecific signals. Although the detachment protocols likely result in an underestimate of total cell numbers, the protocols are suitable for sediments in which the numbers of microorganisms are below 10^5 cells per mL sediment (D´Hondt *et al.*., 2009; Schippers *et al.*., 2010).

The CARD-FISH bacterial cell numbers reflecting living cells were lower than the total cell numbers (Weinbauer *et al.*., 1998) which may indicate a minor proportion of living cells or overlooked CARD-FISH cells due to insufficient cell staining. More evidence for living cells was provided by cultivation experiments (unpublished). In these experiments, fresh samples from various depths were incubated for several months. A strong CO_2 release was observed in aerobic and in anaerobic cultures with or without addition of Fe(III) as a terminal electron acceptor, indicating microbial activity under these conditions. Assays with additional sulfate and nitrate did not show evolution of CO_2 (Michael Siegert and Martin Krüger, personal communication).

In a previous study of the deeper sediments of the terrestrial Chesapeake Bay impact structure (CBIS) at the same drill site total cell counts were obtained after staining with DAPI and exhibited significantly higher numbers, between 10^6 and 10^8 cells per g with high variation over the depth interval 140 - 444 m of the post-impact CBIS sediment (Gohn *et al.*., 2008). The total cell counts increased with depth below 140 m of the post-impact sediment. One explanation for this finding might be the changing lithology connected to a dramatically changing TOC content with depth. The post-impact CBIS sediments from 140 - 444 m consist of a generally fine-grained upper Eocene to upper Miocene sediment with about a 10times higher TOC content than the coarser grained upper Miocene to Pleistocene section above 140 m (Gohn *et al.*., 2009). Most likely the higher TOC content at greater depth sustains significantly more cells than in the upper oligotrophic sediment. These data represent the first observation of a significant increase of cell counts with depth in deep terrestrial sediment. The relevance for a lithological control on the deep biosphere has been previously pointed out for deeply-buried marine sediments (Coolen *et al.*., 2002; Inagaki *et al.*., 2003; Parkes *et al.*., 2005). Below the post-impact CBIS terrestrial sediments in the geologically different zones of sediment breccias, schist, pegmatite and granite (444-1766 m depth) the total cell numbers were considerably lower (10^4 and 10^6 cells per g or not detectable) (Gohn *et al.*., 2008). The novel actinobacterium *Tessaracoccus profundi* was isolated and described from a depth of 940 m (Finster *et al.*., 2009).

The average total cell numbers of about 10^6 cells / g between 20 m and 140 m depth found in this study are in the same order of magnitude or somewhat higher than those found in other deep terrestrial sediments in a similar depth range by total cell counting or by cultivation (Hoos and Schweisfurth, 1982; Balkwill *et al.*., 1989; Fredrickson *et al.*. 1991; Kieft *et al.*., 1995; Takai *et al.*., 2003; Fry *et al.*., 2009). These studies are in agreement with our study, and did not find a decrease in cell numbers with depth. This is in contrast to marine sediments for which a correlation of cell numbers with depth was described (Parkes *et al.*., 1994; 2000; Schippers *et al.*., 2005). The reason for the difference in cell numbers vs. depth in marine and terrestrial sediments is unknown but has considerable importance for the estimation of the global abundance of prokaryotes on Earth (Whitman *et al.*., 1998) as previously stated (Fry *et al.*., 2009).

4.2. Abundance of *Bacteria* versus *Archaea*

This study is the first providing quantitative data on the abundance of *Bacteria* and *Archaea* in deep terrestrial sediment. The dominance of *Bacteria* over *Archaea* in the CBIS post-impact terrestrial sediment was confirmed by Q-PCR and CARD-FISH. The proportions of *Bacteria* and *Archaea* in marine sediments have shown to be highly variable in different sediments and sediment layers and conflicting results have been published for analyses of nucleic-acids (Q-PCR and CARD-FISH) and intact polar lipids (IPL) of cell membranes (Inagaki *et al.*., 2003; 2006; Schippers *et al.*., 2005; 2010; Biddle *et al.*., 2006; Schippers and Neretin, 2006; ; Wilms *et al.*., 2007; Engelen *et al.*., 2008; Lipp *et al.*., 2008; Nunoura *et al.*., 2009; Webster *et al.*., 2009). Schouten *et al.*. (2010) and Logemann *et al.*. (2011) reported about a fossilization of archaeal IPL biomarkers in marine sediments indicating that IPL biomarkers detect fossil signals rather than living *Archaea*, thus putting their proposed dominance in the marine deep biosphere into question.

Another explanation for the conflicting results is given by mismatches of archaeal primers and probes with 16S rRNA gene sequences of the dominant archaeal groups in marine sediments and therefore a potential underestimation of archaeal cell numbers by nucleic-acid based methods (Teske and Sørensen, 2008). A comparison of our archaeal Q-PCR results with our clone library data for the samples at 109 and 125 m revealed a discrepancy of the two methods which used different primers and probes. While in the clone library several different groups were found, Q-PCR did not result in archaeal 16S rRNA gene amplification. For Q-PCR, we used the primers Arch349F and Arch806R, and the TaqMan probe Arch516 (Takai and Horikoshi, 2000). According to Teske and Sørensen (2008) the primer Arch349F has several mismatches within the groups SAGMEG, DHVE6 and MCG. We checked the primer Arch349F against our sequences and found more than five mismatches with some sequences. Similarly, probe Arch516 and primer Arch806R matched only when at least three (probe Arch516F) and two (primer Arch806R) mismatches were allowed. This finding elucidates the necessity for the development of novel archaeal Q-PCR assays.

4.3. Abundance of specific taxa and of functional genes

The detection of the functional gene *cbbL* coding for the large subunit of the form I 'red-like' RubisCO in many samples in relatively high copy numbers in our study indicates that autotrophic *Proteobacteria* are relevant in the deep terrestrial sediments as well. However, their abundance is at least an order of magnitude lower than the 16S rRNA gene copy number of the dominant *Bacteria*, thus heterotrophic bacteria play the mayor role in the deep terrestrial sediment despite the low content of organic carbon. However, heterotrophs were also found in oligotrophic deeply-buried marine sediments (D´Hondt *et al.*., 2004).

The bacterial candidate division JS-1 and the classes *Anaerolineae* and *Caldilineae* of the phylum *Chloroflexi* comprised a higher proportion of the *Bacteria*, but these specific groups with almost no cultivated representatives are less abundant than in marine sediments where almost identical 16S rRNA gene copy numbers for the specific groups and the *Bacteria* were found (Webster *et al.*., 2004; 2011; Blazejak and Schippers, 2010).

Fe(III)-, Mn(IV)-, and sulfate-reducers, methanogens as well as *Eukarya* quantified via general 18S rRNA genes (*Eukarya*), specific 16S rRNA genes (*Geobacteriaceae*) or functional genes (*aprA*, *mcrA*) play a minor or no role in the deep post-impact CBIS terrestrial sediment while these groups were regularly detected in subsurface marine sediments (Schippers and Neretin, 2006; Wilms *et al.*., 2007; Engelen *et al.*., 2008; Nunoura *et al.*., 2009; Webster *et al.*., 2009; Schippers *et al.*., 2010). *Eukarya* and *Geobacteriaceae* were found in the uppermost meter of the CBIS drill site where reactive iron and presumably eukaryotic DNA from farming in the arable soil is available. Deeper eukaryotic DNA was detected in one sample only. Due to the low TOC and sulfate content of the terrestrial sediment, sulfate reduction and methanogenesis are expected to be less relevant than in deeply-buried marine continental margin sediments with a higher TOC content (D´Hondt *et al.*., 2004; Parkes *et al.*., 2005; Schippers *et al.*., 2005; Schippers and Neretin, 2006; Teske, 2006). Both processes are also less relevant in oligotrophic deeply-buried marine sediments (D´Hondt *et al.*., 2004; Sørensen *et al.*., 2004; Teske, 2006; Nunoura *et al.*., 2009) in agreement with our terrestrial study.

4.4. Diversity of *Bacteria* and *Archaea*

The bacterial 16S rRNA gene sequences belong to three classes: *Alphaproteobacteria, Gammaproteobacteria*, and *Actinobacteria*. All bacterial 16S rRNA gene sequences have more than 98.9% similarity to sequences of cultivated heterotrophic bacteria. Almost all of the identified bacteria were previously found in other deep terrestrial sediments (Balkwill *et al.*., 1989; Boivin-Jahns *et al.*., 1996)
The phylogenetic analysis of the *Archaea* identified euryarcheotic as well as crenarcheotic 16S rRNA gene sequences including novel phylogenetic clusters related to lineages that do not yet contain cultivated representatives. The euryarcheotic clone groups E1 and E2 belong to the South African Gold Mine Group (SAGMEG). This group includes 16S rRNA gene sequences found in a South African gold mine and sequences from the deep marine subsurface (Teske and Sørensen, 2008). Fry *et al.*. (2009) also found euryarcheotic sequences belonging to SAGMEG in deep terrestrial sediments including an interbedded coal deposit. Sequences isolated from hot springs (Greece) or dolomite aquifers (South Africa) also belong to the SAGMEG (Fig. 6). In conclusion this group seems not to be restricted to the deep subsurface biosphere, and occurs in marine and terrestrial environments. Similarly, the Deep Sea Hydrothermal Vent Euryarchaeotal Group 6 (DHVE6) to which clone group E3 belongs includes terrestrial and marine sequences. The DHVE6 group defined by Takai and Horikoshi (1999) includes sequences from deep sea hydrothermal vents in the Eastern Pacific Ocean. Successively, sequences from different habitats could be affiliated to this group; examples are from a hydrothermal field at 13°N, 141°W in the South Pacific Rise (Nercessian *et al.*., 2003) and from ODP Site 1231 at the Peru Basin (Sørensen *et al.*., 2004). The closest relative to the group E3 is a sequence from a highly stratified meromictic lake on Ellesmere Island that is characterized by a high salinity in deeper layers (Poliot *et al.*., 2009). Further related 16S rRNA sequences derive from habitats with high salinity: from a hypersaline microbial mat at Guerrero Negro, Mexico (Robertson *et al.*., 2009) and from a commercial gas-water-producing well water in Japan which contains ancient seawater at depths of 347-1132 m (Mochimaru *et al.*., 2007). In conclusion the novel group E3 seems to be related to clones that derive from environments with higher salinity (Fig. 6). The DHVE6 group is affiliated with reduced (metal-) sulfides at vent structures (Takai and Horokoshi, 1999), reduced iron and manganese species

(Sørensen *et al.*., 2004), hydrogen sulfide (Robertson *et al.*., 2009) and/ or high salinity (Robertson *et al.*., 2009; Poliot *et al.*., 2009).

All crenarcheotic clones found in this study belong to the Miscellaneous Crenarcheotic Group (MCG). This group contains a huge number of diverse phylogenetic lineages from different, partially extreme habitats from terrestrial and marine origin (Teske and Sørensen, 2008). We identified clone groups which have closely related sequences from other environments (C5, C8, Fig. 7), and also several novel groups with a relatively high distance to the closest related sequences (C1, C2). As summarized by Teske and Sorensen (2008), the MCG appears to be heterotrophic, which corroborates our Q-PCR data on the dominance of heterotrophic prokaryotes (see above), despite the low TOC content (in particular 0.24 % for 109 m and 0.28 % for 125 m depth).

Conclusions

For the first time quantitative data on the abundance of *Bacteria, Archaea* and *Eukarya* in deep terrestrial sediments are provided using multiple methods (total cell counting, CARD-FISH and Q-PCR). This was done together with the description of the bacterial and archaeal lineages and the quantification of specific taxa and of functional genes. The presence of a significant fraction of rRNA containing, viable bacterial and archaeal cells as revealed by CARD-FISH despite low levels of organic carbon is a relevant finding in this study. The dominance of *Bacteria* over *Archaea* resulted from CARD-FISH and Q-PCR data. Other major findings are the discovery of new sequence clusters within previously described cren- and euryarchaeotal lineages and the presence of high copy numbers of *cbbL* encoding for the large subunit of the form I ‘red-like’ RubisCO suggesting that autotrophic *Proteobacteria* could be relevant in addition to heterotrophs in the terrestrial deep subsurface.

Conflict of interest statement

The research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Table 1 Geochemical solid phase analysis of 48 sediment samples. Mean and (standard deviation) are given in % (w/w).

Total organic C	Total C	Total P	Total S	Total Fe	Reactive Fe
0.18 (0.2)	0.61 (0.62)	0.03 (0.02)	0.65 (0.77)	2.02 (1.6)	0.32 (0.4)

Table 2 Summary of the phylogenetic analysis of bacterial 16S rRNA genes in terrestrial CBIS post-impact sediment.

Clone group	**Number of clones**	**Depth [m]**	**Class**	**Next cultivated neighbour**	**Similarity [%]**
Bact 1	21	101, 109, 125	*Gammaproteobacteria*	*Pseudomonas stutzeri*, strain ATCC 17588, AF094748	> 98.8
Bact 2	2	101, 109	*Gammaproteobacteria*	*Pseudomonas guineae*, strain LMG 24016^{T}, AM491810,	> 99
Bact 3	12	109, 125	*Gammaproteobacteria*	*Acinetobacter lwoffii*, strain DSM 2403, X81665	> 98.8
Bact 4	2	125	*Alphaproteobacteria*	*Mesorhizobium amorphae*, strain ACCC19665, AF041442	> 99
Bact 5	2	75, 101	*Alphaproteobacteria*	*Acidocella aluminidurans*, strain AL46, AB362219	> 98.8
Bact 6	16	109	*Actinobacteria*	*Arthrobacter humicola*, strain KV-653, AB279890	> 99

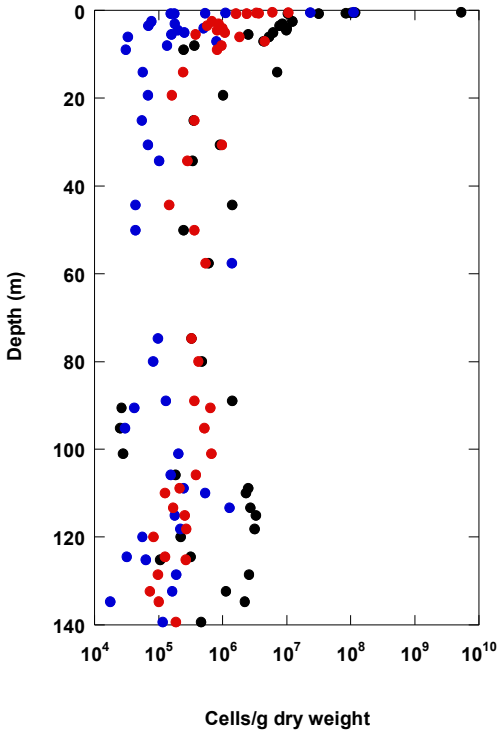

Figure 1: Total cell counts for the terrestrial CBIS post-impact sediment obtained with three different methods (black: method Weinbauer *et al*.., 1998; red: method Kallmeyer *et al*.., 2008; blue: method Lunau *et al*.., 2005).

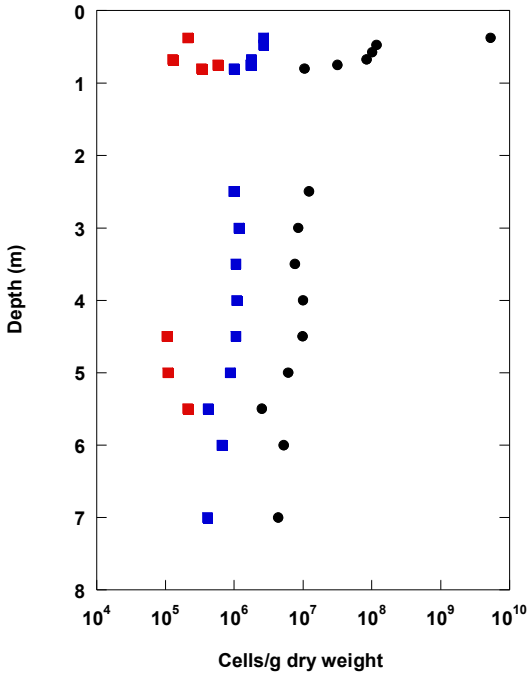

Figure 2: CARD-FISH numbers for *Bacteria* (blue) and *Archaea* (red) displayed with total cell counts (black: Weinbauer *et al*.., 1998) for the uppermost 8 m of the terrestrial CBIS post-impact sediment.

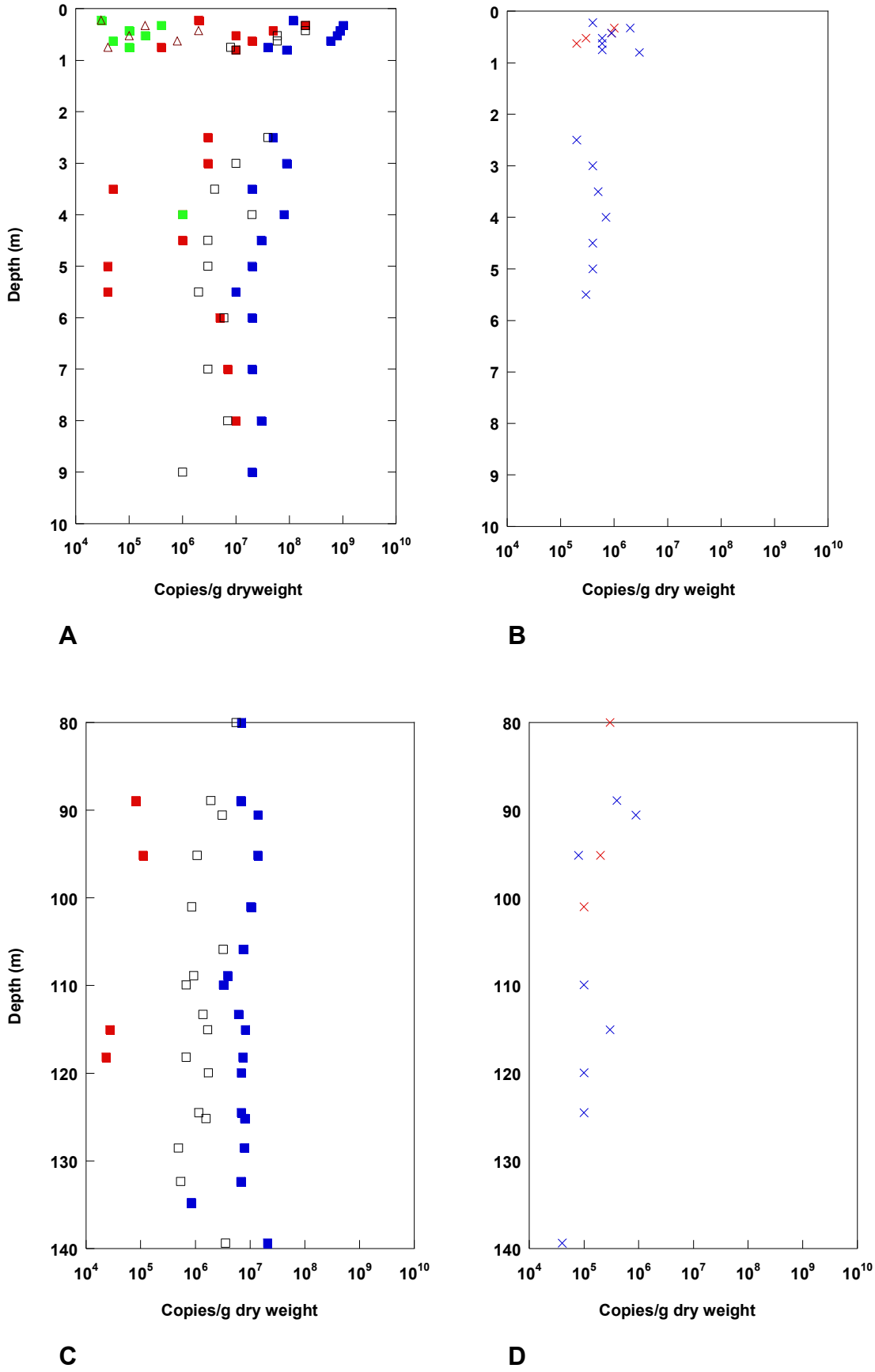

Figure 3: Real-time PCR (Q-PCR) data for the terrestrial CBIS post-impact sediment in the uppermost 10 m depth (A, B), and in 80-140 m depth (C, D). Different phylogenetic groups, blue: Bacteria, red: *Archaea*, green: Eukarya, brown: Geobacteriaceae, black: JS1- Chloroflexi (A, C); functional genes, red: aprA, blue: cbbL (B, D).

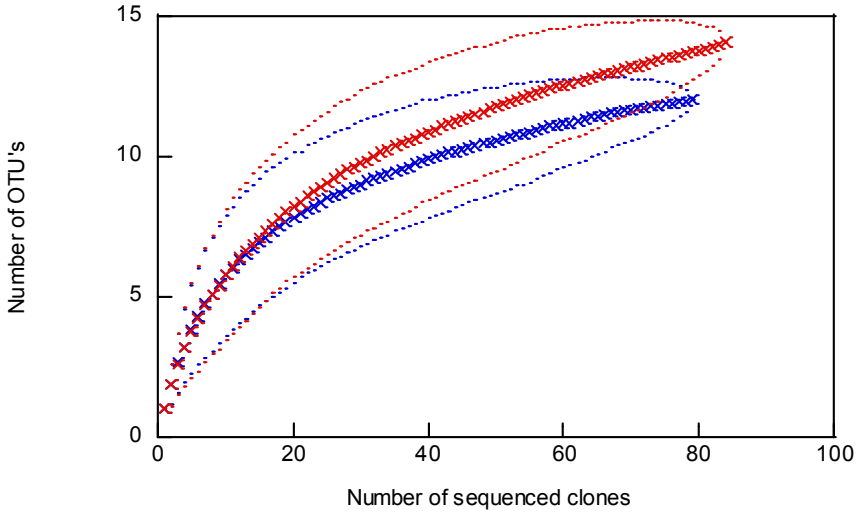

Figure 4: Rarefaction curves for the archaeal 16S rRNA gene sequences from 109 m depth (red) and 125 m depth (blue). Outer and inner lines: high and low confidence interval (95 %). OTU: operational taxonomic unit; cut off of OTU's = 1%.

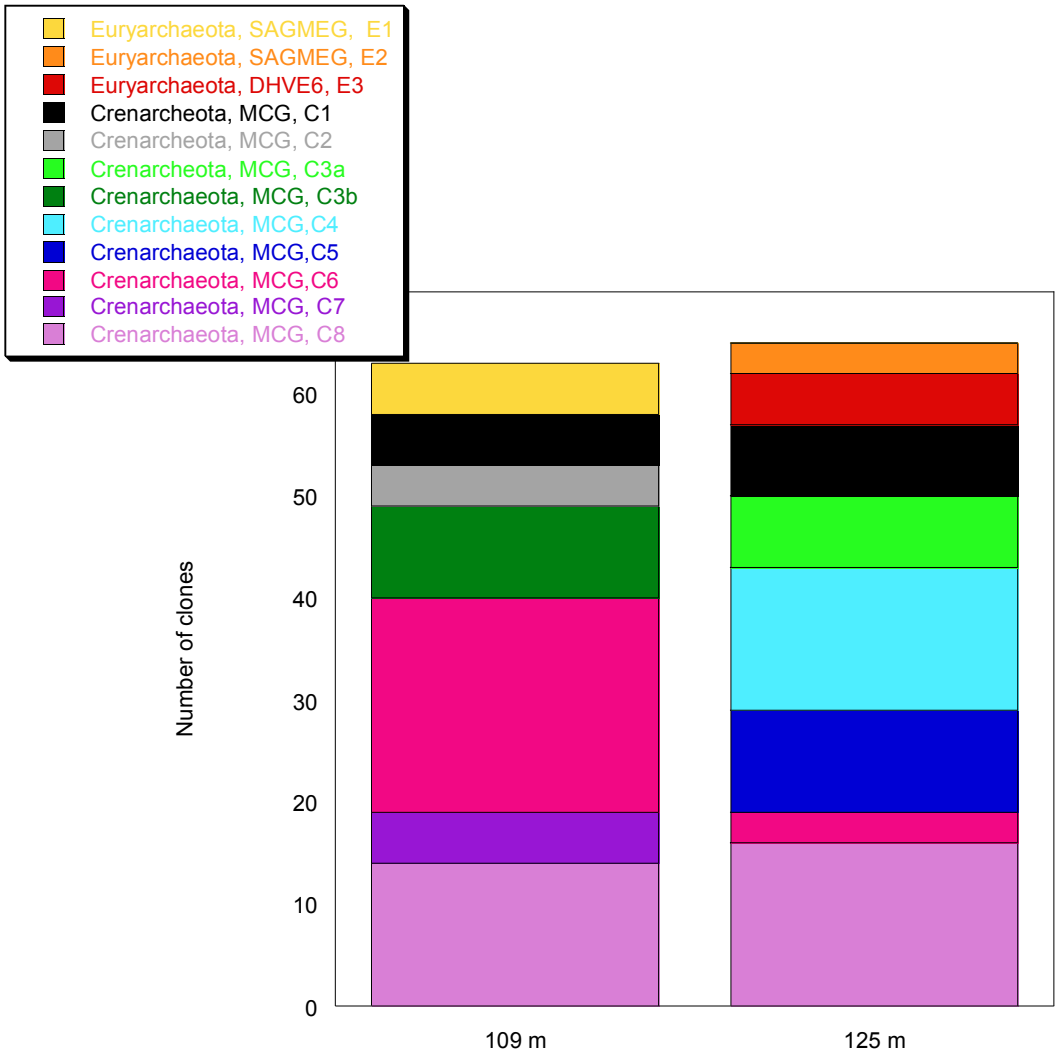

Figure 5 Figure 5: Analysis of the composition of the archaeal community in the terrestrial CBIS post-impact sediment at 109 m and 125 m depth.

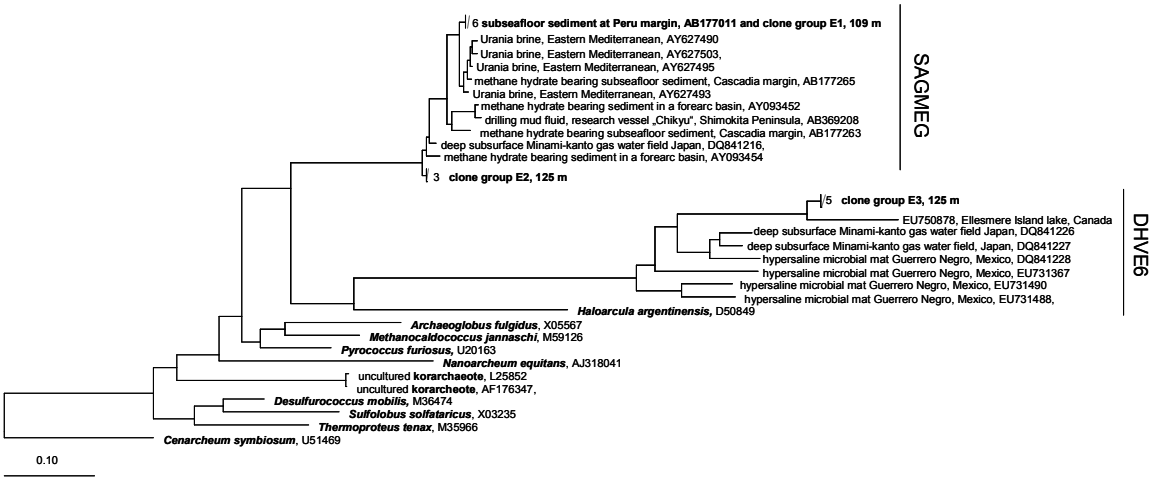

Figure 6: Maximum-Likelihood phylogenetic tree of the *Euryarcheota* showing clades found in terrestrial CBIS post-impact sediment. The tree was rooted with *Cenarcheum symbiosum* as a representative of the deeply branched group "*Thaumarcheota"*. SAGMEG = South African Gold Mine Group, DHVE6 = Deep Sea Hydrothermal Vent Euryarchaeotal Group 6.

Figure 7: Maximum-Likelihood phylogenetic tree of the *Crenarcheota* showing clades found in terrestrial CBIS post-impact sediment. The tree was rooted with Cenarcheum symbiosum as a representative of the deeply branched group "*Thaumarcheota*". MCG = Miscellaneous Crenarcheotic Group.

4.2. Data Report: Total cell counts and qPCR abundance of *Archaea* and *Bacteria* in shallow subsurface marine sediments of North Pond: Gravity cores collected on site survey cruise prior to IODP Expedition 336

Anja Breuker and Axel Schippers*

Federal Institute for Geosciences and Natural Resources (BGR), Stilleweg 2, 30655 Hannover, Germany

*Corresponding author: Phone +49 511 6433103, Fax +49 511 643 2304, e-mail: axel.schippers@bgr.de

Introduction

The total number of prokaryotes in deeply buried marine sediments decreases with sediment depth and varies over orders of magnitude in different areas of the ocean. It is mainly controlled by the content of organic carbon in the sediment as the microbial substrate (Parkes et al., 1994; D´Hondt et al., 2004; Schippers et al., 2005; Edwards et al., 2012; Kallmeyer et al., 2012; Lomstein et al., 2012; Schippers et al., 2012; Hoehler and Jørgensen, 2013). Organic-lean, oligotrophic, and oxic sediments of the Pacific host oxygen-respiring prokaryotes (Røy et al., 2012). Specific archaeal communities for sediments with different trophic states could be detected (Durbin and Teske, 2012). An open question is if *Bacteria* or *Archaea* dominate in oligotrophic sediments as previously discussed for eutrophic sediments based on qPCR analysis (Schippers et al., 2005, 2012). Similar to oligotrophic Pacific sediments, oligotrophic (total organic carbon ~0.15% ± 0.07%) and oxic sediments from the North Pond area in 7 m.y. old western flank of the Mid-Atlantic Ridge 23°N have been shown to contain molecular oxygen down to more than eight meters sediment depth. Aerobic respiration likely dominates organic carbon oxidation (Ziebis et al., 2012). During a site survey cruise prior to the IODP North Pond Expedition 336, sediment cores were collected to 8 m below seafloor. We sampled these cores to count total cells and determine the abundance of *Bacteria* and *Archaea* by pPCR using modified protocols.

Materials and Methods

Organic-lean, oligotrophic, oxic sediments from the North Pond area in 7 m.y. old western flank of the Mid-Atlantic Ridge 23°N were sampled during the IODP Expedition 336 site survey cruise with the German research vessel *Maria S. Merian* in 2009 (MSM 11/1). Sediment cores (12 cm diameter) were successfully retrieved at 6 locations within the North Pond basin between 4040 and 4480 m below sea level by using gravity corers (Ziebis et al., 2012). These cores extended up to 8 m below the seafloor. Once recovered on deck, these cores were quickly cut into 1 m sections that were immediately carried to the cold storage room. Each 1 m section was subsequently split into half-core sections. From one half, samples for microbiological analysis were taken from the interior portion of the half core by excavation with a sterilized spoon, and the outer cm layer was left in the core liner to avoid contamination with seawater. In addition to the gravity cores, an additional 21 sediment samples from the shallow subsurface (0 - 15 cm depth) were taken with the ROV *Jason II* (push cores) during another cruise of the *Maria S. Merian* in 2011 (MSM 20/5).

For total cell counting, 1 mL of each sediment sample was preserved in 9 mL of 0.2 µm sterile filtered 2% formaldehyde in seawater. In the home laboratory, 100 µL of fixed sediment was transferred in a 2 mL Eppendorf tube and suspended in 1 mL 0.1 mM hydrochloric acid (after autoclaving sterile filtered) to dissolve carbonates. Samples were shaken for 5 min and centrifuged for 20 min at 16,000 g. Afterward, 1 mL supernatant was carefully removed. The pellet was suspended in 1 mL TE buffer and centrifuged. This step was repeated. The pellet was suspended with 900 µL TE buffer followed by an ultrasonic treatment for 20 s (Weinbauer et al., 1998). A subsample (100 µL) was stained on filters with SYBR Green as described elsewhere (Lunau et al., 2005). Cells were counted using fluorescence microscopes (Weinbauer et al., 1998).

For qPCR, samples were immediately frozen at –20°C after sampling onboard. Samples were transported and stored frozen in the home laboratory. For DNA extraction from thawed samples, a published protocol for DNA extraction (Webster et al., 2003) with an additional preceding acid treatment step with iodic acid was applied to dissolve carbonates and to improve DNA extraction from cells. Kates et al. (1965) published a protocol to break up ether bonds in cell walls of microorganisms using

iodic acid. According to the described procedure we applied iodic acid, but in a lower concentration and a shorter incubation time (0.1 mol/L vs. ~8 mol/L and 10 min vs. 24h). We assumed that the modified method dissolved the carbonates and/or partially disrupted the cell walls (in particular archaeal ether bondings) but did not lyse the cells. FAST-Prep® tubes without matrix were filled with 0.5 g sediment and centrifuged for 30 s at 14,000 g. Afterward, 1 mL of 0.1 µm filtered 0.1 M iodic acid was added and the pellet was suspended on a shaker for 10 min. The tubes were heated for 10 min at 80°C and centrifuged for 15 min at 16,000 g. A subsample of 1 mL supernatant was removed, and 1 mL TE-buffer was added to the pellet and vortexed. After centrifugation this washing step was repeated. The previously removed matrix was added, and the further procedure followed the protocol of the manufacturer with addition of polyadenine (Webster et al., 2003). Empty tubes were used as a negative control. Extracted DNA was amplified in triplicate by qPCR using the device ABI Prism 7000 (Applied Biosystems). Published assays for the quantification of the 16S rRNA gene copy numbers of *Archaea* (Takai and Horikoshi, 2000) and *Bacteria* (Nadkarni et al., 2002) were applied. 16S rRNA gene copy numbers were converted to cell numbers using conversion factors of 1.5 for *Archaea* and 4.1 for *Bacteria,* as previously done (Schippers et al., 2005).

The reliability of our new protocols was tested in spiking experiments in which known numbers of distinct organisms were added to a sediment sample and the recovery of cells was determined. Different numbers of cells of gram-negative (*Escherichia coli*), and gram-positive (*Bacillus subtilis*) bacteria and archaea (*Methanohalobium evestigatum*), were suspended in sediment samples and the recovery of the cells was determined (Fig. 1). The new procedure considerably improved the cell number recovery. In addition, the acid dissolution of carbonates was confirmed by scanning electron microscopy of samples before and after acid treatment (Fig. 2).

Results and Conclusions

In agreement with published data for other oligotrophic sediments (Kallmeyer et al., 2012), our data show overall low cell numbers decreasing with sediment depth (Fig. 3). An average ~1–2 orders of magnitude higher abundance of *Archaea* than of *Bacteria* was found, in contrast to the previously studied rather organic carbon-rich sediments with a much higher qPCR abundance of *Bacteria* (Schippers et al., 2005, 2012). The near-surface sediment samples up to 15 cm depth showed mean values (and standard deviation) of 2.2 x 10^8 (1.9 x 10^8) cells/mL for *Archaea*, and 1.5 x 10^7 (1.8 x 10^7) cells/mL for *Bacteria*. These qPCR data reflect the dominance of *Archaea* even at the sediment surface. As an explanation for their dominance, *Archaea* are likely better adapted to low energy flux (Valentine, 2007) and have therefore an advantage over *Bacteria* in oligotrophic sediments in contrast to eutrophic sediments (Schippers et al., 2005, 2012). Another explanation is the deposition of archaeal cells from the water column and their preservation in the sediment. Karner et al. (2001) counted pelagic cells of *Crenarchaeota, Euryarchaeota* and *Bacteria* by fluorescence in situ hybridisation in the open ocean up to 5000 m water depth (station in North Pacific subtropical gyre). In their results the fraction of *Crenarchaeota* relative to total DNA containing prokaryotes equaled or exceeded the bacterial fraction below 1000 m. Further work on the composition of the microbial communities should reveal their origin and function in the oligotrophic subsurface sediments.

Acknowledgments

This work is supported by the German Research Foundation (DFG) priority program 527 IODP/ODP grant SCHI 535/7 to A.S. The DFG also provided ship time and financial support for the cruises MSM 11/1 and MSM 20/5 with the German research vessel Maria S. Merian. We thank the chiefs Wolfgang Bach, Katrina Edwards and Heiner Villinger, the crews, and the science party members, especially Anna Blazejak and Beth N. Orcutt for their efforts during sampling.

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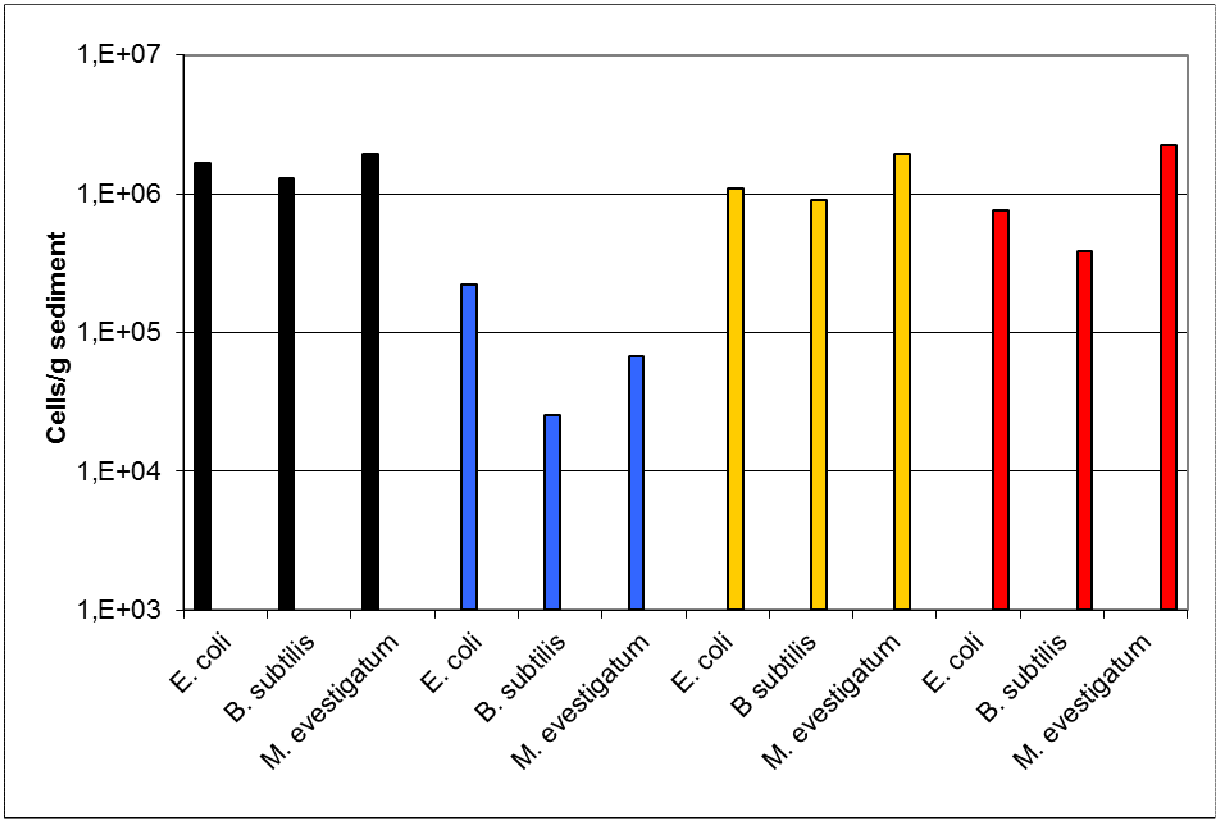

Fig. 1. qPCR analysis of sterilized North Pond sediments spiked with 10^6 cells /g of different species. Black: Total cell counts for comparison; blue: Cell numbers with extraction of DNA after Webster et al. (2003) without acid treatment; yellow: Cell numbers with HI treatment; red: Cell numbers with HCl treatment; 16S rRNA gene copy numbers per cell used for calculation of cell numbers: *Escherichia coli*: 5, *Bacillus subtilis*: 10, *Methanohalobium evestigatum*: 1.5.

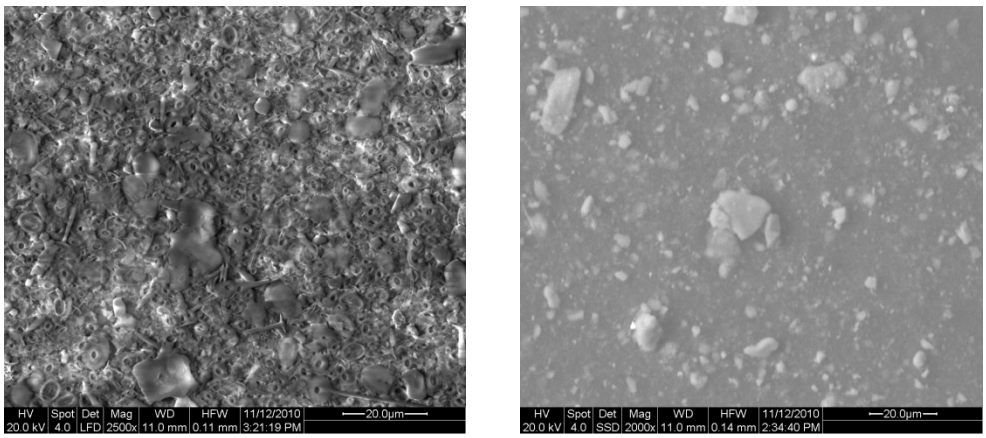

Fig. 2. Scanning electron microscopy images of a North Pond sediment sample before (left) and after (right) acid treatment. On the left image residual skeletons of diatoms and foraminifera can be seen, on the right image the skeletons disappeared.

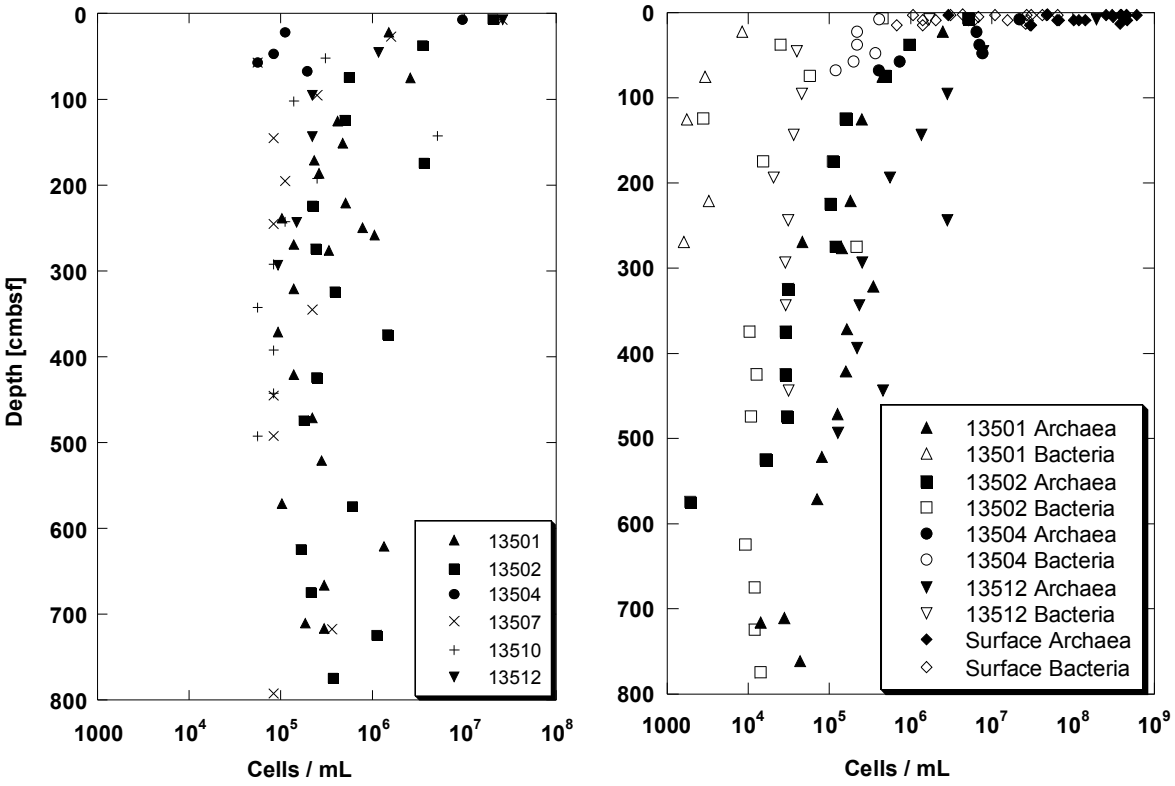

Fig. 3. Total cell counts (left) and qPCR abundance of *Bacteria* and *Archaea* (right) in organic-lean, oligotrophic and oxic North Pond subsurface marine sediments (sampling sites Geob 13501, -2, -4, -7, -10, 12, ref.17; cmbsf: cm below seafloor). Near-surface samples taken with push cores (upper 15 cmbsf) were only analyzed by qPCR.

4.3. Microbial community analysis of deeply buried marine sediments of the New Jersey shallow shelf (IODP Expedition 313)

Anja Breuker, Susanne Stadler and Axel Schippers*

Bundesanstalt für Geowissenschaften und Rohstoffe, Stilleweg 2, D-30655

Hannover, Germany

Running title: Microbiology of New Jersey shallow shelf sediments

Keywords: *Crenarchaeota* / deep biosphere / *Euryarchaeota* / IODP / Miscellaneous Crenarchaeotic Group / real-time PCR / sediments / subsurface

*Correspondence:

Prof. Dr. Axel Schippers
Bundesanstalt für Geowissenschaften und Rohstoffe
Stilleweg 2
30655 Hannover
Germany
axel.schippers@bgr.de
Tel. +49(0)511 643 3103

Abstract

The investigated deeply buried marine sediments of the shallow shelf off New Jersey, USA, are characterized by low organic carbon content and total cell counts of less than 10^7 cells/ mL sediment. The qPCR data for *Bacteria* and *Archaea* were in the same orders of magnitude as the total cell counts. *Archaea* and *Bacteria* occurred in similar 16S rRNA gene copy numbers in the upper part of the sediments, but *Bacteria* dominated in the lowermost part of the analyzed sediment cores down to a maximum analyzed depth of ~ 50 meters below seafloor (mbsf). The bacterial candidate division JS1 and the classes *Anaerolineae* and *Caldinilineae* of the *Chloroflexi* were almost as highly abundant as the total *Bacteria.* Similarly high *dsrA* gene copy numbers were found for sulfate reducers. The abundance of the Fe(III)- and Mn(IV)-reducers comprising *Geobacteraceae* in the upper ~ 15 mbsf correlated with concentrations of manganese and iron in the pore water. The isolated 16S rRNA gene sequences of *Archaea* in clone libraries could be allocated to the phyla *Thaumarchaeota*, *Euryarchaeota* and *Crenarchaeota* with 1 %, 14 % and 85%, respectively. The typical deep subsurface sediment associated groups MBG-B, MBG-D, MCG, and SAGMEG were represented in the sediment community. MCG was the dominant group with a high diversity of the isolated 16S rRNA gene sequences.

Introduction

The deep biosphere in deeply buried, subsurface marine sediments has been explored in various ocean sites mainly within the framework of the Ocean Drilling Program (ODP) and the Integrated Ocean Drilling Program (IODP). Since in the past the focus mainly laid on organic carbon-rich, eutrophic sediments (e.g. along continental margins or in upwelling areas), Whitmann et al. (1998) estimated the total subseafloor sedimentary microbial abundance to be 3.55×10^{30} cells on Earth. A recent study included organic carbon-lean, oligotrophic subsurface sediments and showed that the total cell counts varied between the investigated ocean sites by ca. five orders of magnitude (Kallmeyer et al., 2012). Therefore, the authors proposed a corrected estimation of the values for the global biomass in marine sediments to

$2.9 \cdot 10^{29}$ cells, corresponding to 4.1 petagram (Pg) C and ~0.6% of Earth's total living biomass.

This biomass comprises the three domains of life *Archaea, Bacteria* and *Eukarya*, as well as spores and viruses (Edgcomb et al., 2011; Engelhardt et al., 2012; Lomstein et al., 2012; Schippers et al., 2012; Orsi et al., 2013). Prokaryotes are dominant in the deep biosphere, however conflicting results on the abundance of *Bacteria* versus *Archaea* have been published (Schippers et al., 2005; Biddle et al., 2006; Inagaki et al., 2006; Lipp et al. 2008). Recent studies argue for an overestimation of living *Archaea* based on the quantification of intact polar lipids of their cell membrane and show that the proportion of *Bacteria* versus *Archaea* in marine subsurface sediments is highly variable in different parts of the ocean, resolving the dispute on the dominance of either group in the marine deep biosphere (Schouten *et al*., 2010; Logemann *et al.*, 2011; Schippers et al., 2012).

The analysis of the prokaryotic diversity revealed that the majority of the prokaryotes in subsurface marine sediments belongs to uncultivated phylogenetic lineages (Teske, 2006; Teske and Sørensen, 2008; Fry et al., 2008; Durbin and Teske, 2012; Kubo et al., 2012). Among the *Bacteria*, 16S rRNA gene sequences belonging to the JS-1 group and the *Chloroflexi* were frequently found (Webster et al., 2004; 2007; 2011; Blazejak and Schippers, 2010). Typical groups for the *Archaea* include the Marine Benthic Group B (MBG-B), a deeply branching phylum-level lineage; the Miscellaneous Crenarchaeotal Group (MCG), a frequently detected crenarchaeotal lineage with high intra-group diversity; the South African Gold Mine Euryarchaeotal Group (SAGMEG); and the Marine Benthic Group D (MBG-D), an euryarchaeotal group affiliated with the *Thermoplasmatales* (Durbin and Teske, 2012). The metabolic capabilities of these uncultivated organisms remain unexplored so far. The microbial ecology of the deep biosphere has been mainly studied in organic-rich, eutrophic sediments and relatively few studies focused on organic carbon-lean, oligotrophic subsurface sediments (Inagaki et al., 2001; Sørensen et al., 2004; Nunoura et al. 2009; Roussel et al., 2009; Durbin and Teske, 2011). The analysis of several studies of ultraoligotrophic up to eutrophic marine sediments by Durbin and Teske (2012) revealed that there is a shift in the archaeal community composition from ultraoligotrophic via oligotrophic and mesotrophic to eutrophic sediments. For example the Miscellaneous Crenarchaeotic Group (MCG) has shown to be widespread at all investigated sediments. On the other hand, the occurrence of

a specific group such as the PISA 7 clade was limited to oxic, suboxic or ambiguous sediments. Since organic-lean and/or oligotrophic sediments exhibit specific archaeal diversity patterns, the organic-carbon content is obviously relevant for the natural selection of distinct *Archaea* (Durbin and Teske, 2012).

This study explores the microbial community in deeply buried marine sediments of the New Jersey shallow shelf. These sediments were drilled in IODP Expedition 313 and are characterized by low organic carbon content in the upper sediment (mean 0.47 % from surface down to 50 mbsf) and freshwater intrusions (Mountain et al., 2010; van Geldern et al. 2013). The microbial community analysis included total cell counts, real-time PCR quantification of particular phylogenetic and physiological prokaryotic groups as well as the analysis of the archaeal diversity by 16S rRNA gene cloning and sequencing, following the approach of previous deep biosphere sediment studies (Inagaki et al., 2006; Nunoura et al., 2009; Webster et al., 2009; Breuker et al., 2011). The phylogenetic analyses focussed on *Archaea* and in particular on the MCG (Kubo et al., 2012). The intention of the study was to give insights into the microbial community in the uppermost 50 mbsf sediment depths, on the background of alternating marine and freshwater influence on the sediment geochemistry.

Materials and Methods

Site and sediment description

The New Jersey Atlantic shelf and New Jersey's coastal plain have been the locations of several drilling campaigns including Deep Sea Drilling Project (DSDP) Leg 95, ODP Legs 150, 150X, 174A, and 174AX, and the Atlantic Margin Coring Project (AMCOR; Mountain et al., 2010; van Geldern et al., 2013). The sediments investigated here were cored in summer 2009 during IODP Expedition 313 at site M0027 (Fig. 1). The sediment is generally characterized by terrigenous input of organic material. The dominantly sandy sediment investigated in the presented study is interstratified by various types of sediments. Concerning the uppermost 50 mbsf, there is a sandy layer down to 14 mbsf with a mean total organic carbon of 0.13 %, and a clay dominated layer from ~ 15-22 mbsf with higher concentrations of total organic carbon (mean TOC 0.61 %, Fig. 2A, Table 1). From 23 mbsf to 50 mbsf the

sandy sediment contains thin clay layers, and the mean TOC is 0.88 %. According to Durbin and Teske (2012), the New Jersey shallow shelf sediment down to 50 mbsf can be characterized as organic-lean (TOC < 1 %).

Pore water chemical analyses (Fig. 2B and C) of chloride revealed a complex pattern of alternating, sharply separated, fresh and salt water intervals beneath the shelf, explained by the intrusion of meteoric or meteoric-like freshwater (van Geldern et al., 2013). The sulfate/ chloride ratios at different depths are close to that of marine seawater (0.05). At ~ 17 - 29 mbsf both chloride and sulfate concentrations are lower than in seawater but sulfate is more depleted than chloride (ratio of 0.02-0.03). This fact might indicate microbial sulfate reduction because at this depths the alkalinity is higher, which could reflect the following reaction: 2 CH_2O + SO_4^{2-} -> 2 HCO_3^- + H_2S. However, a definite discrimination between sulfate reduction and freshwater intrusion is not possible based on the available data (van Geldern et al., 2013).
Depth gradients of Fe(II) and Mn(II) concentrations in the pore water did not strictly follow the chloride profiles suggesting biogeochemical Fe(III)- and Mn(VI)-reduction. The porosity of the sediment fluctuated between ~30 and ~ 50 % in the upper 50 mbsf (Mountain et al., 2010). This range of porosity does not limit prokaryotic life in subsurface sediments (Parkes et al., 2000). Concerning the depth profiles of gas concentrations, elevated methane concentrations could not be detected in the uppermost 50 mbsf (van Geldern et al., 2013).

Sediment sampling

The cores from site M0027 were sampled for analyzing the microbial ecology. 53 sediment samples were taken from the surface down to a maximum depth of 621 mbsf. Samples down to a depth of ~ 50 m were taken via piston coring shown to be robust against contamination (House et al., 2003). Samples for microbiological analysis (Table 1) were taken from the centre of each sediment core. Samples from deeper sediments were obtained mainly by other drilling techniques and many of them were shown to be potentially contaminated using fluorescent microspheres of bacterial size (Smith et al., 2000; House et al., 2003). Thus, these samples were not included in this microbiological study.

For total cell counts with fluorescence microscopy, a 1 mL sediment plug (mini-core) from a syringe was extruded into a sterile 15 mL-screw capped tube

containing 9 mL of 2 % (v/v) filter sterilized (0.2 µm) formaldehyde in 3.5% NaCl-solution. The vial was closed and shaken vigorously to disperse the sediment particles. The fixed samples were stored and transported cooled (< 10 °C) to BGR (Bundesanstalt für Geowissenschaften und Rohstoffe, Hannover, Germany). For DNA based molecular analysis at least 3-4 cm long mini-cores were transferred into a sterile 50 mL-screw capped tube using a sterile spatula or sterile spoon. The DNA-samples were frozen at below – 20°C, and were transported frozen on dry ice to BGR and were afterwards stored frozen until analysis.

Total cell counts

Total cell counts were determined in formaldehyde fixed samples (2 % final concentration) by staining with SYBR Green I after Weinbauer et al. (1998). For cell staining the protocol after Lunau et al. (2005) with a moviol mounting medium was used. Cells were counted on two filters per sample and the mean values are displayed.

DNA-extraction

High-molecular-weight DNA was extracted from 0.5 g of a frozen sediment sample following a modified FastDNA Spin Kit for Soil (Bio101) protocol (Webster et al., 2003). DNA extracts from blank tubes (no sediment added) were used as negative control in the extraction procedure.

Quantitative microbial community analysis by qPCR

Extracted DNA was amplified by qPCR using the device ABI Prism 7000 (Applied Biosystems) and master mixes from the companies Applied Biosystems, Eurogentec or Invitrogen. Each DNA extract was measured in triplicate. Correlation coefficients of standard curves were > 0.99. Data were included if at least two data points matched with a standard deviation of less than 0.5. Mean values are displayed. After each qPCR, melting curves were measured for SYBR Green I assays. The copy numbers of the 16S rRNA gene were quantified for *Archaea* (Takai and Horikoshi, 2000), *Bacteria* (Nadkarni et al., 2002), the JS-1-related bacteria and the classes

Anaerolineae and *Caldinilineae* of the *Chloroflexi* (Blazejak and Schippers, 2010), and the Fe(III)- and Mn(IV)-reducing family *Geobacteraceae* (Holmes et al., 2002). Functional genes were quantified as described: *mcrA* for methyl coenzyme M reductase (Wilms et al., 2007), *dsrA* for dissimilatory sulfite (bi)reductase (Schippers and Neretin, 2006), *aprA* for adenosine 5´-phosphosulfate reductase (Blazejak and Schippers, 2011), and *cbbL* for the enzyme Rubisco (Selesi et al., 2007).

Archaeal diversity analysis

High-molecular-weight DNA was extracted from 0.5 g frozen sediment samples from various depths as described above. PCR for the amplification of the 16S rRNA gene of *Archaea* was carried out with a one base modified forward primer 21f (TTC CGG ATG ATC CYG CCG GA (De Long, 1992)) and reverse primer 958r (YCC GGC GTT GAM TCC AAT T, (De Long, 1992)). The following thermocycling conditions were used: one cycle at 95 °C for 5 min; 30-38 cycles at 95°C for 1 min, 52°C for 1 min, and 72°C for 1.5 min; and one cycle at 72°C for 6 min. For four different depths below seafloor (8.9 m, 10.2 m, 13 m and 16.7 m) of site M0027 PCR amplification products were obtained. The negative controls (DNA-extraction) did not result in a PCR product. Clone libraries were constructed and isolated 16S rRNA genes were sequenced by the company Microsynth AG (Balgach, Switzerland). From each sample 181-192 16S rRNA gene sequences were analyzed. Sequences were edited by using the Geneious programme (www.geneious.com). Chimera check was done with the Greengenes Bellerophon programme (greengenes.lbl.gov/cgi-bin/nph-index.cgi). The >800 bp segments were checked against sequences in the GenBank database (www.ncbi.nlm.nih.gov) each by using BLAST for similarity searches. Nearest neighbors which were not found in the ARB database (SSURef_108_silva_09_09_11_opt.arb; Ludwig et al., 2004; Pruesse et al., 2007) were imported and aligned using the integrated aligner and manually curated.

All archaeal sequences were aligned by using the SINA Webaligner (www.arb-silva.de/aligner) or the integrated aligner of the ARB software (www.arb-home.de; Ludwig et al., 2004) and manually curated. Rarefaction curves were calculated with the Mothur software (version 1.27, www.mothur.org; Schloss et al., 2009) for operational taxonomic units (OTUs) for archaeal 16S rRNA gene sequences at 3 %

level. The richness estimators Chao1 and ACE were also calculated with the mother software (3 % level).

Phylogenetic trees were calculated by Maximum Likelihood analysis via ARB software. For tree reconstruction only 16S rRNA gene sequences with more than 1000 bp were used. Shorter sequences were added to the tree by the Maximum Parsimony method. Representatives of the major taxonomic groups (according to Baker et al., 2003) and nearest neighbors were displayed in the trees. The phylogenetic groups were arranged according to Sørensen et al. (2004), Teske and Sørensen (2008) and Spang et al. (2010). According to Kubo et al. (2012) 441 16S rRNA gene sequences belonging to the MCG group were grouped with the ARB software and analyzed.

The 16S rRNA gene sequences obtained in this study were submitted to GenBank nucleotide databases under the accession numbers KC003479 - KC004012.

Phylogenetic distance analysis

To measure the phylogenetic distances between the four samples according to depths, a UniFrac analysis (http://bmf2.colorado.edu, Lozupone and Knight, 2005) was carried out with all 16S rRNA gene sequences from the New Jersey shallow shelf sediment. The 16S rRNA gene sequences were added to a maximum likelihood tree with *Nostoc commune* as outgroup with the ARB programme. The tree was exported and uploaded online. Principal coordinates analysis (PCoA) was run online without abundance weights. The geochemical data of corresponding depths were extrapolated from the data for depths above and below if not available for the exact depths.

Results and Discussion

In this study the microbial ecology of deeply buried sediments of the New Jersey shallow shelf, characterized by low organic carbon content and freshwater intrusions, was explored by total cell counting, quantitative real-time PCR (qPCR) as well as by 16S rRNA gene cloning and sequencing for the *Archaea*. The sediments of the New

Jersey shallow shelf were organic-lean in the studied depth range from the sediment surface down to about ~ 50 mbsf, except for two sediment depths around 30 mbsf, for which a higher content of total organic carbon was measured (Fig. 2A, Table 1). We consider sediments with an organic carbon content of less than 1 % as organic-lean according to Durbin and Teske (2012). However, the organic carbon concentration is not a directly proportional index of the sediment trophic state, as substrate availability and organic carbon residence time can vary between sediments with similar organic carbon contents (Durbin and Teske, 2012). Consequently, Durbin and Teske also defined other parameters such as the sedimentation rate, the penetration depth of electron acceptors such as sulfate, and the ammonium concentration to characterize the trophic state of sediments. Concerning the New Jersey shallow shelf sediments, the ammonium concentrations were up to 700 µM in the studied depth range. Depletion of sulfate could either be explained by freshwater intrusions (van Geldern et al., 2013) or by sulfate reduction (Fig. 2B). Overall, the sediment can be characterized as oligotrophic-mesotrophic and falls in the same group as e.g. the sediments from ODP sites 1225, 1226 (D´Hondt et al. 2004).

Total cell counts

Total counts of prokaryotic cells generally correlate with the content of organic carbon in marine sediments (D´Hondt et al., 2004; Kallmeyer et al., 2012; Schippers et al., 2012). The depth profile of the total cell counts for site M0027 is shown in Fig. 3A, the values are given in Table 1.

The total cell counts decreased with sediment depth, which is typical for subsurface marine sediments (Parkes et al., 2000; D´Hondt et al. 2004; Kallmeyer et al., 2012). Maximum cell counts did not exceed 10^7 cells/mL sediment reflecting the low organic carbon content. The total organic carbon content was higher between ~10 mbsf and ~35 mbsf (clay layers) than in the upper ~10 mbsf which explains why the maximum cell counts were not detected in the near-surface sediment as usually found for marine sediments. The impact of lithological features of sediments on total cell numbers and qPCR data has been shown previously (Inagaki et al., 2003; Parkes et al, 2005; Schippers et al., 2012), and we can assume that clay layers provide more substrate for the microorganisms than the bulk sediment. However, the highest TOC values around 30 mbsf do not correspond to high total cell counts. This

can be explained by thin clay layers analyzed for TOC while total counts were analyzed for not exactly the same depth layers (different rather sandy sediment samples). Generally, the total cells counts were low and in agreement with data for other organic-lean marine sediments (D´Hondt et al., 2004; Nunoura et al., 2009; Kallmeyer et al., 2012; Schippers et al., 2012).

Quantitative microbial community analysis by qPCR

Maximal gene copy numbers were detected between ~ 10 and ~ 20 mbsf (Fig. 3), corresponding to the elevated organic carbon content, and not in near-surface sediments as usually found (Schippers et al., 2010; 2012). The qPCR data of site M0027 for *Bacteria* and *Archaea* (Fig. 3 B) were in the same orders of magnitude as the total cell counts (Fig. 3 A). *Archaea* and *Bacteria* had similar copy numbers up to a depth of 20 mbsf. In the deeper part of the analyzed core *Bacteria* dominated. For comparison we compiled published qPCR data of several sediment studies which show that the proportion of *Bacteria* versus *Archaea* in marine sediments is highly variable in different parts of the ocean (Table 2). The ratio of *Archaea* versus *Bacteria* seems to be variable depending on the type of sediment. Using qPCR, an almost equal abundance of *Bacteria* and *Archaea* has also been found for the Porcupine Seabight (IODP Exp. 307; Webster et al., 2009), the northeast Pacific ridge-flank (IODP Exp. 301; Engelen et al., 2008), Sumatra forearc basins (Schippers et al., 2010), sediments of the Black Sea and the Benguela upwelling system off the Atlantic coast of Namibia (Schippers et al., 2012). By contrast, *Bacteria* dominated other sediments such as the Sea of Okhotsk (Inagaki et al., 2003), the Gulf of Mexico (IODP Exp. 308; Nunoura et al., 2009), the Peru continental margin and the equatorial Pacific sediments (ODP Leg 201; Schippers et al., 2005; Inagaki et al., 2006), as well as gas-hydrate bearing sediments from the Cascadia margin (ODP Leg 204; Inagaki et al., 2006). Most data on the abundance of *Bacteria* and *Archaea* in deeply buried marine sediments originate from qPCR analysis of rather organic carbon-rich, eutrophic sediments with organic carbon contents of more than 1 %. Here, *Bacteria* either dominated or an overall equal proportion of *Bacteria* and *Archaea* was determined. *Archaea* were found to be dominant by qPCR only in the organic-lean, oligotrophic sediments from “North Pond” (Breuker and Schippers,

2013). Further research should include more oligotrophic sediments and investigate which parameters control the ratio of *Bacteria* versus *Archaea*.

Particular phylogenetic and physiological groups were additionally revealed by qPCR in this study (Fig. 3 B and 3 C), as was the case in previous marine subsurface sediment studies (Schippers and Neretin, 2006; Leloup et al., 2007; Wilms et al., 2007; Engelen et al., 2008; Nunoura et al., 2009; Webster et al., 2009; Blazejak and Schippers, 2010; 2011; Schippers et al., 2010; 2012). The 16S rRNA gene copy numbers of the bacterial candidate division JS1 and the classes *Anaerolineae* and *Caldinilineae* of the *Chloroflexi* were detectable in the upper 30 mbsf with more than 10^5 copies per mL sediment. The bacterial 16S rRNA gene copy numbers were higher in most sediment layers, however JS1 and the two classes of *Chloroflexi* comprised a significant part of the bacterial community. Previous studies for the sediments off Sumatra, of the Peru margin, the Benguela upwelling system off Namibia and of the Black Sea showed that the bacterial candidate division JS-1 and the classes *Anaerolineae* and *Caldilineae* of the phylum *Chloroflexi* were even as highly abundant as *Bacteria* (Blazejak and Schippers, 2010; Schippers et al., 2012). Obviously, these groups play a dominant role in subsurface marine sediments as already discussed elsewhere (Webster et al., 2004; Teske, 2006; Fry et al., 2008), although the physiology of these presumably heterotrophic bacterial groups remains almost unexplored (Webster et al., 2011).

The *Geobacteraceae* comprising Fe(III)- and Mn(IV)-reducers were found in the upper ~ 15 mbsf with increasing 16S rRNA gene copy numbers with depth up to 10^5 copies / mL sediment. These data correlated with increasing concentrations of manganese and iron in the pore water (Fig. 2 C, maximum of 56 µM Fe at 14.7 mbsf and of 16.5 µM Mn at 17.9 mbsf). These results suggest that organic matter degradation via Fe(III)- and Mn(IV)-reduction is a relevant biogeochemical process in the New Jersey shallow shelf sediments, as previously found for sediments of the Sumatra forearc basins (Schippers et al., 2010).

Another important biogeochemical process is sulfate reduction as often found for subsurface marine sediments (Leloup et al., 2007; Blazejak and Schippers, 2011; Schippers et al., 2010; 2012). A high occurrence was found for the functional gene *dsrA* (dissimilatory sulfite reductase gene) of sulfate-reducers up to a depth of ~ 30 mbsf with maximum gene copy numbers at 20 mbsf (Fig. 3 C) in the sediment layer in which a decline of the porewater sulfate concentration and an increase of the total

organic carbon concentration was observed (Fig. 2). However, it cannot be clearly discriminated between sulfate reduction and/or freshwater intrusion at this depth as mentioned above. The second functional gene *aprA* (adenosine5-phosphosulfate reductase gene) of this group was detectable in a few samples only. The gene *mcrA* of methanogens was not detectable. The lack of methanogens is in accordance with the virtual absence of methane in the uppermost 50 mbsf (van Geldern et al., 2013) and the low total organic carbon content.

The copy numbers of the functional gene *cbbL* encoding for the large subunit of ribulose 1.5-bisphosphate carboxylase/oxygenase (Rubisco) of some autotrophic microorganisms (Fig. 3 C) were about two orders of magnitude lower than the bacterial 16S rRNA gene copy numbers and occurred only in a distinct depth range of 15 to 25 mbsf. This gene was previously also detected in low numbers in marine sediments of the Black Sea and the Benguela upwelling system off the Atlantic coast of Namibia (Schippers et al., 2012). A higher abundance of the same *cbbL* gene than in this study was detected in the organic carbon-poor terrestrial subsurface sediments in the Chesapeake Bay area, Virginia, USA (Breuker et al., 2011). Only targeting *cbbL* in our study does not reflect a comprehensive analysis of autotrophs, likely other CO_2-fixing enzymes than Rubisco are more important in this environment. The data just show that autotrophs exist, considering that marine sediments are dominated by heterotrophs (Parkes et al., 2000; Biddle et al., 2006).

Archaeal diversity

The phylogenetic analysis of *Archaea* from four depths (8.9 mbsf, 10.2 mbsf, 13 mbsf and 16.7 mbsf) of site M0027 was carried out via clone libraries and 16S rRNA gene sequencing. For each depth 124 -162 different clones were achieved. The clones could be allocated to the phyla *Thaumarchaeota*, *Euryarchaeota* and *Crenarchaeota* with an overall mean abundance of 1 %, 14 % and 85 %, respectively. Richness estimators CHAO 1 and ACE for a 3% OTU level are given in Table 3 and reveal a decreasing diversity with depth. The composition of the microbial community of all 16S rRNA gene sequences as percentage of all received 16S rRNA gene sequences is displayed in Fig. 4 A. Trees for euryarchaeotal and crenarcheaotal 16S rRNA gene sequences are shown in Fig. 5 and Fig. 6.

The overall abundance of crenarchaeotal 16S rRNA gene sequences in the clone libraries was 70%-100% (Fig. 4 A). Within the *Crenarchaeota,* the Miscellaneous Crenarchaeotic Group (MCG) was the dominant one. In addition to the MCG group, the former group C3 (included into the MCG group by Kubo et al., 2012) is the only other group found in a depth of 16.7 mbsf. The MCG is found in a wide range of habitats (Vetriani et al., 1999). In subsurface sediments the MCG may utilize buried organic carbon and is often found but not restricted to anaerobic sediments (Biddle et al., 2006; Durbin and Teske, 2012). Kubo et al. (2012) concluded that MCG are not likely to be methanotrophs which is in accordance with the absence of methane and the gene *mcrA* of methanotrophs in the New Jersey shallow shelf sediments in the upper ~ 50 mbsf (van Geldern et al., 2013). Recent results by Lloyd et al. (2013) showed that MCG are among the most numerous archaea in subsurface marine sediments. Single-cell genomic sequencing of one cell of MCG revealed encoding of extracellular protein-degrading enzymes indicating that MCG play a role in protein remineralization in anoxic sediments.

The intragroup diversity of the achieved 16S rRNA gene sequences belonging to the MCG group was high. After grouping 16S rRNA sequences with 3% similarity to clone groups, 105 different clone groups remained. The depth-dependent distribution of all MCG related 16S rRNA gene sequences is displayed in Fig. 4 B. No 16S rRNA sequences of the New Jersey shallow sediment could be allocated to the groups MCG 1, 4, 5, 7, 11 and 16 (Kubo et al., 2012). The proportion of 16S rRNA gene sequences of MCG 8 was increasing with depth whereas the proportion of 16S rRNA gene sequences of MCG 3 was decreasing with depth (Fig. 4 B). Few 16S rRNA gene sequences belonging to MCG 2 were only found at 8.9 mbsf and one sequence belonging to MCG 6 could only be detected at 10.2 mbsf. Also 16S rRNA gene sequences belonging to MCG 9 and MCG 10 could only be detected at 10.2 mbsf and 16.7 mbsf in low abundance. The groups MCG 12, MCG 13 and MCG 14 were weakly represented at all depths with a maximum abundance at 13 mbsf. 16S rRNA gene sequences allocated to MCG 15 (former group C3) occurred similarly frequent at 8.9 mbsf and 13 mbsf (12.6% and 14.3% of all sequences, respectively). This group was represented at 10.2 mbsf with somewhat lower abundance (7.8%) and at 16.7 mbsf with only 1.6% of the whole MCG related sequences. Group MCG 17 was represented with a sequences abundance of 2% - 7% with a maximum at 10.2 mbsf.

Although the biogeochemical features of the MCG are unknown, this study illustrates a depth dependent distribution of the MCG and its subgroups and therefore contributes ecological information about the co-occurrence with well described deep subsurface archaeal lineages described below.

The crenarchaeotal Marine Benthic Group B (MBG-B) was only found at 8.9 mbsf, the shallowest analyzed depth. Teske and Sørensen (2008) described that MBG-B were detected amongst other *Archaea* by several researchers in different habitats such as deep marine sediments, coastal and intertidal sediments as well as hydrothermal vent sites. They also pointed out the fact that MBG-B archaea were found in some studies in correlation within the methane-sulfate transition zone where they may benefit directly or indirectly from anaerobic methane oxidation. Biddle et al. (2006) showed that such zones are dominated by MBG-B and MCG archaea. Due to the fact that methane is virtually absent in the here studied sediment interval up to ~ 50 mbsf (van Geldern et al., 2013), a contribution to methane oxidation by MBG-B and MCG cannot be inferred.

Some sequences belonging to the deeply branching Terrestrial Hot Spring Crenarchaeotal Group (THSCG) were detected as well at 8.9, 10.2 and 13 mbsf. The THSCG was originally named by Takai & Horikoshi (1999) and divided in two subgroups (I, II). The groups included 16S rRNA gene sequences from a hydrothermal field in the Okinawa trough and 16S rRNA gene sequences from a hot spring area in the Yellowstone National Park, Wyoming (Barns et al., 1994) and were not found to be monophyletic. The polyphyletic structure was confirmed by the addition of 16S rRNA gene sequences from a sulfide chimney on the Juan de Fuca Ridge (Schrenk et al., 2003). The clusters of the THSCG of our study do not show a close relationship to any of these previously described 16S rRNA sequences. The closest related neighbors of the New Jersey shallow shelf 16S rRNA gene sequences are derived from different locations such as White Oak river sediments (acc. nr. JN605142, Kubo et al, 2012), a tropical marine sediment (acc. nr. JQ258758, Ratnagiri, Arabian Sea coast, unpublished) and hypersaline groundwater (acc. nr. JF747774, Manantial del Toro, Dominican Republic, unpublished).

Regarding the phylum *Thaumarchaeota*, some 16S rRNA gene sequences belonging to Marine Group 1 α (MG 1) could be exclusively found at 13 mbsf (Fig. 4 A, Fig. 6). The detected 16S rRNA gene sequences are closely related to the subgroup MG 1 α and therefore to *Nitrosopumilus maritimus*, the first cultivated

ammonia-oxidizing archaeum (Könnecke et al., 2005). The MG 1 was described by Massana et al. (2000) and subsequently expanded into several subgroups (Sørensen et al., 2004; Takai et al., 2004; Durbin and Teske, 2010). This group is usually found in the water column or at the sediment surface. However, MG-I archaea also appear occasionally in deep sediment samples where they possibly represent seawater contamination (Inagaki et al., 2006; Durbin and Teske, 2012).

Regarding the *Euryarchaeota*, their overall percentage of all sequences was highest at 13 mbsf. Interestingly, at a depth of 16.7 mbsf euryarchaeotal 16S rRNA gene sequences were not detected. The major part within the *Euryarchaeota* was formed by the Marine Benthic Group D / Deep Hydrothermal Vent Euryarchaeotal Group 1 (MBG-D / DHVEG-1). MBG-D is not found in the water column and seems to be benthic, sediment dwelling archaea (Teske and Sorensen, 2008). Recent results by Lloyd et al. (2013) showed that MBG-D are among the most numerous archaea in subsurface marine sediments. Single-cell genomic sequencing of three cells of MBG-D revealed encoding of extracellular protein-degrading enzymes indicating that MBG-D play a role in protein remineralization in anoxic sediments, as discussed above for MCG (Lloyd et al., 2013).

Some sequences of small subgroups were found in particular depths (Fig. 4 A): 16S rRNA gene sequences of the small euryarchaeotal groups belonging to the Thermoplasmatales, namely AMOS1A-4113-D04 and Ant06-05, were detected at 8.9 mbsf. 16S rRNA gene sequences of the AMOS1A-4113-D04 group were first of all isolated from a continuous flow bioreactor containing anaerobic methanotrophic archaea. Other representatives of this group originate from deep sea hydrothermal vents, the Kazan mud Volcano in the Eastern Mediterrean Sea, the Western Pacific Ocean or the Madovi estuary sediment (west coast of India). Concerning the group Ant06-05, the first isolation source was the Nankai Trough (Japan). Other related 16S rRNA gene sequences were derived from a hot spring in Kamchatka (Russia).

16S rRNA gene sequences of group 20a-9 were found at 10.2 mbsf and 13 mbsf. At 10.2 mbsf one sequence of group 20c-4 could be detected whereas at 13 mbsf one sequence of group CCA47 was found. These groups comprise few sequences too but related 16S rRNA gene sequences are derived from very different isolation sources all over the world. Group 20c-4 contains 16S rRNA gene sequences from sediments of the Aegean Sea (Greece), the Southern Okinawa Trough, salt marsh sediments and a natural gas field. Group CCA47 contains 16S

rRNA gene sequences from oxygen depleted marine environments, methane seep sediments, marine sediments from the tropical Western Pacific or methane seep influenced sediments off Taiwan. 16S rRNA gene sequences of group 20a-9 are derived from different marine sediments (Yonaguni Knoll IV at Southern Okinawa Trough, Aegean Sea, Yung-An Ridge (Taiwan), anoxic hypersaline sediment at Salton Sea (California), Lake Taihu (China) and estuary sediments (Madovi, India).

The small Group SM1-K20 is related to the Deep Sea Euryarchaeotal Group (DSEG), which is also commonly found in deep marine sediments. The third euryarchaeotic lineage comprises the widespread South African Gold Mine Euryarchaeotal Group (SAGMEG) and its small sister group 20a-9. The SAGMEG group spans over a wide range from terrestrial to marine environments (Takai et al., 2001, Inagaki et al., 2003; 2006).

UniFrac analysis

The UniFrac analysis (Fig. 7) revealed that ~ 46 % variation can be explained by clustering the samples from 8.9 mbsf, 10.2 mbsf and 13 mbsf together versus the sample from 16.7 mbsf. 29 % variation can be explained by clustering the samples of the two upper depths against the samples of the two deeper depths. Interestingly, another 25 % variation can be explained by clustering the samples of the depths of 8.9 mbsf, 13 mbsf and 16.7 mbsf versus the sample of 10.2 mbsf. The main variation explained that P1 correlates well with lower amounts of TOC, manganese, iron, ammonium and calcium. It also correlates with higher amounts of chloride, bromide, sodium, boron, lithium, strontium, potassium and magnesium in the pore water (data from Mountain et al. 2010, not shown). Concerning the lithological features P1 also correlates with the amounts of dolomite/ankerite, kaolinite, pyrite, siderite and total sulfur. P2 correlates with the amounts of sulfate and phosphorus in the pore water with (in relative terms) higher sulfate and lower phosphorus values for the upper samples and the absence of kaolinite and chlorite for the upper samples. P3 correlates with calcite and total inorganic carbon. Overall, the UniFrac analysis data indicate that the observed differences in the microbial community composition go along with variations between the four samples based on their mineralogical and geochemical properties.

In summary, the microbial community in deeply buried marine sediments of the shallow shelf off New Jersey, USA, is characterized by comparably low total cell counts reflecting the oligotrophic-mesotrophic nature of the sediments. *Archaea* and *Bacteria* occurred in similar 16S rRNA gene copy numbers, except in the lowermost part of the analyzed sediment at 40 to 50 mbsf where *Bacteria* dominated. Highly abundant were the bacterial candidate division JS1 and the classes *Anaerolineae* and *Caldinilineae* of the *Chloroflexi* as well as sulfate reducers. The abundance of the Fe(III)- and Mn(IV)-reducing group *Geobacteraceae* correlated with concentrations of manganese and iron in the pore water. Sulfate, Fe(III)- and Mn(IV)-reduction but not methanogenesis seem to be important biogeochemical processes in the here studied depth range. Typical deep subsurface sediment associated archaeal groups such as MBG-B, MBG-D, MCG, and SAGMEG are well represented in the microbial community, with MCG as the most dominant, highly diverse group. The subgroup MCG 8 tends to increase in sequence abundance with depth while MCG 3 decreases in sequence abundance with depth.

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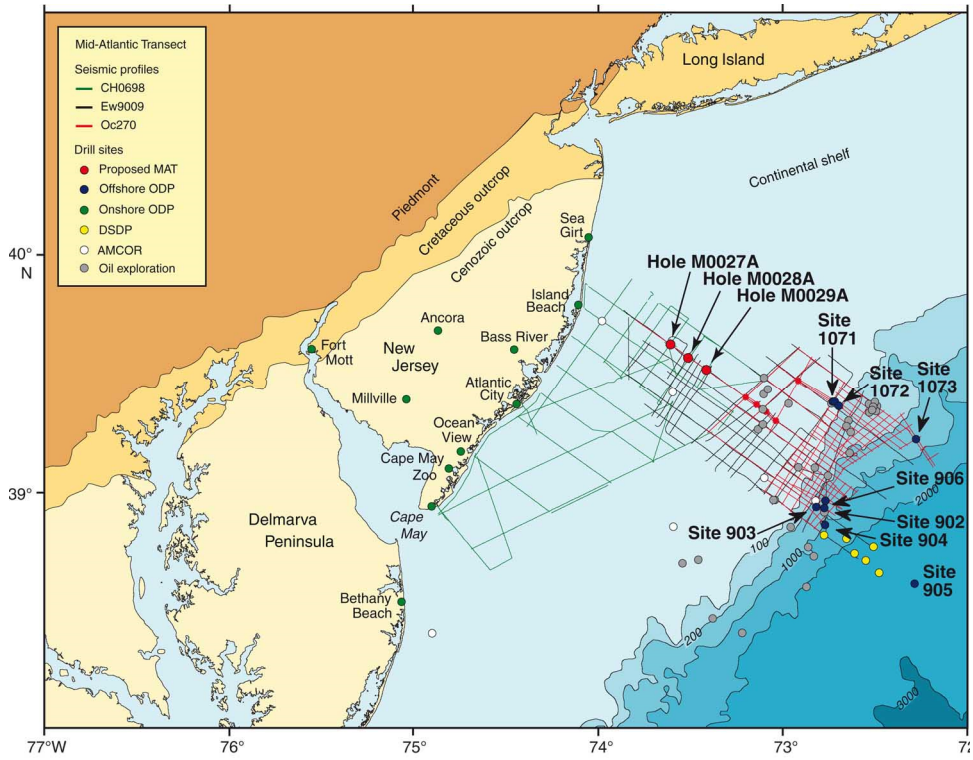


Figure 1: Map of the New Jersey shallow shelf showing site M0027 (hole M0027A) along with other completed boreholes as well as tracks of reconnaissance seismic lines (from Mountain et al., 2010).

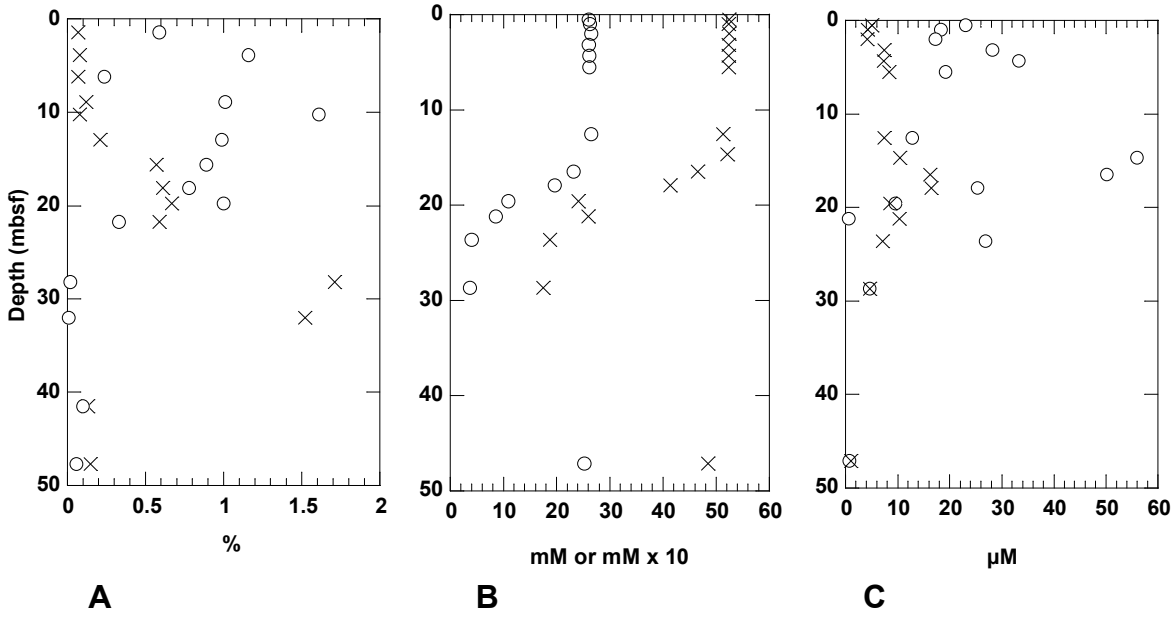


Figure 2: Geochemistry of New Jersey shallow shelf sediments of Site M0027. A: Concentrations of total organic carbon (TOC, x, %) and total inorganic carbon (TIC, o, %); B: Interstitial water concentrations of chloride (x, mM x 10); sulfate (o, mM); C: Interstitial water concentrations of manganese (x, µM) and iron (o, µM). Data from Mountain et al. (2010).

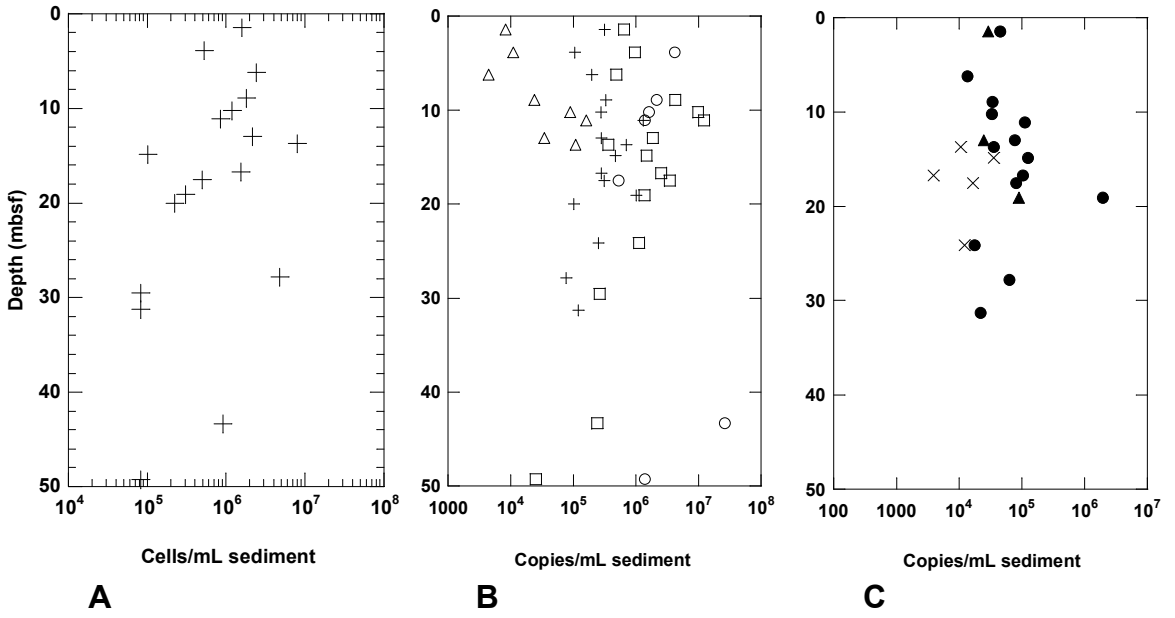
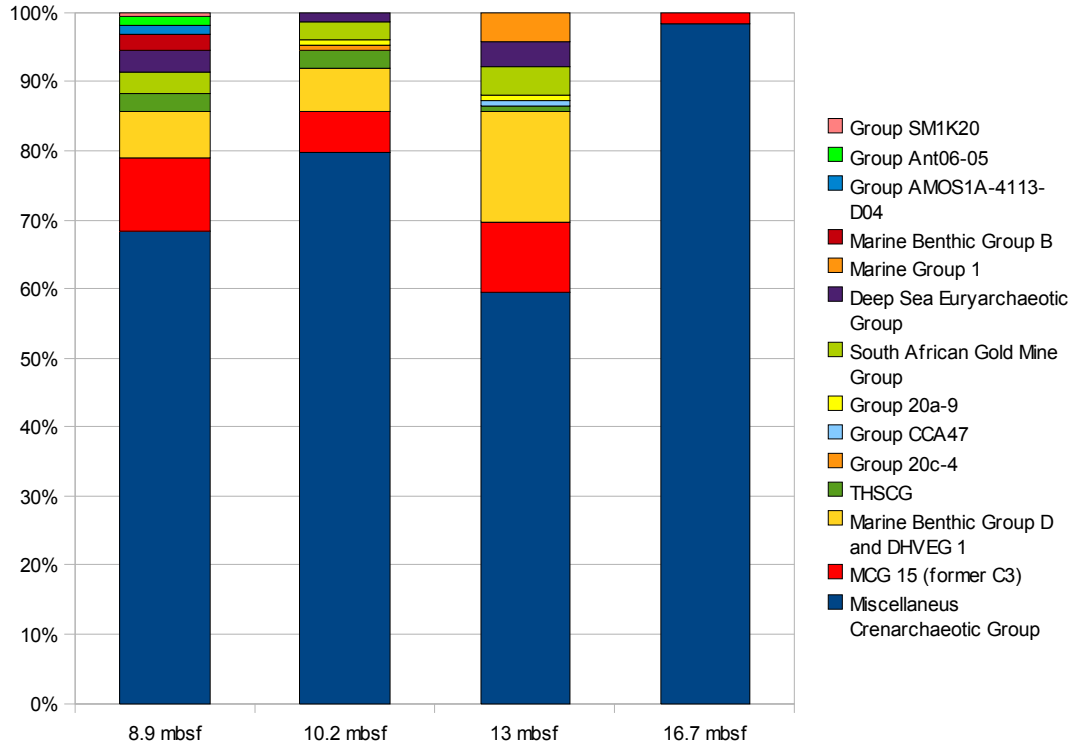
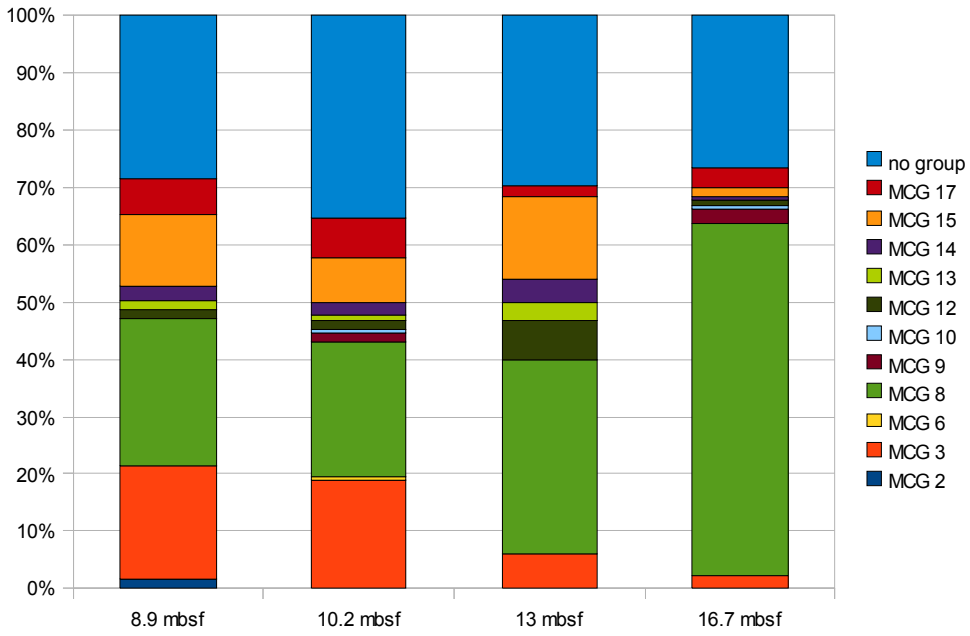


Figure 3: Total cell counts and real-time PCR (qPCR) data of New Jersey shallow shelf sediments of site M0027. A: Total cell counts; B: Quantification of 16S rRNA genes of different phylogenetic groups, *Bacteria*, o; *Archaea*, □; *Geobacteraceae*, Δ; JS1 and *Chloroflexi*, +; C: Quantification of functional genes of sulfate reducers (*apr,* ▲; *dsr,* ●) and autotrophic microorganisms (*cbbL*, x).

Figure 4



A



B

Figure 4: Diversity of the archaeal community in New Jersey shallow shelf sediments of site M0027. A: Overview of the abundance of archaeal groups in the clone libraries from four depths; B: Abundance of subgroups of the Miscellaneous Crenarchaeotic Group.

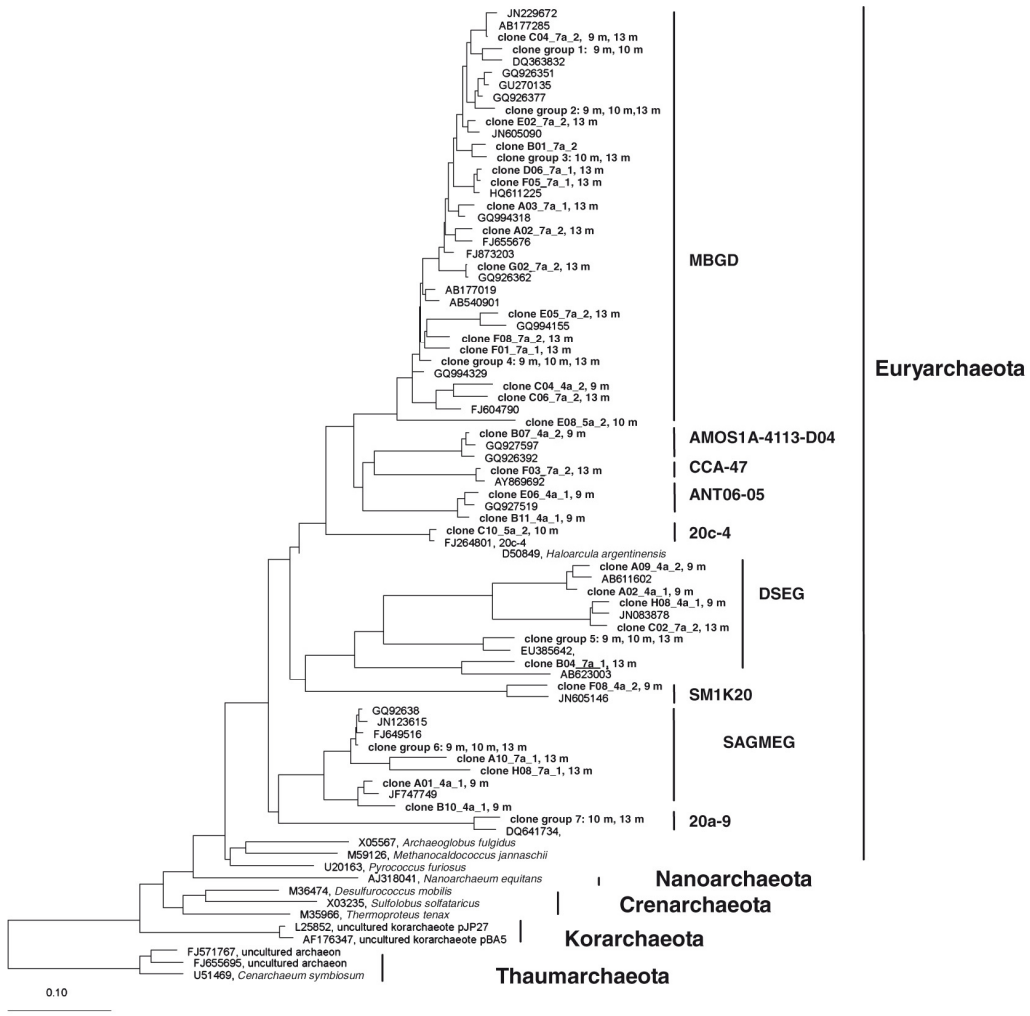


Figure 5: Maximum Likelihood phylogenetic tree of the *Euryarcheota* in New Jersey shallow shelf sediments of site M0027 from four depths.

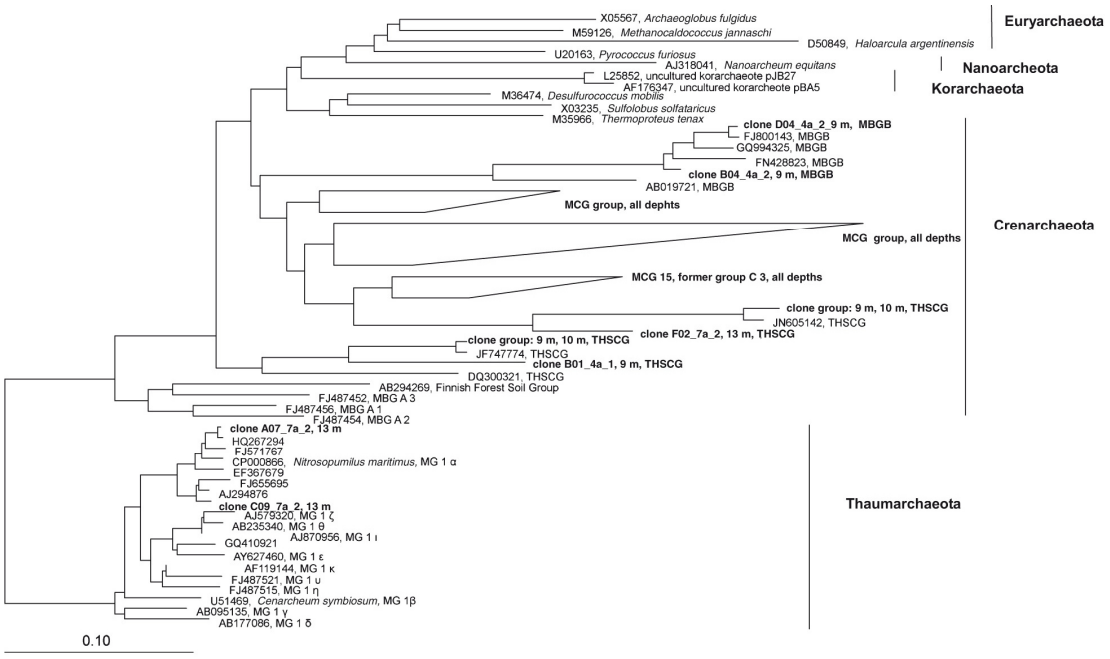


Figure 6: Maximum Likelihood phylogenetic tree of the *Crenarcheota* and *Thaumarchaeota* in New Jersey shallow shelf sediments of site M0027 from four depths.

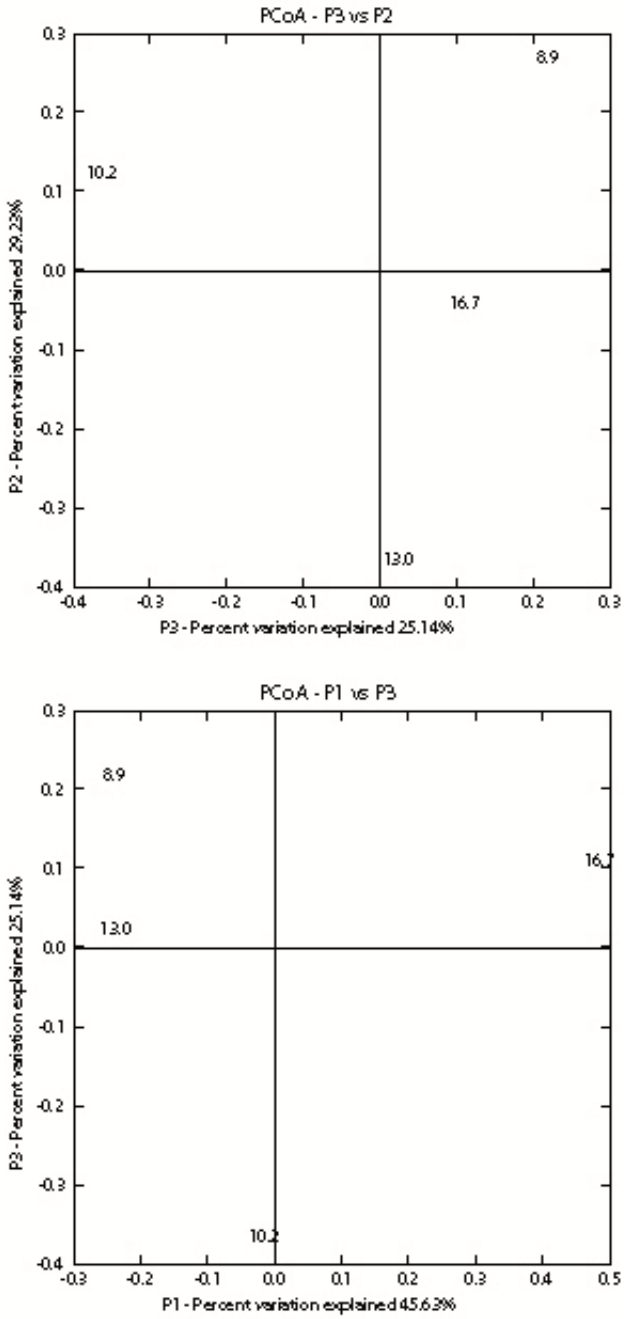


Figure 7: UniFrac analysis data.

Table 1 Table 1: Main lithology, total organic carbon (TOC in % w/w) and total cell counts (TCC in cells/mL sediment) for the uppermost 50 msbf (data are partly from van Geldern et al., 2013).

	depth below seafloor (m)	TOC	TCC		
sandy layer TOC mean: 0.13%	1.44	0.07	1.28E+06		
	3.87	0.08	6.65E+05		
	6.21	0.07	2.11E+06		
	8.94	0.12	2.00E+06		
	10.23	0.08	1.28E+06		
	11.1		9.43E+05		
	12.99	0.21	2.16E+06		
	13.71		8.06E+06		
sediment with thin clay layers (14.73,	14.86		1.02E+05		
	15.62	0.57			
15.33, 17.72 mbsf)	16.71		1.54E+06		
	17.55		4.99E+05		
and a thicker clay layer	18.13	0.61			
from 19.5 - 22.68 mbsf	19.09		3.05E+05		
	19.78	0.67			
Mean TOC: 0.61%	20.01		2.22E+05		freshwater intrusion
	21.77	0.59			
sediment with	24.13		2.77E+04		Mean TOC: 1.12%
thin clay layers (27.39,	27.84		4.80E+06		
27.98 and 28.15 mbsf)	28.18	1.71			
	29.53		8.32E+04		
	31.28		8.32E+04		
	31.99	1.52			
	41.48	0.13			
Mean TOC: 0.88%	43.37		9.15E+05		
	47.69	0.15			
	49.28		8.32E+04		

Table 2 Compilation of published mean total cell counts and qPCR abundance of *Bacteria* and *Archaea* in the depth range of 1–10 and 10–200 mbsf (meter below seafloor) in subsurface marine sediments (cells / mL sediment; nd: not determined).

Expedition/area	1 – 10 mbsf			10 – 200 mbsf			Reference
	Total counts	Bacteria	Archaea	Total counts	Bacteria	Archaea	
ODP Leg 201 Peru margin	10^7-10^8	10^7	10^4-10^7	10^7	10^6	10^3-10^5	Schippers et al. 2005
ODP Leg 201 Peru margin	10^7-10^8	> 90 %	< 10 %	10^7	> 99 %	< 1 %	Inagaki et al. 2006
ODP Leg 204 Cascadia margin	10^7	> 70 %	< 30 %	10^6	> 70 %	< 30 %	Inagaki et al. 2006
ODP / IODP	nd	~ 60 %	~ 40 %	nd	~ 60 %	~ 40 %	Lipp et al. 2008
IODP Exp. 301 Juan de Fuca	10^8-10^9	10^6-10^8	10^5-10^6	10^8	10^6	10^6	Engelen et al. 2008
IODP Exp. 307 Porcupine Seamount	nd	nd	nd	10^6-10^7	10^5-10^6	10^4-10^5	Webster et al. 2009
IODP Exp. 308 Gulf of Mexico	10^5-10^6	10^5-10^6	10^5	10^4-10^5	10^4	< 10^2	Nunoura et al. 2009
Sea of Okhotsk	10^6-10^7	10^4-10^5	< 10^4	10^6-10^7	10^4-10^5	< 10^4	Inagaki et al. 2003
North Sea tidal flat	10^7-10^8	10^7	10^6	nd	nd	nd	Wilms et al. 2007
SO189 Forearc off Sumatra	10^7-10^8	10^7-10^8	10^7-10^8	nd	nd	nd	Schippers et al. 2010
M72-5 Black Sea	10^7-10^8	10^5-10^6	10^5-10^6	nd	nd	nd	Schippers et al. 2012
M76-1 Benguela Upwelling	10^7-10^9	10^6-10^8	10^6-10^9	nd	nd	nd	Schippers et al. 2012
MSM11-1 “North Pond”	10^5-10^6	10^4	10^5-10^6	nd	nd	nd	Breuker and Schippers 2013
IODP Exp. 313 New Jersey shallow shelf	10^6	10^6	10^6	10^6	10^6	10^5-10^6	This study

Table 3: Richness estimators CHAO 1 and ACE for a 3% OTU level.

	8.9 m	10.2 m	13 m	16.7 m
Clones per sample	177	172	171	173
Chao 1	172	103	109	41
Clones in relation to CHAO 1 estimator	103 %	167 %	157 %	422 %
ACE	198	187	112	66
Clones in relation to ACE estimator	89 %	92 %	153 %	262 %

4.4. Microbial community stratification controlled by the subseafloor fluid flow and geothermal gradient at the Iheya North hydrothermal field in the Mid-Okinawa Trough (IODP Expedition 331)

Katsunori Yanagawa[1,2*], Anja Breuker[3], Axel Schippers[3], Manabu Nishizawa[4], Akira Ijiri[5], Miho Hirai[6], Yoshihiro Takaki[1], Michinari Sunamura[2], Tetsuro Urabe[2], Takuro Nunoura[6], and Ken Takai[1]

[1]Department of Subsurface Geobiological Analysis and Research (D-SUGAR), Japan Agency for Marine-Earth Science and Technology (JAMSTEC), Yokosuka, Japan
[2]Department of Earth and Planetary Science, University of Tokyo, Tokyo, Japan
[3]Geomicrobiology, Federal Institute for Geosciences and Natural Resources (BGR), Hannover, Germany
[4]Laboratory of Ocean-Earth Life Evolution Research (OELE), Japan Agency for Marine-Earth Science and Technology (JAMSTEC), Yokosuka, Japan
[5]Geomicrobiology Group, Kochi Institute for Core Sample Research, Japan Agency for Marine-Earth Science and Technology (JAMSTEC), Kochi, Japan
[6]Marine Functional Biology Group, Research and Development (R&D) Center for Marine Biosciences, Japan Agency for Marine-Earth Science and Technology (JAMSTEC), Yokosuka, Japan

*****Corresponding author: Katsunori Yanagawa, Department of Subsurface Geobiological Analysis and Research (D-SUGAR), Japan Agency for Marine-Earth Science and Technology (JAMSTEC), 2-15 Natsushima-cho, Yokosuka 237-0061, Japan. Tel.: +81 46 867 9717; fax: +81 46 867 9715; e-mail: kyanagawa@jamstec.go.jp

Running Title: Microbial ecology of a subseafloor hydrothermal field

Keywords: hydrothermal recharge zone, Marine Group I, Hot Water Crenarchaotic Group IV (HWCGIV), subseafloor biosphere, IODP Expedition

ABSTRACT

The impacts of lithological structure and geothermal gradient on subseafloor microbial communities were investigated at a marginal site of the Iheya North hydrothermal field in the Mid-Okinawa Trough. Subsurface marine sediments composed of hemipelagic muds and volcaniclastic deposits were recovered through a depth of 151 m below the seafloor at Site C0017 during the Integrated Ocean Drilling Program Expedition 331. Microbial communities inferred from 16S rRNA gene clone sequencing in low-temperature hemipelagic sediments were mainly composed of members of *Chloroflexi* and Deep Sea Archaeal Group. In contrast, 16S rRNA gene sequences of Marine Group I *Thaumarchaeota* dominated the microbial phylotype communities in the coarse-grained pumiceous gravels interbedded between the hemipelagic sediments. Based on the physical properties of sediments such as temperature and permeability, the porewater chemistry and the microbial phylotype compositions, the shift in the physical properties of the sediments is suggested to induce a potential subseafloor recharging flow of oxygenated seawater in the permeable zone, leading to the generation of variable chemical environments and microbial communities in the subseafloor habitats. In addition, the deepest section of sediments under high-temperature conditions (~90˚C) harbored the sequences of an uncultivated archaeal lineage of Hot Water Crenarchaeotic Group IV that may be associated with the high-temperature hydrothermal fluid flow. These results indicate that the subseafloor microbial community compositions and functions at the marginal site of the hydrothermal field are highly affected by the complex fluid flow structure, such as recharging seawater and underlying hydrothermal fluids, coupled with the lithologic transition of sediments.

INTRODUCTION

Numerous scientific expeditions have investigated the marine subsurface biosphere via molecular biological analyses targeting 16S rRNA and functional genes as well as by metagenomics, metatranscriptomics, microscopic analyses, metabolic activity measurements and cultivation experiments (15, 45, 66). These previous studies demonstrated that the subseafloor biosphere is composed of a vast number of microbial cells, including uncultivated, phylogenetically diverse and physiologically unknown components. Subseafloor microbiology over the past two decades indicates that buried organic matter is the most important energy and carbon source in deep

subsurface environments that affects microbial abundance and the community composition (23, 45, 47). Thus, organic-rich subseafloor sediments of continental margins and the eastern equatorial Pacific Ocean harbor a larger microbial biomass (3, 8, 19, 48) than oligotrophic sediments, where an extremely low microbial cell abundance is observed (23). On the other hand, the lithologic control of subseafloor microbial community development has also been suggested (20, 47) as the physical properties of sediments, such as porosity and permeability, highly affect the subseafloor hydrogeologic structures and the spatial, energetic and nutritional habitability of the subseafloor microbial community (5, 7, 52).

Subseafloor hydrothermal fluid flow regimes have been predicted to provide spatially expansive and physicochemically variable habitats for the phylogenetic and functional diversity of microorganisms (10, 43, 58, 59, 61). The mixing of high-temperature reduced hydrothermal fluids and low-temperature interstitial fluids forms a wide range of physical and chemical gradients in the subseafloor environment. Currently, the variability of 16S rRNA gene phylotype communities has been investigated in several deep-sea hydrothermal sediments (11, 39, 42, 65, 69). Although the hydrothermal fluid discharging zones around active hydrothermal systems have been established to often host the local recharge flows of oxygenated deep-sea water (13, 27), the relationship between microbial community development and the physico-chemical conditions influenced by the hydrothermal discharging and recharging fluid flows in the subseafloor environments remains poorly understood.

In this study, we sought to determine the pattern in subseafloor microbial community development along with the lithostratigraphic transition and physico-chemical gradient in a deep-sea hydrothermal system, the Iheya North Knoll in the Mid-Okinawa Trough, during the Integrated Ocean Drilling Program (IODP) Expedition 331 using the D/V *Chikyu* (63). The drilling and coring operations at IODP Site C0017 located at the margin of the hydrothermal field indicated the potential recharge flow of low-temperature seawater into the subseafloor sediments, which was likely caused by the complex hydrogeologic structure and the underlying high-temperature hydrothermal fluid flow (63). The anomalously low heat flows around Site C0017 estimated from temperature gradients of surface sediments suggested the zonation of seawater recharge (34), and the downhole temperature measurement during IODP Expedition 331 showed a concave downward temperature profile (Fig. 1A), which indicated the lateral flows of recharged seawater in particular lithological

layers of coarse-grained pumiceous gravels and breccias (63). The oxygenated seawater flows in the porous layers would supply relatively abundant electron acceptors to the anoxic subseafloor sedimentary habitats. The lithostratigraphic transition between hemipelagic sediments and pyroclastic deposits has been predicted by the seismic reflection signals at Site C0017 (63, 67). In deeper sections of sediments at Site C0017, a higher temperature gradient was found by the downhole temperature measurement during IODP Expedition 331, and the temperature in the deepest part was estimated to be 90°C (63). Here, we report the variability of subseafloor microbial phylotype communities and functional gene distribution (*dsrA*, *aprA* and *amoA*) in the sedimentary habitats influenced by the hydrogeologic structure and the temperature gradient near a deep-sea hydrothermal system.

MATERIALS AND METHODS

Site description and sediment sampling

IODP Expedition 331 was conducted at the Iheya North hydrothermal field in the Mid-Okinawa Trough using the D/V *Chikyu* in September 2010 (63). IODP Site C0017 was located at 1550 m east of the hydrothermal activity center of the Iheya North field and was covered with thick terrigenous sediments, hemi-pelagic sediments and pumiceous deposits (63). Coring operations retrieved sediments down to 151 m below the seafloor (mbsf). The extended shoe coring system (ESCS) were used for the coring at 95.0, 108.2, 130.1, 141.1 mbsf and the hydraulic piston coring system (HPCS) were used for the rest of the cores (63). Core samples collected at Site C0017 were composed of pumiceous volcaniclastic gravels, breccias and hemipelagic mud and were lithostratigraphically classified into four units (63). The upper Unit I (0 to 18.5 mbsf) was predominantly composed of hemipelagic mud, and Units II and III (19.1 to 36.2 and 61.1 to 78.8 mbsf, respectively) consisted of pumiceous gravel-dominant layers with minor hemipelagic mud and volcaniclastic sediment. Sections from 36.2 to 61.1 mbsf and 78.8 to 94.3 mbsf were not recovered. The deepest section (Unit IV), a cored interval from 94.3 to 144.7 mbsf, was dominated by hemipelagic mud. *In situ* temperatures at Site C0017 were measured using an advanced piston corer temperature tool (APCT-3) and thermoseal strips (Nichiyu Giken Co., Ltd., Kawagoe, Japan) (Fig. 1A).

The retrieved cores were immediately cut into 1.5-m-long sections on deck, and whole round cores (WRCs) for microbiological study (approx. 10-20 cm in length) were then subsampled from the short sections. The microbiological samples were obtained from the inner parts of the WRCs with a sterilized spatula and immediately stored at -80°C in heat-sealed laminated foil bags containing an oxygen scavenger. The subsamples for geochemical analyses were collected from the sections juxtaposed to the WRCs for microbiology.

Geochemical analysis

Porewater was obtained from 10- to 20-cm-long WRCs; the total alkalinity and the ammonium, sulfate and methane concentrations were determined previously (63). The nitrate concentration was measured by ion chromatography using a high-capacity anion exchanger (TSK-gel SAX column, Tosoh) with UV detection (LC-10Ai and SPD-10A, Shimadzu) (33, 40). The lower detection limit of the nitrate concentration was 0.3 μM, and the reproducibility was better than 10%. Subsamples for dissolved organic carbon analysis were stored frozen at -20°C in precombusted 10-ml glass vials, each with a Teflon-coated septum and screw caps. The acetate concentration and stable carbon isotopic composition ($\delta^{13}C_{acetate}$) were determined by isotope ratio monitoring-liquid chromatography-mass spectrometry, as previously described (18).

DNA extraction and 16S rRNA gene clone analysis

DNA was extracted from approximately 2 g of the frozen innermost parts of the WRCs using the PowerMAX Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA) according to the manufacturer's protocol, with minor modifications. A blank water sample was also used simultaneously as a negative control during the DNA extraction process. The 16S rRNA gene fragments were amplified by PCR using an universal primer set, Uni530F-907R (44), for all samples, and an archaea-specific primer set composed of Arch_530F, Arch2_530F, Nano_530F (44) and Arc958R (9) was used for samples in which indigenous microbial populations were not detected using the universal primer set. PCR amplification with LA Taq polymerase (TaKaRa Bio Inc., Otsu, Japan) was performed using the following cycle conditions: 40 cycles of denaturation at 96°C for 25 s, annealing at 50°C for 45 s, and extension at 72°C for 60 s. PCR amplification of the negative control for DNA extraction was used to

assess experimental contamination. Cloning and sequencing of the PCR products were performed as described previously (62). Approximately 400-bp 16S rRNA gene sequences with more than 97% sequence identity were assigned to the same phylotype. Representative sequences were aligned using the SINA aligner (51). Phylogenetic affiliations were identified by the maximum parsimony method using the SILVA SSU Ref 111 Database in the ARB software program (32). The same method was previously applied to the drilling fluids, and the 16S rRNA gene sequences from the WRCs displaying greater than 97% identity with those of the drilling fluids were defined as potential contaminants (70). The 16S rRNA gene clone libraries were compared by Jackknife environment cluster analysis and principal component analysis (PCA) in the UniFrac program (http://bmf.colorado.edu/unifrac/) (31).

Archaeal *amoA* gene clone analysis

The PCR amplification of *amoA*, encoding ammonia monooxygenase subunit A, was conducted using the primer set of Arch-amoAF and Arch-amoAR (14) and Ex Taq polymerase (TaKaRa Bio Inc., Shiga, Japan) with Mg^{2+} buffer, as previously described (41). The amplification condition was 40 cycles of denaturation at 96°C for 25 s, annealing at 52°C for 30 s and extension at 72°C for 60 s. The PCR products were cloned and sequenced as described above. Sequences presenting ≥95% identity were assigned to the same phylotype. Representative sequences were aligned with closely related *amoA* gene sequences deposited in public databases using the CLUSTALW program, and the ambiguous nucleotide positions were corrected manually. Phylogenetic affiliations were assigned based on phylogenetic trees constructed by the neighbor-joining method in the ARB software. Bootstrap analysis was performed with 1000 replicates.

Quantitative fluorescent PCR

Quantitative fluorescent PCR (Q-PCR) for 16S rRNA genes was performed as described previously (4, 6, 71). Copy numbers of the 16S rRNA genes were determined using a universal primer-probe set (60), an archaea-specific primer-probe set (60) and a bacteria-specific primer-probe set (37). Functional genes *dsrA* and *aprA*, which encode dissimilatory sulfite reductase and adenosine 5'-phosphosulfate reductase subunit A, respectively, were quantified as described elsewhere (4, 54), using specific primer sets (28, 35). The primers and probes in this study are

summarized in Table 1. After each Q-PCR, melting curves were measured for SYBR Green I assays. The sizes of the PCR products were further confirmed by gel electrophoresis. All Q-PCR assays were run in triplicate.

Nucleotide sequence accession numbers

The nucleic acid sequences obtained in this study have been deposited in the DDBJ/EMBL/GenBank databases under the following accession numbers: AB824899-AB825952 (16S rDNA) and AB936820-AB936831 (*amoA*).

RESULTS

Thermal and geochemical variation associated with sediment lithology

At IODP Expedition 331 Site C0017, located 1550 m east of the activity center of the Iheya North hydrothermal field, *in situ* temperature measurements did not show a significant temperature change in Units I and II, whereas a marked temperature increase was observed in Unit III; the temperature at 69 mbsf was 25°C (Fig. 1A). At the deepest section of Unit IV, the temperature increased up to 90°C.

In the Unit I layer, which mainly consisted of hemipelagic sediments (Fig. 1B and C), the porewater alkalinity and ammonium concentration increased from 3.2 to 7.3 mM and from 0.02 to 0.45 mM, respectively, whereas the sulfate concentration slightly decreased as the depth increased (Fig. 2). The nitrate concentration was less than 2 μM throughout the unit. These geochemical features were indicative of a typical anaerobic sedimentary environment, where oxygen was presumably consumed by microbial respiration within the uppermost sediment. Units II and III consisted of coarse-grained porous volcaniclastic pumiceous deposits (Fig. 1B and C). The Unit II layer presented an inverse trend of alkalinity and ammonium and sulfate concentrations compared with Unit I (Fig. 2). Furthermore, the nitrate concentration significantly increased up to 34 μM at the bottom of the unit. The nitrate concentration and alkalinity value are similar to those in the deep seawater of the East China Sea (ca. 38 μM for nitrate and 2.5 mM for alkalinity) (25, 36). Hence, the chemical characteristics suggest that porewater with little influence of early diagenesis of infiltrated seawater exists in coarse-grained pumiceous gravels and breccias localized at 26.6-30.0 mbsf in Unit II. Given the anomalously low thermal gradient in this area (34), a lateral flow would be caused by recharging of the oxygenated and low-temperature bottom seawater. At the bottom of this hole (Unit

IV), we observed no apparent chemical signature for hydrothermal fluid input in the porewater chemistry. Throughout the sediment column, the methane concentrations were quite low, mostly below 1 μM (63).

Acetate is a key intermediate substance of various microbial metabolic pathways in anaerobic environments (29). Biogeochemical processes via acetate in subseafloor sediments at Site C0017 were examined by the stable carbon isotopic analysis of acetate in the porewater (Fig. 2). The acetate concentration highly fluctuated, possibly due to the difference in sediment lithology. High acetate concentrations of up to 80.6 μM were detected at certain depths in Units I, II and III, where the sediment is mostly hemipelagic clay. In contrast, the concentration in the layers of volcaniclastic sand ranged from 8.2 to 22.6 μM. A similar variation was also found in the total organic carbon content (63). Throughout the sediment column, the isotopic composition of acetate ($\delta^{13}C_{acetate}$) ranged from -37.6‰ to -32.2‰. The local maximal stable carbon isotopic compositions of porewater acetate were observed at the interface of Units I and II.

Total cell counts and Q-PCR analysis

The microbial cell abundance at Site C0017 decreased logarithmically with sediment depth from 3.2×10^7 cells per ml of sediment at 0.7 mbsf to less than the detection limit of approximately 6.5×10^5 cells per ml of sediment at 68 mbsf (63). Similar depth profiles were obtained from the 16S rRNA gene-targeted Q-PCR analysis (Fig. 3A). The 16S rRNA gene copy number ranged from 8.8×10^5 to 8.7×10^7 genes g^{-1} sediment for total prokaryotes, from 5.7×10^4 to 2.7×10^7 genes g^{-1} sediment for bacteria and from 6.4×10^4 to 8.9×10^6 genes g^{-1} sediment for archaea. The highest cell count and 16S rRNA gene number were detected at a depth of 6.4 mbsf. To reveal the spatial distribution of particular physiological microbial groups, we quantified the copy numbers of functional genes *dsrA* and *aprA* for potential sulfate reducers that encoded dissimilatory sulfite reductase and adenosine 5'-phosphosulfate reductase subunit A, respectively. They were less abundant than the 16S rRNA genes but detectable in most of the samples (Fig. 3B).

16S rRNA gene phylotype community

In the shallow depths of Unit I above 14.8 mbsf, the 16S rRNA gene phylotype communities were dominated by previously uncultivated sequences of typical

subseafloor lineages. At 6.4mbsf, bacterial phylotypes affiliated with phylum *Chloroflexi* comprised 27.1% in the 16S rRNA gene clone library (23/86 clones) (Fig. 4). Members of the Deep Sea Archaeal Group (DSAG), also referred to as Marine Benthic Group B (MBG-B), accounted for 26.4% (23/87 clones) at 0.7 mbsf. The 16S rRNA gene communities in Unit II, except for those from the upper depth (20.1 mbsf), were markedly dominated by members of Marine Group I (MG-I) *Thaumarchaeota* and *Alphaproteobacteria* (Fig. 4). Archaeal sequences belonging to the Miscellaneous Crenarchaeotic Group (MCG) were predominantly detected at depths of 63.6 and 68.1 mbsf in Unit III. Data from the deep layer in Unit IV showed that the 16S rRNA gene communities using a universal primer set likely reflected highly biased compositions by external contamination rather than the indigenous compositions in these deep sedimentary habitats (data not shown). Due to the markedly low microbial cell abundances (Fig. 3A), these samples would be susceptible to microbiological contamination from the drilling fluid used for the operation during IODP Expedition 331 (70). As no detectable archaeal 16S rRNA gene sequences were obtained from the drilling fluid (70), we further constructed archaeal 16S rRNA gene clone libraries in the deep sediment samples of Unit IV. Notably, we found a drastic transition of the archaeal 16S rRNA gene phylotype composition in Unit IV. At a depth of 95.0 mbsf, sequences of South Africa Gold Mine Euryarchaeotic Group (SAGMEG) and AK8 predominated the archaeal phylotype composition, whereas Hot Water Crenarchaeotic Group IV (HWCGIV) were dominant at the depth of 141.1 mbsf, representing 29.0% of the clonal frequency (Fig. 4).

Based on the phylogenetic distance and abundance of each 16S rRNA gene phylotype, we compared differences in the microbial phylotype composition among all samples using UniFrac analysis. The results of the cluster analysis and PCA revealed the evident compositional variability among the 11 sediment samples (Fig. 5A and B). The microbial phylotype compositions in three samples from the permeable zone in Unit II were similar to one another and were significantly distinct from those in the other sample layers. The predominant phylotypes commonly found in the samples within this cluster were the members of MG-I *Thaumarchaeota* and *Alphaproteobacteria*, as described above. Similarly, the microbial phylotype compositions in the shallow sediments of Unit I were also closely related to one another.

Detection of archaeal *amoA* genes from the permeable pumice-rich layer

Previous studies of microbial communities in marine and soil environments showed that most of the MG-I *Thaumarchaeota* harbor the *amoA* gene, encoding ammonia monooxygenase subunit A, and can oxidize ammonia to nitrite (50). We could amplify archaeal *amoA* gene fragments from a permeable pumice-rich layer at a depth of 30.0 mbsf. Forty-three sequences of the archaeal *amoA* gene were evaluated and classified into 12 phylotypes. All the archaeal *amoA* phylotypes were phylogenetically related to sequences detected in ocean waters and sediments (Fig. S1 in the supplemental material). Co-occurrence of the MG-I 16S rRNA and archaeal *amoA* genes suggested the potential contribution of ammonia oxidation in the permeable layer, where the recharged seawater flows may have created the aerobic habitats.

DISCUSSION

Variation of microbial communities in the subseafloor sediments

In this study, microbial cell populations in subsurface marine sediments at Site C0017 were quantified using both microscopic observation and Q-PCR. The 16S rRNA gene numbers showed a pattern similar to the total cell counts, indicting a high reliability of the data produced by both quantification methods (Fig. 3A). Relatively low microbial cell abundances in the subseafloor sediments at this site, compared with the abundances in other subseafloor sedimentary habitats, would be explained by the relatively low productivity of the overlying oligotrophic ocean and the distance from land (23).

In the Unit I sediment, the increase of the porewater alkalinity and ammonium, the decrease of sulfate and the low nitrate concentration are considered as the result of anaerobic microbial processes (Fig. 2). Furthermore, it seems likely that the anaerobic microbial activity provides the ^{13}C enrichment of acetate at the interface of Units I and II. One conceivable explanation would be that slight carbon isotopic fractionation occurs during fermentative acetate production (17, 49). However, judging from the fact that the maximal stable carbon isotopic compositions of acetate are associated with the local minimum concentrations of acetate, the ^{13}C enrichment of acetate is a potential signature of microbial consumption as a substrate. This is supported by previous laboratory experiments with pure cultures of acetotrophic sulfate reducers, which showed that acetate was enriched in ^{13}C up to 19.3‰ (16).

The 16S rRNA gene phylotype communities in the Unit I sediments were dominated by the members of *Chloroflexi* and DSAG. Both are widely distributed in many deep-sea sediments and, in some cases, represent more than half of the 16S rRNA gene clone libraries (15). However, no representatives of marine subsurface *Chloroflexi* (*Dehalococcoidetes*) and DSAG have so far been cultured, and thus their metabolic pathways remain elusive. Currently, a single cell genomic approach for *Dehalococcoidetes* from marine subsurface sediments suggests they are strictly anaerobic organotrophs or lithotrophs (24, 68). The members of DSAG are considered to be involved in the biogeochemical cycling of organic carbon, iron oxide and/or manganese (21, 22).

The profiles of the *dsrA* and *aprA* gene copy numbers suggested that the relative abundance of sulfate-reducing bacteria was higher at shallower depths than in deeper sections (Fig. 3B), which was comparable with the findings of other sediments (4). Indeed, a current biogeochemical modeling of microbial sulfate reduction using the concentration and multiple sulfur isotopic compositions of porewater sulfate has strongly suggested the occurrence of active microbial sulfate reduction in the shallowest zone of sediments down to 20 mbsf (Unit I) (1). Correspondingly, the 16S rRNA gene phylotypes affiliated with *Deltaproteobacteria* were detected from sediments above 74.9 mbsf (Fig. 4). These results suggest that microbial sulfate reduction may be an important metabolic function that supports subseafloor microbial production in anaerobic hemipelagic mud habitats.

The 16S rRNA gene phylotype composition drastically changed in the deeper sediments, potentially influenced by the subsurface flow of recharged seawater in the permeable pumiceous zone. The cluster analysis and PCA also indicated that the physical and/or chemical properties associated with the lithological structure would differentiate the possible subseafloor microbial community composition responding to the lithostratigraphic transition (Fig. 5). A key component of the microbial phylotype composition in the permeable pumice-rich sediments was the predominance of the MG-I members in *Thaumarchaeota* (Fig. 4). Several studies have previously reported that thaumarchaeal phylotypes are frequently obtained from the aerobic and oligotrophic sediments (12) and presumably from the anoxic subsurface sediments (19, 21, 53). Although the dissolved oxygen concentration was not measured in this study, relatively high concentrations of nitrate in the permeable samples were indicative of the presence of dissolved oxygen (46) and were distinct from the typical

anaerobic sedimentary environments (Fig. 2). The chemical conditions would provide the feasibility of microbial ammonia oxidation in the permeable zone. Correspondingly, the members of MG-I *Thaumarchaeota* are known to possess the dissimilatory ammonia oxidation pathway (56). In fact, we successfully detected archaeal *amoA* genes from this zone (Fig. S1 in the supplemental material). The Q-PCR results also indicated the distinct composition of microbial components in the permeable layer. The comparison between archaeal and total 16S rRNA gene copy numbers revealed that the archaeal 16S rRNA gene abundance accounted for 25.4-39.5% of the total prokaryotic 16S rRNA gene abundance in the permeable layer, whereas the archaeal population remained as 4.11-24.6% of the total prokaryotic 16S rRNA gene population at any other sample depth. Thus, the distinct microbial community development in the permeable layer might be caused by the dominance of potential ammonia-oxidizing MG-I *Thaumarchaeota* in the possible indigenous microbial communities. Although another explanation (e.g., certain Q-PCR biases due to a few mismatches of the ARCH516 TaqMan probe with the archaeal phylotypes typically found in the anoxic subseafloor sediments (30, 66)) cannot be completely excluded, the estimated 16S rRNA gene abundances of MG-I *Thaumarchaeota* in the specific subseafloor habitats are an order of magnitude higher than the MG-I cell abundances in the ambient bottom seawater of the Iheya North hydrothermal field (55). Therefore, the MG-I *Thaumarchaeota* likely represent one of the predominant indigenous microbial populations in the specific layer rather than only being contaminants from the potentially recharged seawater.

Abundant incidence of HWCGIV in high-temperature sediments

Based on the microbial community surveys of hydrothermal mixing zones of habitats at the seafloor of the Iheya North hydrothermal field, metabolically diverse microbial communities (including psychrophilic to hyperthermophilic chemolithotrophs) were expected to be distributed abundantly and widely in the subseafloor environments beneath the hydrothermal field (38). However, the culture-dependent attempts for potentially indigenous microbial populations associated with hydrothermal activity (such as *Thermococcales*, *Aquificales* and *Epsilonproteobacteria*) were unsuccessful throughout the sediments at Site C0017 (63). The temperature in the deepest sample at 151 mbsf of up to 90°C was below the upper temperature limit of life (122°C) (64). The archaeal 16S rRNA gene

community composition in the deepest (141 mbsf) and hottest core for the microbiological analysis was dominated by the HWCGIV (also known as Terrestrial Hot Spring Crenarchaeotic Group [THSCG] or UCII) and MCG (Fig. 4), whereas we found no *Thermococcales*-, *Methanococcales*- and *Archaeoglobale*s-related sequences throughout the sediments that were previously detected in the hydrothermal chimney structures of the Iheya North field (38). The HWCGIV have been reported in microbial habitats associated with deep-sea hydrothermal vents (42, 55, 71). The biological thermometer estimation using the GC contents of the 16S rRNA gene sequences proposed by Kimura et al. (26) indicates that the potential optimal and maximum growth temperatures of the HWCGIV found in this study are 74°C and 83°C, respectively. These predicted values are consistent with *in situ* temperatures measured with thermoseal strips (Fig. 1A). Accordingly, the HWCGIV likely represent an active and indigenous population in the subseafloor sediments under the high temperature conditions at Site C0017. Nevertheless, the temperature condition does not explain why the hyperthermophilic chemolithotrophic populations, such as *Thermococcales, Methanococcales* and *Archaeoglobales*, which can grow above 70°C (57), were absent. The porewater chemistry in the deepest sediments revealed no evident chemical input of hydrothermal fluids. Not only the temperature but also the substantial chemical fluxes from hydrothermal fluid flow may be required for hyperthermophilic chemolithotrophic microbial community development in the subseafloor environments.

Oxic fluid circulation within deep biosphere

In hypothetical models of the total hydrothermal circulation of the Iheya North field, proposed by Kawagucci et al. (25) and Tsuji et al. (67), the hydrothermal circulation would begin with the bottom seawater recharge in the sediments along the faults of the Okinawa Trough basin far distant from the hydrothermal field. Particularly, this model assumes that the seawater recharge occur not only in the Central Valley (estimated to be 2-km square area), but also in the spatially abundant and widespread basin-filling sediments surrounding the Iheya North Knoll. During the long spatial and temporal migration in the sediments at the recharge stage, the microbially produced methane, ammonium and other compounds are likely added to the source fluids (25). However, the recharged seawater flow discovered in this study is substantially different from such a great spatial and temporal scale of hydrothermal

circulation and is spatially and temporally limited. Indeed, the porewater sulfate is partially utilized by potential functions of the indigenous subseafloor sulfate-reducers (1), but the concentration is relatively constant throughout the sediments (Fig. 2). In addition, none of the methanogen-related sequences were detected in any of the 16S rRNA gene clone libraries at Site C00017 (Fig. 4). These results suggest that the sediments and porewater in core samples are less affected by the geochemical and microbiological alterations than by the large scale of seawater recharge and alteration processes. The existence of high concentrations of porewater nitrate is an important chemical signature of the oxidative (potentially aerobic) condition and the relatively fresh seawater input in the Unit II layer (Fig. 2). The microbial community development pattern also suggests the drastic transition within Unit II (Fig. 5). Although Units II and III showed quite similar lithologic characteristics, there was a hard layer boundary (almost no core recovered) between Units II and III, and the unrecovered hard layer would serve as an impermeable layer to prevent possible vertical fluid exchange (63). The microbial community compositions inferred from the 16S rRNA gene clone sequences were also significantly different between Units II and III, and the sequences affiliated with MCG dominated the phylotype compositions in the Unit III samples, contrasting with the MG-I *Thaumarchaeota* members in Unit II. Thus, the highly permeable layer in Unit II would provide a novel habitat of the subseafloor biosphere that has been unexplored in the previous scientific ocean drilling expeditions. The seismic reflection survey and its interpretation revealed horizontally widespread and vertically multiple distributions of porous and permeable layers in the sediments around the Iheya North hydrothermal field (67). If some of these permeable layers host the horizontal recharge flow of relatively fresh seawater, the microbial community stratification estimated in the sediments of Site C0017 may be a common pattern of subseafloor microbial community development in the marginal sedimentary environment of the Iheya North field.

CONCLUSIONS

This study reports the potential microbial community stratification associated with the complex fluid flow structure, such as recharging seawater and underlying hydrothermal fluids, coupled with the lithologic transition of sediments at Site C0017 in the Iheya North hydrothermal field of the Mid-Okinawa Trough. Uncultivated microbial components, which are frequently detected in subseafloor sedimentary

environments, populated the shallow sections, whereas members of MG-I *Thaumarchaeota* dominated the 16S rRNA gene phylotype communities in the pyroclastic deposits. The sharp transition of the potential microbial community is most likely controlled by different physical properties of sediments, such as the permeability of hemipelagic muds and volcaniclastic sediments, which are further related to the hydrogeologic structure and geothermal gradient of the subseafloor environment. Our results reveal the dynamics of biogeochemical and microbiological processes in the subseafloor sediments, directly and indirectly associated with local fluid flows such as fresh seawater recharge and hydrothermal fluid discharge. A great spatial and temporal scale of hydrothermal circulation has been extensively investigated in crustal aquifers at mid-ocean ridge flanks, which also potentially supplies oxidants through the basaltic basement and has a significant role in biogeochemical cycles and crustal rock alteration (2, 46). The drilling operation during IODP Expedition 331 was unsuccessful in reaching the volcanic basement, which might exist at ~450 mbsf at Site C0017 (63). However, this study implies that the oxidants transported through the local seawater circulation associated with hydrothermal activity are important for generating variable chemical environments and microbial communities in the subseafloor sedimentary habitats and even in potentially deeper sediment-basement interface habitats.

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TABLE 1. List of primers and probes used in the Q-PCR assays

Target gene	Primer/probe	Sequence (5'-3')	Reference
Prokaryotic universal 16S rRNA gene	Uni340F	CCTACGGGRBGCASCAG	(60)
	Uni806R	GGACTACNNGGGTATCTAAT	(60)
	Uni516F (TaqMan probe)	TGYCAGCMGCCGCGGTAAHACVNRS	(60)
Archaeal 16S rRNA gene	Arch349F	GYGCASCAGKCGMGAAW	(60)
	Arch806R	GGACTACVSGGGTATCTAAT	(60)
	Arch516F (TaqMan probe)	TGYCAGCCGCCGCGGTAAHACCVGC	(60)
Bacterial 16S rRNA gene	331F	TCCTACGGGAGGCAGCAGT	(37)
	797R	GGACTACCAGGGTATCTAATCCTGTT	(37)
	TaqMan probe	CGTATTACCGCGGCTGCTGGCAC	(37)
Dissimilatory sulfite reductase (*dsrA*)	DSR-1F+	ACSCACTGGAAGCACGGCGG	(28)
	DSR-R	GGTTRKACGTGCCRMGGTG	(28)
Adenosine 5'-phosphosulfate reductase subunit A (*aprA*)	AprA-1-FW	TGGCAGATCATGATYMAYGG	(35)
	AprA-5-RV	GCGCCAACYGGRCCRTA	(35)

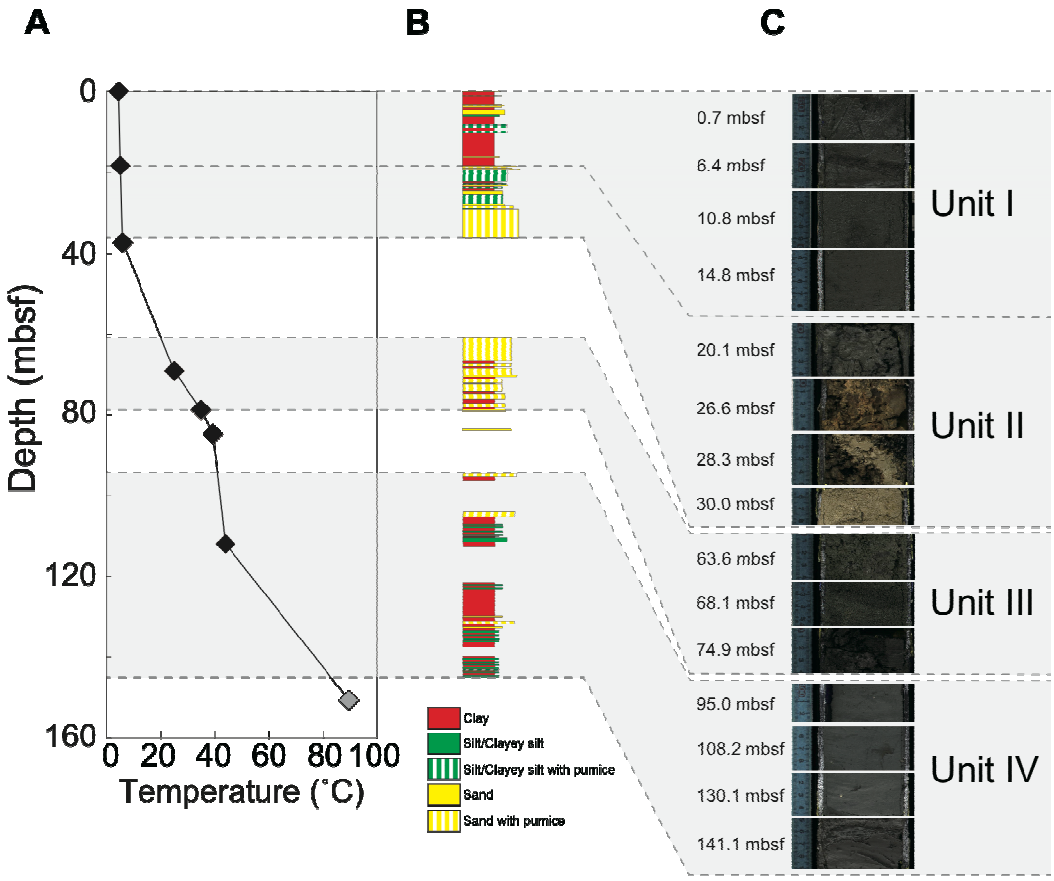

FIG 1 Temperature profile (A), lithostratigraphic transition (B) and core photograph (C) of sediment samples at IODP Expedition 331 Site C0017, which were originally published elsewhere (63). The black diamonds indicate *in situ* temperature measured by the APCT-3 temperature shoe. The grey diamond indicates the exposed minimum temperature determined by a thermoseal strip taped to the outer surface of the core liner. The lithologic description was roughly modified to show the entire sedimentary structure of the core samples. The photographs were taken from the section closest to the microbiology samples used in this study.

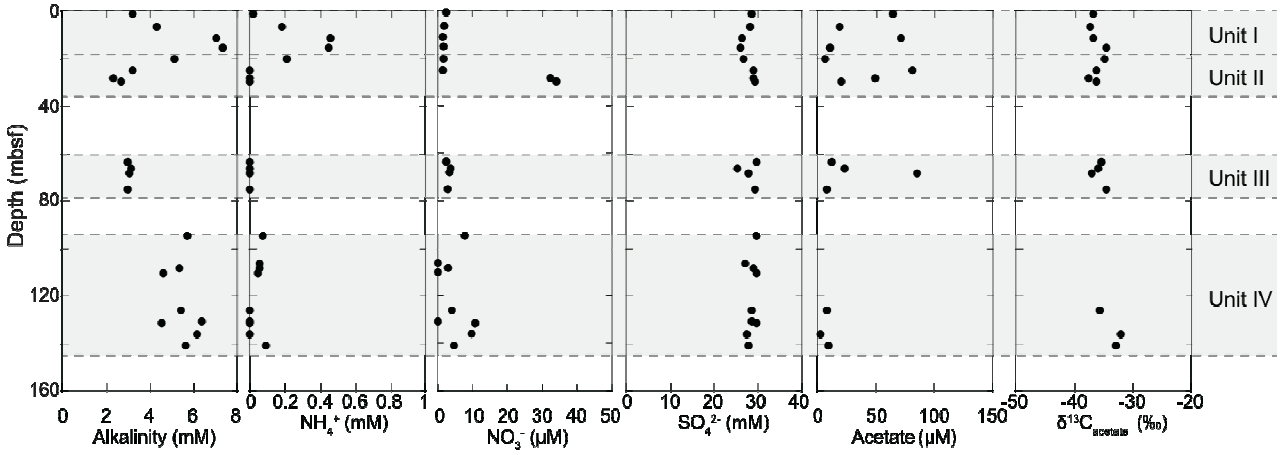

FIG 2 Depth profile of porewater alkalinity, the ammonium, nitrate, sulfate, and acetate concentrations and the carbon isotopic composition of acetate in the core samples at Site C0017. The original data regarding alkalinity, ammonium, nitrate and sulfate were published elsewhere (63).

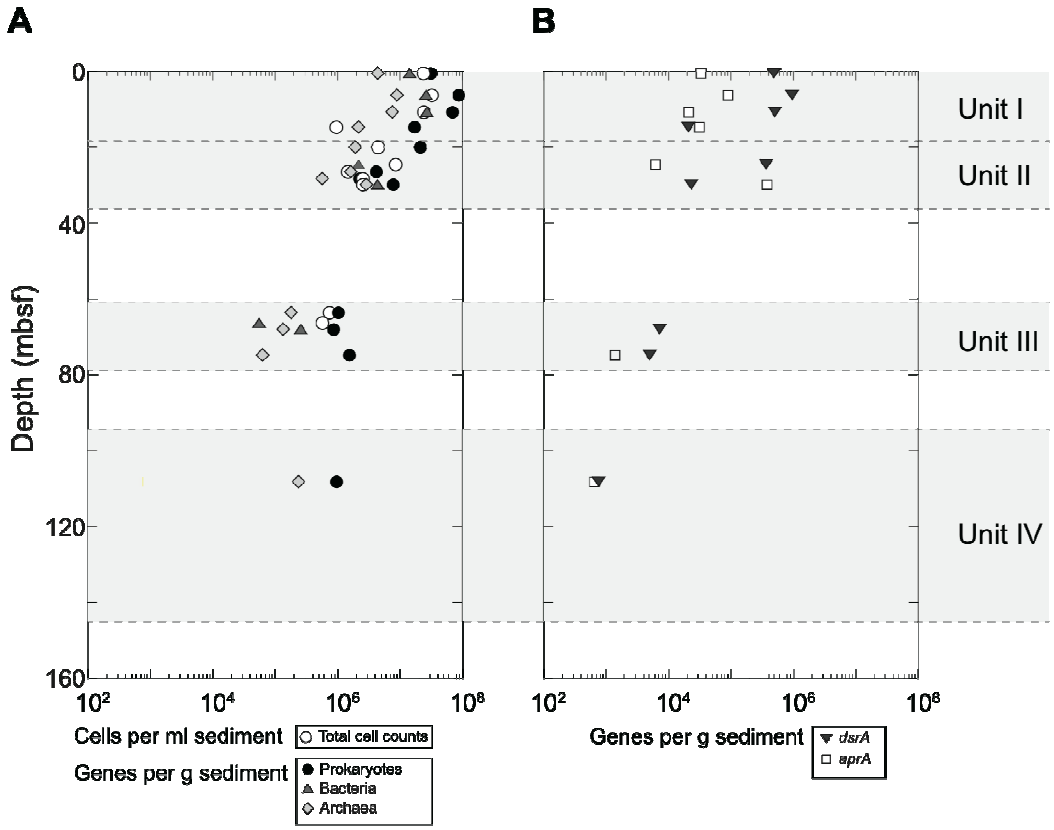

FIG 3 Total cell counts and numbers of 16S rRNA and functional genes in the subseafloor core samples at Site C0017. (A) Total cell counts (open circles) and the 16S rRNA gene numbers of prokaryotes (black circles), bacteria (dark gray triangles) and archaea (light gray diamonds) quantified by Q-PCR. The total cell counts were originally reported by Takai et al. (63). (B) Numbers of functional genes *dsrA* (dark gray triangles) and *aprA* (open squares).

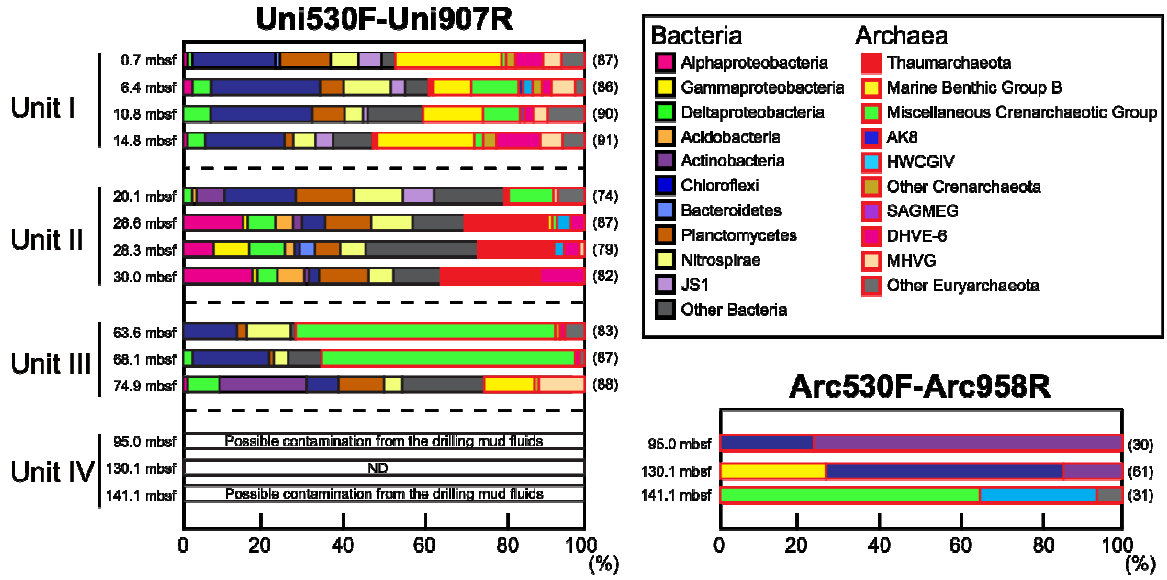

FIG 4 The 16S rRNA gene phylotype compositions in the sediments at Site C0017. The 16S rRNA gene fragments were amplified with the universal and archaea-specific primer sets of Uni530F-907R and Arc530F-Arc958R, respectively. The numbers in parentheses indicate the number of clones.

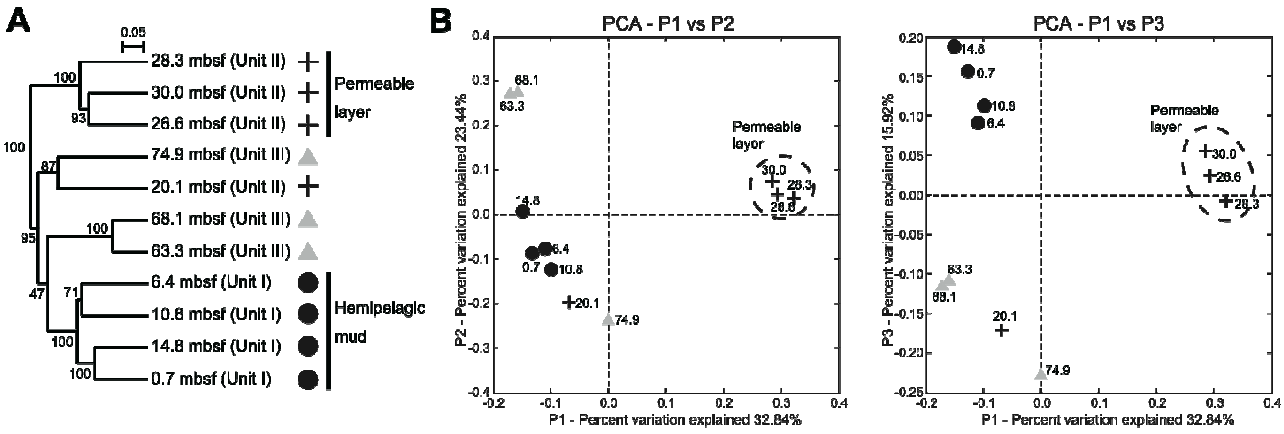

FIG 5 Jackknife environment cluster analysis (A) and PCA (B) in the UniFrac program. The jackknife values were estimated using 100 permutations and are shown in the nodes of the dendrogram. Each axis of the PCA plot indicates the fraction of the variance in the data. The black circles, plus signs and triangles indicate the sediment samples of Units I, II and III, respectively.

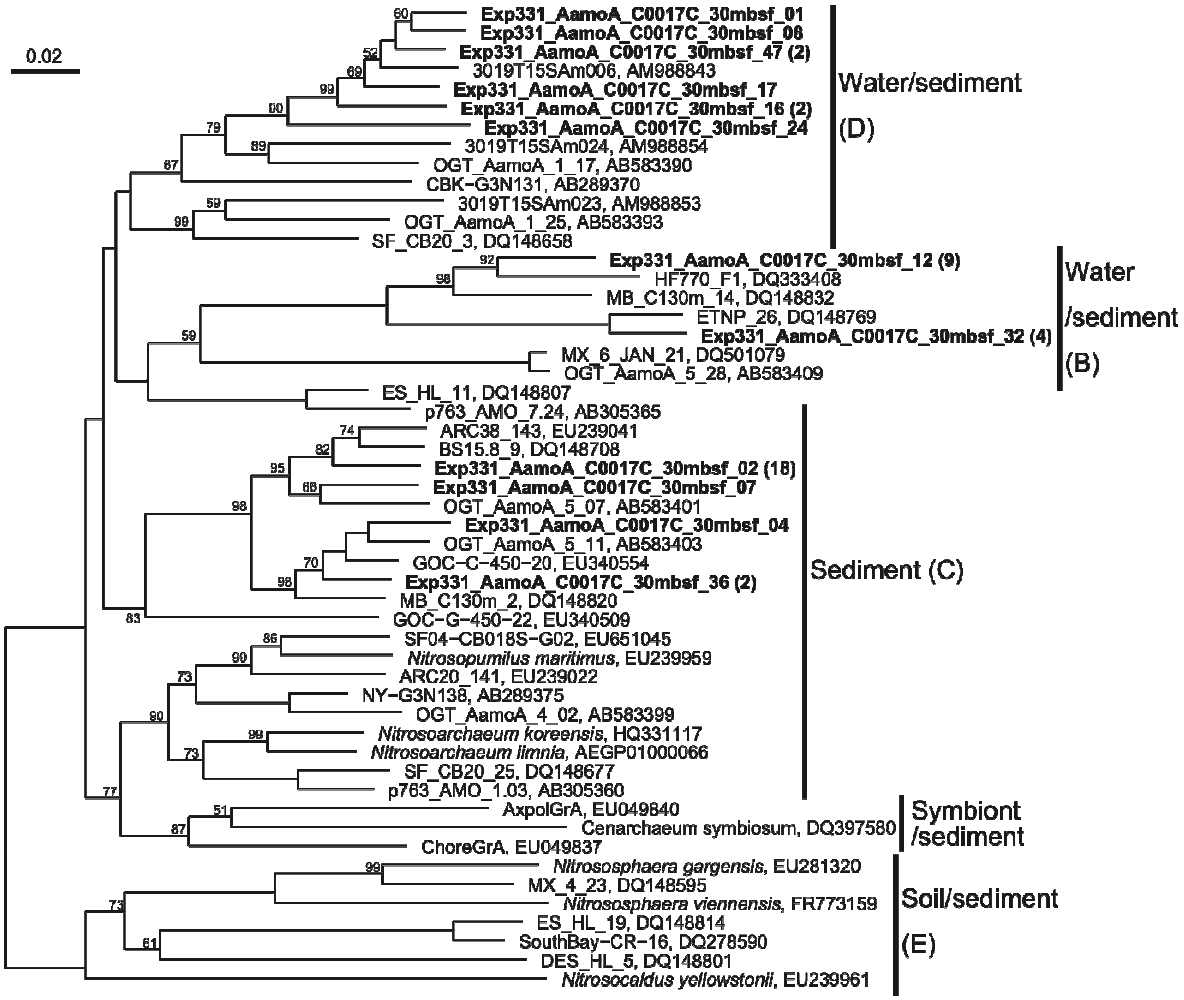

Fig. S1. Phylogenetic tree of the archaeal *amoA* gene sequences obtained from the permeable layer at the depth of 30.0 mbsf. Boldface type indicates the sequences obtained in this study. The parenthetic numbers show the total number of phylotypes. Bootstrap values are expressed as percentages of 1000 trials. The values at the nodes represent the scores greater than 50%. The scale bar represents 2% estimated sequence divergence.

24.5. Defining boundaries for the distribution of microbial communities beneath the sediment-buried, hydrothermally active seafloor

Running Title: Biosphere limits beneath a deep-sea vent
Classification: Biological sciences/Environmental sciences, Microbiology

Katsunori Yanagawa[a,b,c,*], Akira Ijiri[d], Anja Breuker[e], Sanae Sakai[a], Youko Miyoshi[f], Shinsuke Kawagucci[a], Takuroh Noguchi[g], Miho Hirai[h], Axel Schippers[e], Jun-ichiro Ishibashi[f], Yoshihiro Takaki[a], Michinari Sunamura[b], Tetsuro Urabe[b], Takuro Nunoura[h], and Ken Takai[a]

[a]Department of Subsurface Geobiological Analysis and Research (D-SUGAR), Japan Agency for Marine-Earth Science and Technology (JAMSTEC), Yokosuka 237-0061, Japan
[b]Department of Earth and Planetary Science, University of Tokyo, Tokyo 113-0033, Japan
[c]Faculty of Social and Cultural Studies, Kyushu University, Fukuoka 819-0395, Japan
[d]Geomicrobiology Group, Kochi Institute for Core Sample Research, Japan Agency for Marine-Earth Science and Technology (JAMSTEC), Kochi 783-8502, Japan
[e]Geomicrobiology, Federal Institute for Geosciences and Natural Resources (BGR), Stilleweg 2, 30655 Hannover, Germany
[f]Department of Earth and Planetary Sciences, Faculty of Science, Kyushu University, Higashi-ku, Fukuoka 812-8581, Japan
[g]Interdisciplinary Science Unit, Multidisciplinary Science Cluster, Research and Education Faculty, Kochi University, Kochi, 783-8502, Japan
[h]Marine Functional Biology Group, Research and Development Center for Marine Biosciences, Japan Agency for Marine-Earth Science and Technology (JAMSTEC), Yokosuka 237-0061, Japan
[*]Corresponding author: Katsunori Yanagawa, Faculty of Social and Cultural Studies, Kyushu University. 744, Motooka, Nishi-ku, Fukuoka 819-0395, Japan. Tel.: +81 92 802 5676; fax: +81 92 802 5603; e-mail: kyanagawa@scs.kyushu-u.ac.jp

Subject Categories: Microbial ecology and functional diversity of natural habitats.

Abstract

Subseafloor microbes beneath active hydrothermal vents are thought to live near the upper temperature limit for life on Earth. We drilled and cored the Iheya North hydrothermal field in the Mid-Okinawa Trough, and examined the phylogenetic compositions and the products of metabolic functions of sub-vent microbial communities. We detected microbial cells, metabolic activities and molecular signatures only in the shallow sediments down to 15.8 m below the seafloor at a moderately distant drilling site from the active hydrothermal vents (450 m). At the drilling site, the profiles of methane and sulfate concentrations and the $\delta^{13}C$ and δD isotopic compositions of methane suggested the laterally flowing hydrothermal fluids and the *in situ* microbial anaerobic methane oxidation. *In situ* measurements during the drilling constrain the current bottom temperature of the microbially habitable zone to ~45 °C. However, in the past, higher temperatures of 106-198 °C were possible at the depth, as estimated from geochemical thermometry on hydrothermally altered clay minerals. The 16S rRNA gene phylotypes found in the deepest habitable zone are related to those of thermophiles, although sequences typical of known hyperthermophilic microbes were absent from the entire core. Overall our results shed new light on the distribution and composition of the boundary microbial community close to the high-temperature limit for habitability in the subseafloor environment of a hydrothermal field.

Introduction

Deep-sea hydrothermal vents are extreme environments, especially with respect to the high temperatures, which create the limits of life on Earth. Several studies have further addressed the potential microbial habitats beneath the seafloor hydrothermal vents, the so-called “sub-vent biosphere”, and have indicated the possible occurrence of functionally active and metabolically diverse (hyper-)thermophilic microbial communities associated with shallow subseafloor hydrothermal fluids and mineral deposits (Deming and Baross, 1993; Delaney *et al.*, 1998; Summit and Baross, 1998; Huber *et al.*, 2002, 2003). The compositions and functions of the sub-vent microbial communities have been inferred from culture-dependent and culture-independent analyses of microbial communities in (i) *in situ* growth chambers placed in hydrothermal fluid flows (Karl *et al.*, 1988; Reysenbach *et al.*, 2000; Corre *et al.*, 2001; Takai *et al.*, 2004), (ii) crustal fluids collected directly from the shallow

subseafloor environments (~10 m below the seafloor [mbsf]) via seafloor drilling or probe insertion (Cowen *et al.*, 2003; Higashi *et al.*, 2004; Huber *et al.*, 2006; Kato *et al.*, 2009; Orcutt *et al.*, 2011), (iii) hydrothermal sediments (Teske *et al.*, 2002; Dhillon *et al.*, 2005; Nunoura *et al.*, 2010; Teske *et al.*, 2014; Dowell *et al.*, 2016; Teske *et al.*, 2016) and (iv) chimney structures of active hydrothermal vents (Takai *et al.*, 2001; Schrenk *et al.*, 2003; Nakagawa *et al.*, 2005). These studies found that mesophilic, thermophilic and hyperthermophilic members of *Epsilonproteobacteria*, *Gammaproteobacteria*, *Aquificales*, *Thermococcales* and *Methanococales* were the potentially predominant microbial components in the sub-vent biosphere. Additionally, previous studies associated with international scientific ocean drilling projects have also indicated the existence of microbial cells in deep subsurface sedimentary and rocky habitats in the hydrothermal vent systems on the Juan de Fuca Ridge [Ocean Drilling Program (ODP) Leg 139 (Cragg and Parkes, 1994) and 169 (Cragg *et al.*, 2000; Summit *et al.*, 2000) and Integrated Ocean Drilling Program (IODP) Expedition 301 (Lever *et al.*, 2013)] and the Manus Basin [ODP Leg 193 (Kimura *et al.*, 2003)]. However, these microbial explorations associated with scientific ocean drilling projects have not successfully provided data on the compositions and functions of potential subseafloor microbial communities. In particular, it is unclear how local hydrothermal flow may influence the uneven distribution of sub-vent microbial community. Complex subsurface hydrothermal flow may limit or stimulate the sub-vent microbial activities.

The IODP Expedition 331 by the D/V *Chikyu* (Takai *et al.*, 2011) provided a good opportunity for direct investigation of the sub-vent biosphere at the Iheya North hydrothermal field in the Mid-Okinawa Trough. The Iheya North field is located in a continental-margin backarc basin, and the hydrothermal activity is highly influenced by trough-filling terrigenous sediment and knoll-covering volcaniclastic deposits (Kawagucci *et al.*, 2011). The subseafloor geochemical and microbiological processes associated with buried organic matter and hydrothermal circulation produce unique hydrothermal fluid compositions that are enriched with high concentrations of methane and ammonia (Sakai *et al.*, 1990; Kawagucci *et al.*, 2011). During IODP Exp. 331, we conducted drilling and coring operations at five sites, located at 0 m (C0016), 100 m (C0013), 450 m (C0014) and 1,550 m (C0017) east and 600 m (C0015) northwest of the most active hydrothermal mound (Takai *et al.*, 2011). Previously, we have reported downhole changes in the microbial community

corresponding to variations in the subseafloor hydrogeologic and lithostratigraphic structures that affected the recharging seawater and hydrothermal fluid input at Site C0017 (Yanagawa *et al.*, 2014). That study obtained the 16S rRNA gene sequences of putative thermophiles only from the deepest sediments at 141 mbsf, where the *in situ* temperature was estimated to be approximately 90 °C, lower than the previously reported upper growth temperature limit of hyperthermophiles (122 °C) (Takai *et al.*, 2008). The results suggested that the deepest sediment at Site C0017 is inhabited by potential thermophilic populations and that the habitable limit of the subseafloor microbial community lies at a depth greater than that of the bottom of the drilled hole (Yanagawa *et al.*, 2014).

In addition to Site C0017, this study investigated the subseafloor environments at two additional drilling sites (Sites C0013 and C0014) that have higher thermal gradients and are closer (100 and 450 m, respectively) to the vigorous hydrothermal vents. We chose these vents to clarify the distribution, composition and function of microbial communities occurring adjacent to the high-temperature biosphere limit associated with high-temperature hydrothermal fluids. The drilling operations at these sites successfully penetrated the subseafloor hydrothermal fluid reservoirs and created artificial hydrothermal fluid vents with temperatures of up to 311 °C (Kawagucci *et al.*, 2013a). Hence, the recovered core samples were potentially exposed to the *in situ* temperature range of 4°C to >300 °C and provide important insights into the boundary microbial community and the limits of the biosphere.

Materials and Methods

Sampling Sites and Sample Collection

Subseafloor drilling and coring operations were conducted at the Iheya North hydrothermal field in the Mid-Okinawa Trough during IODP Expedition 331 (Takai *et al.*, 2011). Coring and *in situ* temperature measurements were conducted at IODP Site C0013 and Site C0014 as previously described (Takai *et al.*, 2011). Sediment samples for geochemical and microbiological analyses were collected as previously described (Yanagawa *et al.*, 2013b; Yanagawa *et al.*, 2014). Details on the sample collections are provided in Supplementary Information Materials and Methods.

Geochemical Analyses

The carbon and hydrogen isotopic compositions of methane were determined via continuous-flow isotope ratio mass spectrometry as previously described (Umezawa

et al., 2009; Kawagucci *et al.*, 2013a). The oxygen isotopic compositions of hydrothermally altered mud were determined as previously described (Miyoshi, 2013). The concentration and isotopic composition of DIC was determined as previously described (Miyajima *et al.*, 1995; Toki *et al.*, 2004; Noguchi *et al.*, 2013). Details are provided in Supplementary Information Materials and Methods.

Molecular Analyses of 16S rRNA Genes and Functional Genes

DNA was extracted from core subsamples using the PowerMAX Soil DNA isolation kit (MoBio Laboratories, Carlsbad, CA). Before the physical cell disruption, the samples were incubated at 65 °C for 5 min. Then, mechanical shaking was performed for 10 min with a ShakeMaster (BioMedical Science, Tokyo, Japan). Other subsequent steps were performed according to the manufacturer's protocol. The extracted DNA was stored at -80 °C.

Q-PCR was performed for prokaryotic and archaeal 16S rRNA genes and the functional genes of *dsrA*, *aprA* and *mcrA* as previously described (Nunoura *et al.*, 2008; Blazejak and Schippers, 2011; Yoshida-Takashima *et al.*, 2012; Breuker *et al.*, 2013). The primers, probes and amplification conditions used in this study are summarized in Supplementary Table S2.

For the clone analysis of 16S rRNA genes, the gene fragments were amplified by PCR using a universal and archaea-specific primer set (Nunoura *et al.*, 2012). The *mcrA* gene fragments were also amplified using specific primers. Details for primers and PCR amplification conditions are described in Supplementary Table S2. The amplified 16S rRNA and *mcrA* genes were cloned, sequenced and aligned as previously described (Yanagawa *et al.*, 2013b). The phylogenetic affiliations were identified using the SILVA SSU Ref 111 Database (Ludwig *et al.*, 2004) and phylogenetic trees were constructed. A detailed description is provided in Supplementary Information Materials and Methods. Sequences have been deposited in the GenBank database under accession numbers: AB824899-AB825952 for 16S rRNA gene sequences and LC061224-LC061266 for *mcrA* gene sequences.

Cultivation Tests

Serial dilution cultivations for anaerobic heterotrophs, methanogens, and sulfate reducers were performed to quantify the abundance of populations that can be cultivated in the laboratory using sediment slurries from a variety of depths. The cultivated population abundance of aerobic heterotrophs was quantified by colony-forming units on marine agar 2216 (BD). Details for cultivation conditions are

provided in Supplementary Information Materials and Methods and Supplementary Table S3.

Microbial Activity Measurements

The potential rates of methane production and oxidation and acetate oxidation were determined through radioisotope tracer incubation experiments (Tasumi *et al.*, 2015). The sediment slurry samples were incubated with appropriate radiotracers ($^{14}CH_4$, $H^{14}CO_3^-$ and [2-^{14}C] acetate) for 1 month at 30 °C and 60 °C based on the *in situ* temperatures in Supplementary Figure S1. The radioactivity of a portion of the reaction products in the headspace was measured using a gas chromatograph (Shimadzu GC-2014, Shimadzu, Kyoto, Japan) and a high-sensitivity radioactivity detector RAGA Star (Raytest, Straubenhart, Germany), as shown in Supplementary Information Materials and Methods. The potential activity was calculated based on the proportion of radioactive ^{14}C product to total radioactive substrate.

Results and Discussion

Temperature in the Sub-vent Environment

Sediment core samples analyzed in this study were obtained from two holes at IODP Site C0013 (Holes C0013B and C0013D) and four holes at Site C0014 (Holes C0014B, C0014D, C0014E and C0014G). The depths of the deepest holes were 35 and 137 mbsf at Site C0013 (Hole C0013D) and Site C0014 (Hole C0014G), respectively. Although *in situ* temperatures were not measured during the drilling at Site C0013, it could be estimated from the fact that most of the plastic core liners melted several meters below the seafloor (Yanagawa *et al.*, 2013b). The melting point of the plastic is >70 °C. The subseafloor environment at Site C0014, at which most of the deep coring operations were conducted by using aluminum core liners, exhibited a steep temperature gradient of 3 °C/m based on the temperatures measured during the drilling operation. At this site, the temperatures increased from 4.5 °C at the seafloor to >210 °C at 50 mbsf (Takai *et al.*, 2011) (Supplementary Figure S1).

Hydrothermally altered mud with elemental sulfur and sulfide grit was found in shallow sediments at 0.8 and 4.5 mbsf in Holes C0013B and C0013D, respectively (Miyoshi *et al.*, 2015). The clay minerals in the hydrothermal mud yielded oxygen isotopic values of +8.0 and +9.6‰ (Miyoshi, 2013). These values indicated that the clay minerals formed at >138 °C (Supplementary Table S1). At Site C0014, the

shallow sediments were not affected by hydrothermal alteration at depths above approximately 12 mbsf, whereas the deeper >12 mbsf sediments were hydrothermally altered (Miyoshi *et al.*, 2015). The oxygen isotopic values of the hydrothermally altered clays at 12.8 and 15.1 mbsf in Hole C0014B were +8.7 and +12.5‰, respectively (Miyoshi, 2013). This indicated that the clay formation temperatures were >106 °C at Site C0014 (Supplementary Table S1) (Miyoshi, 2013). The geochemical thermometer estimate based on the oxygen isotopic compositions of hydrothermally altered clay minerals suggests that at some point in time, even the shallow sediments at Sites C0013 and C0014 have experienced high-temperature conditions that are close to the known temperature limit of life at 122 °C (Takai *et al.*, 2008). Indeed, any of the microbial cell observations, metabolic activity measurements, cultivation tests and prokaryotic 16S rRNA gene quantifications described below provided positive signatures for the existence of microbial populations only in the near-surface sediments at Site C0013. Thus, most of the results and discussion in the following sections are described with a focus on Site C0014.

A rapid increase in the pore-water potassium (K) concentration was observed just below the seafloor at 12.1 mbsf in Hole C0014B, 11.5 mbsf in Hole C0014D and 17.7 mbsf in Hole C0014G (Supplementary Figure S2). These discontinuities in K concentrations could be explained by the possible occurrence of impermeable layers in the subseafloor environment (Figure 1) and hydrothermal fluid flows just below the layers. The distribution pattern of the hydrothermally altered mud supports this interpretation (Supplementary Table S1) (Miyoshi *et al.*, 2015). In addition, because potassium was enriched in the endmember 310 °C hydrothermal fluids (80 mM) but low in the ambient 4 °C seawater (10 mM) (Kawagucci *et al.*, 2011), the K concentration is an indicator of the degree of mixing between hydrothermal fluids and infiltrated seawater and/or ambient pore-water. Although the magnesium concentration is also an excellent indicator of mixing between ambient seawater and hydrothermal fluids, the pore-water magnesium concentration could be affected by not only mixing but also association and dissociation processes related to hydrothermally altered minerals (Takai *et al.*, 2011; Miyoshi *et al.*, 2015). Thus, in this study, the pore-water K concentration is likely more reliable than the magnesium concentration. Assuming a bimodal mixing between endmember hydrothermal fluid and seawater without conductive cooling, K concentrations lower than 37 mM

correspond to the temperature range of sediments lower than 122 °C, the potential habitable temperature range of previously cultivated microorganisms. In other words, K concentrations higher than 37 mM indicate that the sediments are exposed to high temperatures exceeding the potentially habitable temperature range.

Geochemical Evidence of Hydrothermal Fluid Input and Methane Oxidation

The total alkalinity and dissolved inorganic carbon (DIC) concentrations featured significant peaks at shallow depths in Holes C0014B and C0014D (Supplementary Figure S2). Based on the stable carbon isotope compositions, this enrichment was probably caused by CO_2 in the endmember hydrothermal fluids. However, the DIC concentration peaks did not match the vertical K concentration profiles in each of the holes even though both components could have been provided by the hydrothermal fluids (Supplementary Figure S2). The different profiles for these two components (DIC and K) indicated that at least two different hydrothermal fluid sources fed the different lithostratigraphic horizons, such as the depth zones of 1-8.4 mbsf and >12.1 mbsf in the case of Hole C0014B. The hydrothermal fluid input in the shallower sediments (1-8.4 mbsf in Hole C0014B and 0-2.2 mbsf at C0014D) consisted primarily of low-temperature liquid CO_2 and/or enriched gas components, whereas the input in the deeper zones (>12.1, >11.5 and >17.7 mbsf in Holes C0014B, C0014DE and C0014G, respectively) was derived directly from high-temperature vapor-lost fluid. Both of these fluids could result from the subseafloor phase-separation and phase-partitioning processes of hydrothermal fluids (Von Damm and Bischoff, 1987).

The methane and sulfate concentrations exhibited more complex vertical variations than the DIC and K concentrations (Figure 1). The sulfate concentration exceeding the seawater level at Holes C0013B and D (Supplementary Figure S2) was probably due to the dissolution of anhydrite with decreasing temperature during the core recovery and sampling processes (Takai *et al.*, 2011). In contrast, sulfate depletion and methane enrichment (relative to seawater levels) were apparent even within the shallow depths for each hole at Site C0014 (Figure 1a). Interestingly, a clear inverse relationship existed between methane and sulfate above 14 mbsf in Hole C0014B. This indicated the lateral flows of methane-rich hydrothermal fluids in the sulfate-rich subseafloor environment. In addition to the lithostratigraphically controlled hydrothermal fluid inputs, the $\delta^{13}C_{CH4}$ and δD_{CH4} profiles suggested the contribution of microbial community functions. The patterns in the $\delta^{13}C_{CH4}$ values

along the vertical extent of the core were synchronized with δD_{CH4} values, and isotopically positive shifts in both the $\delta^{13}C$ and δD values of methane were observed only within the shallow low-temperature zones (7.8-10.7 mbsf in Hole C0014B and <15.9 mbsf in Hole C0014G), where the methane concentration was extremely low (Figure 1a and Figure 2). These isotopically heavier shifts in methane were not directly derived from the ^{13}C- and D-depleted methane in the high-temperature hydrothermal fluids (Kawagucci, 2015). The concurrent isotopic changes in methane ($\Delta\delta D_{CH4}/\Delta\delta^{13}C_{CH4}$) in the shallow low-temperature zones fell into a range bracketed by slopes of 7 to 19 (Figure 2), which have been reported to be representative values for microbial methane oxidation (Alperin *et al.*, 1988; Kessler *et al.*, 2006; Holler *et al.*, 2009; Feisthauer *et al.*, 2011).

The vertical patterns of the geochemical signals differed among the holes (Figures 1ab and Supplementary Figure S2). The spatial heterogeneity in the subseafloor pore-water geochemistry was likely affected by the local input of hydrothermal fluids, and the observed patterns, particularly in Hole C0014B, indicated vertical differences in fluid chemistry due to different sources of laterally flowing hydrothermal fluids and their mixing behavior with infiltrated seawater and ambient pore-water. In combination with the complex variations in the lithostratigraphic and physical properties of the core samples, the phase separation, flow patterns and mixing behavior of subseafloor hydrothermal fluids are hypothesized to be controlled by the lithostratigraphy, such as fresh hemipelagic sediments, porous pumiceous deposits and hard impermeable layers (Takai *et al.*, 2011).

Abundance Estimation of Microbial Cellular and rRNA Gene Populations

The microbial cell abundance at Site C0014 decreased dramatically with depth from approximately 1×10^8 cells per ml of sediment just below the seafloor to less than the detection limit of approximately 6.5×10^5 cells per ml of sediment (Takai *et al.*, 2011) (Figure 1c). A quantitative real-time PCR (Q-PCR) analysis for whole prokaryotic and archaeal 16S rRNA genes suggested that microbial populations were present only in the shallow sediments above 14.3, 10.2 and 15.8 mbsf in Holes C0014B, C0014E and C0014G, respectively (Figure 1c). The abundance of the whole prokaryotic 16S rRNA genes with depth showed similar values and profiles to the microbial cell abundance in all of the holes. However, several shallow sediment depths in Holes C0014B and C0014G exhibited large data gaps between the

microbial cell abundance and the whole prokaryotic 16S rRNA gene abundance due to the relatively higher detection limit of the microbial cell count, as shown in Figure 1c, or due to the technical limitations of the Q-PCR (Hoshino and Inagaki, 2012; Lloyd *et al.*, 2013; Morono *et al.*, 2014). The relative abundance of archaeal 16S rRNA genes increased with depth and represented approximately half of the whole prokaryotic 16S rRNA gene assemblages in the deepest zones where Q-PCR could detect 16S rRNA genes (Supplementary Figure S3).

Cultivation and Activity Measurements for Heterotrophs

Successful enrichments of heterotrophs were obtained from most of the shallow sediment samples from Hole C0014B at a temperature of 30 °C, and subsequent quantitative cultivation analyses were conducted on these samples. Aerobic heterotrophs, anaerobic heterotrophs and sulfate reducers were detected at depths above 8.5, 12.2 and 3.6 mbsf, respectively (Supplementary Figure S4). In contrast, 60 °C incubations and onboard cultivation experiments for (hyper-) thermophilic heterotrophs and chemolithoautotrophs did not yield any cultivable population at any depth (Takai *et al.*, 2011). The cultivated populations ranged up to 9.6×10^5 CFU g^{-1} sediment for aerobic heterotrophs, 5.5×10^7 cells g^{-1} sediment for anaerobic heterotrophs and 5.5×10^2 cells g^{-1} sediment for sulfate reducers. The 16S rRNA gene sequences of the isolated aerobic heterotrophs, anaerobic heterotrophs and sulfate reducers were highly similar to those of *Geofilum rubicundum* (100% similarity) [NR_112717], *Clostridium* sp. S710(0)-1 (98% similarity) [GU136592] and *Desulfomicrobium norvegicum* (100% similarity) [NR_025407], respectively (Supplementary Table S3). Potential anaerobic heterotrophic activities were also detected in the sediment samples from which the anaerobic heterotrophs were successfully cultivated (Figure 1e). The activity, defined as the oxidation of the methyl group of ^{14}C-labeled acetate to $^{14}CO_2$, gradually decreased with increasing sediment depth. Notably, all of the estimations of cultivation-dependent viable heterotrophic populations, ^{14}C-tracer heterotrophic activities and prokaryotic 16S rRNA gene quantification provided independent detectable signatures for the occurrence of a microbial community in the same depth zone of the subseafloor environment (Figures 1c and e and Supplementary Figure S4).

16S rRNA Gene Community Structures

The primer set of Uni530F/Uni907R for the universal prokaryotic 16S rRNA gene (Nunoura *et al.*, 2012) was applied to DNA assemblages extracted from the

sediment samples. Potentially indigenous 16S rRNA gene communities were obtained from the sediment samples from depths above 8.5, 10.2 and 15.8 mbsf in Holes C0014B, C0014D and C0014G, respectively (Figure 3a). None of the 16S rRNA genes were amplified from environmental DNA assemblages below these depths. The 16S rRNA gene phylotype compositions changed significantly with transitions in the geochemical and lithostratigraphical environments and/or the elevated temperatures. Typical uncultivated microbial members in the marine sedimentary environment, such as *Deltaproteobacteria, Chloroflexi*, JS1 group in *Candidatus* (*Ca*.) Atribacteria, Deep-sea Hydrothermal Vent Euryarchaeotic Group 6 (DHVEG-6) and Miscellaneous Crenarchaeotic Group (MCG), were detected in the 16S rRNA gene clone communities at all depths. Certain members closely related to potentially thermophilic bacterial taxa/divisions, such as *Thermotogae, Thermodesulfobacteria* and OP1 (*Ca*. Acetothermia), were detected as minor populations in certain deeper sections (Supplementary Data S1).

Members of the HotSeep-1 group composed 60-100% of the total number of deltaproteobacterial 16S rRNA gene sequences from the deeper sediment samples at 8.5 mbsf in Hole C0014B, 10.2 mbsf in Hole C0014D and 15.8 mbsf in Hole C0014G (Supplementary Data S1). The HotSeep-1 group was previously detected in hydrothermal sediments in the Guaymas Basin (Teske *et al.*, 2002; Kniemeyer *et al.*, 2007; Dowell *et al.*, 2016) and in an enrichment culture of anaerobic methane oxidizers under high-temperature conditions (Holler *et al.*, 2011). Hence, the HotSeep-1 group is thought to be responsible for high-temperature sulfate reduction, coupling with the anaerobic oxidation of methane (AOM) by a deeply branching, putatively thermophilic group of methanotrophic archaea (ANME-1-Guaymas I) (Holler *et al.*, 2011). Although the sequences related to anaerobic methanotrophs (ANMEs) were found in certain depth horizons, the members closely related to ANME-1-Guaymas I did not co-exist with the bacterial HotSeep-1 group. Notably, the ANME-1-Guaymas I phylotypes were detected only at 14.3 mbsf in Hole C0014B, representing only 2% of the archaeal clone library (Supplementary Data S1).

Archaeal 16S rRNA gene amplicons were obtained from samples from the deepest zones at Site C0014, at which many positive signals for the existence of microbial communities were detected. These archaeal 16S rRNA gene amplicons were detected not by using a universal primer set but by using the archaea-specific primer set Arch530F/Arch958R (Figure 3b). The archaeal 16S rRNA gene

communities in these sediments were dominated (42-97% of total archaeal sequences) by the Hot Water Crenarchaeotic Group IV (HWCGIV; also known as UCII or a subcluster of the Terrestrial Hot Spring Crenarchaeotic Group [THSCG]) (Supplementary Data S1). They were also detected as the predominant population (99% of the total archaeal sequences) in the near-surface sediment at 1 mbsf in Hole C0013B, which is the only sample containing detectable 16S rRNA genes at Site C0013 (data not shown). The HWCGIV sequences have previously been reported in deep-sea hydrothermal vent habitats (Schrenk *et al.*, 2003; Nunoura *et al.*, 2010; Yoshida-Takashima *et al.*, 2012) and in a high-temperature zone at 141.1 mbsf at Site C0017 (Yanagawa *et al.*, 2014).

Microbial Functions of Methanogenesis and AOM

The metabolic activities of hydrogenotrophic methanogenesis, acetoclastic methanogenesis and AOM were traced using radioisotope-labeled substrates at *in situ* temperatures. Hydrogenotrophic and acetoclastic methanogenic activities were detected at certain depths in the relatively shallow zones at Site C0014, whereas AOM activity occurred widely in the subseafloor environment, from which positive signals of the existence of microbial communities were obtained (Figure 4). Although the hydrogenotrophic methanogenesis activity outcompeted the AOM activity in the shallower zone and the AOM activity dominated in the deeper zone, the opposing microbial processes of methane production and consumption occurred at the same depths (0.3, 3.6 and 5.0 mbsf in Hole C0014B, 0.2 mbsf in Hole C0014D and 0.3, 4.1 and 7.8 mbsf in Hole C0014G). In addition, relatively high AOM activities and extremely low pore-water sulfate concentrations were observed at the approximate depths of 3.6, 8.6 and 15.8 mbsf in Holes C0014B, C0014D and C0014G, respectively (Figure 1a).

Phylogenetic diversity and the abundance of functional genes related to methanogenesis and AOM were also characterized via both Q-PCR for genes associated with methyl coenzyme M reductase (*mcrA*), dissimilatory sulfite reductase (*dsrA*) and adenosine 5'-phosphosulfate reductase (*aprA*) and clone analysis for *mcrA* (Figure 1d). The Q-PCR data indicated that the *mcrA*, *dsrA* and *aprA* genes were widely distributed in most of the shallow sediments that yielded many positive signals for the existence of microbial communities, and the abundances decreased with increasing depth in the sediment. This pattern also indicated that the complex biogeochemical processes associated with the microbial methane- and sulfate-

related metabolisms co-occur in the sub-vent biosphere at Site C0014. The abundances of these functional genes were one to two orders of magnitude lower than those of the prokaryotic 16S rRNA genes in the same samples.

Based on the sequences of the *mcrA* genes obtained from the clone analysis, the *mcrA* genes were conventionally classified into the types derived from potential methanogenic and methanotrophic populations. The methanogenic type of *mcrA* genes were obtained only from the uppermost sediment at 0.3 mbsf in Hole C0014B (Figure 4) and were phylogenetically related to the *mcrA* genes of *Methanococcoides* (Supplementary Figure S5). The *mcrA* group a-b genes, which were derived from the ANME-1 (Knittel and Boetius, 2009), were most frequently obtained throughout the entire depth of the potential microbial habitable zone at Site C0014. In contrast, most of the *mcrA* gene sequences of groups c-d, e and f, hosted by ANME-2a, -2c and -3 (Knittel and Boetius, 2009), respectively, were found in the shallower depths of sediments (Figure 4). Molecular ecological studies have indicated that the ANME-1 populations occur in deeper, more reductive and more sulfate-depleted habitats than the ANME-2 populations (Knittel *et al.*, 2005; Krüger *et al.*, 2008; Nunoura *et al.*, 2008; Rossel *et al.*, 2011; Yanagawa *et al.*, 2011). Furthermore, the potentially thermophilic ANME-1 group has been recognized as a key component in certain hydrothermal ecosystems, such as Guaymas Basin and Juan de Fuca Ridge (Biddle *et al.*, 2012; Lever *et al.*, 2013; Merkel *et al.*, 2013). This *mcrA* group of thermophilic ANME-1 is defined as Hydrothermal ANME-1 Cluster II (Lever *et al.*, 2013) [alternatively classified as *mcrA*-Guaymas (Biddle *et al.*, 2012) or ANME-1GBa (Merkel *et al.*, 2013)]. The optimal growth temperatures of the thermophilic ANME-1 in the Guaymas site have been estimated to be above 70 °C (Merkel *et al.*, 2013). We detected the same group of *mcrA* gene sequences at 6.7 mbsf in Hole C0014D and 15.8 mbsf in Hole C0014G (Figure 4 and Supplementary Figure S5). The bacterial 16S rRNA gene sequences of potential thermophiles (*Thermotogae* and OP1) were also detected at these sediment depths (Supplementary Data S1). Thus, the possible host archaeal populations of the Hydrothermal ANME-1 Cluster II *mcrA* genes may be thermophilic. Indeed, the radioisotope-tracer AOM activity measurements showed relatively high activity, 3.1 pmol cm^{-3} d^{-1}, at 60 °C at 15.8 mbsf in Hole C0014G (Figure 4).

Synthesis and Interpretation

Development of Methane-Consuming Microbial Communities

Previous studies of microbial communities associated with the hydrothermal vent fluids and chimney deposits in the Iheya North hydrothermal field have hypothesized the existence of active sub-vent microbial communities that are potentially dominated by hyperthermophilic *Thermococcales* and chemolithoautotrophs with H_2- and/or sulfur-compound metabolisms. These metabolisms were extrapolated from the variability in the microbial communities in the most interior and/or reductive seafloor habitats that are highly affected by the hydrothermal fluid input (Nakagawa *et al.*, 2005; Takai *et al.*, 2006). However, several thermodynamic estimates of chemolithotrophic microbial community development in hydrothermal mixing zones have suggested that the populations that couple anaerobic methanotrophy with sulfate reduction would energetically dominate the microbial communities in the sediment-hosted seafloor and subseafloor habitats that are highly affected by hydrothermal fluid inputs (Takai and Nakamura, 2011; Nakamura and Takai, 2014; Takai *et al.*, 2014). In this study, all of the pore-water geochemical analyses, metabolic activity measurements and cellular and molecular microbial community analyses indicated the occurrence of functionally active microbial communities dominated by AOM populations in the relatively shallow subseafloor habitats down to 15.8 mbsf in Hole C0014G. Several studies have examined the abundance, phylogenetic diversity and function of AOM populations associated with seafloor hydrothermal activity in the Guaymas Basin and Yonaguni Knoll IV fields (Teske *et al.*, 2002; Nunoura *et al.*, 2010; Yanagawa *et al.*, 2013a; Dowell *et al.*, 2016). Because these investigations have focused on the shallow sediments just beneath the seafloor, where the diffusive mixing of hydrothermal fluids and seawater likely characterizes the geochemical environments, there remains a lack of knowledge on the subseafloor AOM communities associated with hydrogeologically controlled advection and the partitioning and mixing processes of hydrothermal fluids and infiltrated seawater near deep-sea vents.

The vertical profiles of pore-water methane and sulfate concentrations (Figure 1a) and stable isotopic values of $\delta^{13}C_{CH4}$ and δD_{CH4} (Figure 2) indicated the abundant occurrence of potentially sulfate-reducing AOM functions in several specific horizons within the microbially habitable subseafloor environment (e.g., depths of 0-11.7 mbsf in Hole C0014B and 0-10.2 and 0-15.8 mbsf in Hole C0014G). In addition, previous

study of significant ^{34}S enrichment in pore-water sulfate strongly suggested the presence of microbial sulfate reduction at several depths within the microbially habitable terrain, i.e., approximately 5 mbsf in Hole C0014B and 16.1 mbsf in Hole C0014G (Aoyama *et al.*, 2014). These compositional and isotopic profiles of pore-water methane and sulfate are largely dependent on the mass balance of hydrothermal fluid and seawater inputs and *in situ* microbial consumption. Thus, the geochemically identified peaks and valleys in the potential microbial methane oxidation and sulfate reduction are not necessarily equivalent to the abundance of *in situ* microbial functions. Indeed, the potential *in situ* microbial activity profile of AOM, as determined via radioisotope-tracer experiments, did not match the geochemically identified peaks and valleys of microbial methane oxidation (Figure 4), and the *mcrA* and *dsrA* gene abundances gradually decreased with increasing depth (Figure 1d). However, overall, the pore-water geochemistry, the *in situ* metabolic activities and the 16S rRNA and functional gene distributions indicated a significant association between subseafloor AOM activity and sulfate reduction. On the other hand, previous studies have suggested that microbial AOM activity might be coupled to the reduction of iron (hydr)oxide minerals but not sulfate reduction in metalliferous hydrothermal sediments (Wankel *et al.*, 2012). The physical and chemical variations in the subseafloor environment resulting from advective hydrothermal fluid and seawater flows related to the lithostratigraphic and hydrogeologic conditions would create diverse biogeochemical processes.

Constraints on Microbial Community Development in the Sub-Vent Biosphere

All of the direct microscopic observations, PCR-based molecular analyses, cultivation tests and metabolic activity measurements indicated that functionally active, metabolically diverse microbial communities developed in the shallow zones of subseafloor sediments associated with hydrothermal fluid flows. The positive signals of the existence of microbial communities were obtained from the sediments down to 14.3, 10.2 and 15.8 mbsf in Holes C0014B, C0014D and C0014G, respectively. Microbial populations were not detected in the deeper, high-temperature hydrothermal fluid regimes, due to the limit of microbial habitability and/or the methodological detection limit in this study. The Q-PCR and clone library analyses for 16S rRNA genes showed that the abundance of archaeal 16S rRNA gene phylotypes increased with depth, whereas bacterial phylotype populations dominated the microbial communities at shallower depths (Supplementary Figure S3). However,

neither the culture-dependent nor the culture-independent analyses detected the existence of certain previously cultivated hyperthermophilic populations, such as *Thermococcales*, *Methanococcales*, *Archaeoglobales* and members of *Crenarchaota*, even though such hyperthermophilic archaeal populations are known to dominate the microbial communities in other high-temperature hydrothermal fluid and chimney habitats (Nakagawa *et al.*, 2005; Takai *et al.*, 2006). One conceivable explanation for this may relate to unstable temperature conditions for their growth due to fluctuating hydrothermal fluids, as described below. In the 16S rRNA gene clone libraries obtained from the deepest sediments of the microbially habitable terrain in the subseafloor environment, putative thermophilic phylotypes, such as members of HWCGIV, thermophilic ANME-1, OP1 and *Thermotogae*, were found (Figure 3 and Supplementary Data S1). According to the molecular thermometer calculation based on the GC content of the 16S rRNA gene sequences (Kimura *et al.*, 2010), the potential growth temperature range was estimated to be the highest (50 °C to 76 °C) for members of HWCGIV among the putative thermophilic populations.

The relationship between microbial habitability and the *in situ* temperature of the subseafloor biosphere has rarely been investigated. Recently, an active subseafloor microbial community has been discovered in deeply buried terrigenous sediments with a temperature of <60 °C at 2,458 mbsf (Inagaki *et al.*, 2015). The low abundance of this community was attributed to the increase in energy used for the repair of essential biomolecules such as amino acid and DNA. The energetic costs of amino acid racemization and DNA depurination increase exponentially with temperature (Lever *et al.*, 2015). However, the physical and chemical conditions that form the boundary between habitable and uninhabitable terrains have not been directly explored in subseafloor environments (Takai *et al.*, 2014). Sub-vent microbial communities are believed to live near the upper temperature limits for life on this planet. However, based on the predicted thermal gradient from the temperatures measured during the drilling operation (Takai *et al.*, 2011), the temperature near the detection limit of microbial populations, activities and molecules in Hole C0014B (14.3 mbsf) was estimated to be ca. 45 °C. In contrast, the geochemical thermometer estimate based on the oxygen isotopic compositions of hydrothermally altered clay minerals from 12.8-15.1 mbsf in Hole C0014B suggested that the boundary habitat experienced higher temperatures (>106 °C) (Supplementary Table S1 and

Supplementary Figure S1). The former temperature (ca. 45 °C) and latter (>106 °C) temperature are far below and similar to the upper temperature limit for microbial growth, respectively (Takai *et al.*, 2008). Additionally, the molecular thermometer estimate based on the potentially most thermophilic 16S rRNA gene phylotype (HWCGIV) population at the depth limit yields a growth temperature range of 50 °C to 76 °C. It remains uncertain which of the estimated temperatures most accurately represents a realistic *in situ* temperature range because the bottom temperature of present microbial habitable zone was not directly measured during the drilling operation, and the geochemical and molecular thermometer estimates do not necessarily reflect the current *in situ* temperature range. One plausible interpretation is that the deepest microbial habitat in Hole C0014B has been exposed to considerably high temperatures (>106 °C) in the past and presently experiences fluctuating temperatures that are induced by ever-varying degrees of mixing between the subseafloor high-temperature hydrothermal fluids, diffusive and/or advective seawater flows and conductive cooling. Similarly, the hydrothermally altered sediments that were observed in the shallow sediments at Site C0013 and the oxygen isotopic values of the clay minerals indicate formation temperatures of >138 °C (Miyoshi, 2013) (Supplementary Table S1). The excess levels of pore-water sulfate concentrations (relative to the sulfate concentrations of seawater) in these sediments were attributed to anhydrite dissolution related to cooling during core recovery and sampling (Supplementary Figure S2). Because anhydrite is stable only under high temperatures (>150 °C) (Gieskes *et al.*, 2002; Takai *et al.*, 2011), the subseafloor sediments at Site C0013 that contain excess pore-water sulfate concentrations (relative to seawater) are likely exposed to high temperatures that exceed the microbially habitable range at the present. The microbial habitability in the sub-vent biosphere is probably highly constrained by the latest temperature history and/or the present temperature conditions induced by spatiotemporally variable high-temperature hydrothermal fluid input.

Furthermore, the physical properties of the sediments are another important factor constraining the microbially habitable terrain in the sub-vent environment. The possible impermeable layers, which are predicted from the discontinuities in the pore-water chemical compositions, serve as not only shields limiting vertical flow and diffusion of fluids but also as barriers limiting vertical migration of microbial cells. Interestingly, the potential limit of microbial community development was always

located above or near the possible impermeable layers at Site C0014 (Figure 1). Fluctuations in temperature in the boundary habitat probably occasionally sterilize the microbial community via exposure to high-temperature hydrothermal fluids, and this is followed by a gradual return to microbially habitable temperatures. However, the existence of impermeable layers may contribute to the slow recolonization of the active microbial community after sterilization because the impermeable layer limits migration of viable populations from refugia. Additionally, the impermeable layers may also serve as the possible microbial refugia from the lethal temperature fluctuations associated with the occasional exposure to high-temperature hydrothermal fluids. The slow but successful recolonization of the active microbial community after a temporary sterilization may be initiated from viable microbial cells disseminated throughout the safe interior of impermeable layers. Although the permeable and impermeable inter-layer sequences of cores are often difficult to recover in IODP-like drilling operations, these hypotheses of the limit and recolonization of the sub-vent biosphere should be clarified in future research.

Conflict of Interest

The authors declare no conflict of interest.

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Figure 1 (next site) Pore-water concentrations of methane and sulfate (Takai *et al.*, 2011) (**a**), the carbon and hydrogen isotopic composition of methane (**b**), the numbers of 16S rRNA and functional genes (**c** and **d**) and heterotrophic activity (**e**) in the subseafloor core samples at IODP Site C0014. Q-PCR was used to quantify numbers of 16S rRNA genes of prokaryotes (black circles) and archaea (red diamonds), and functional genes of *mcrA* (purple diamonds), *dsrA* (green triangles) and *aprA* (orange squares). The total cell counts (gray crosses) were originally reported by Takai *et al.* (2011). Heterotrophic activity was determined based on the potential activity of the anaerobic oxidation of acetate. The incubations were conducted at 30 °C and 60 °C based on the *in situ* temperatures in Supplementary Figure S1. The samples incubated at 60 °C were indicated with parentheses, next to each symbol. Values below the detection limit for the Q-PCR data and heterotrophic activity are plotted as open symbols on the left axes. The red-shaded layers indicate the depth range of the limits for microbes based on Q-PCR data and heterotrophic activity. These depth ranges correspond to the depths of 14.3-17.2 mbsf in Hole C0014B, 10.2-11.4 mbsf in Hole C0014D and 15.8-17.6 mbsf in Hole C0014G. The possible impermeable layers in Supplementary Figure S2 are indicated by the blue dashed lines.

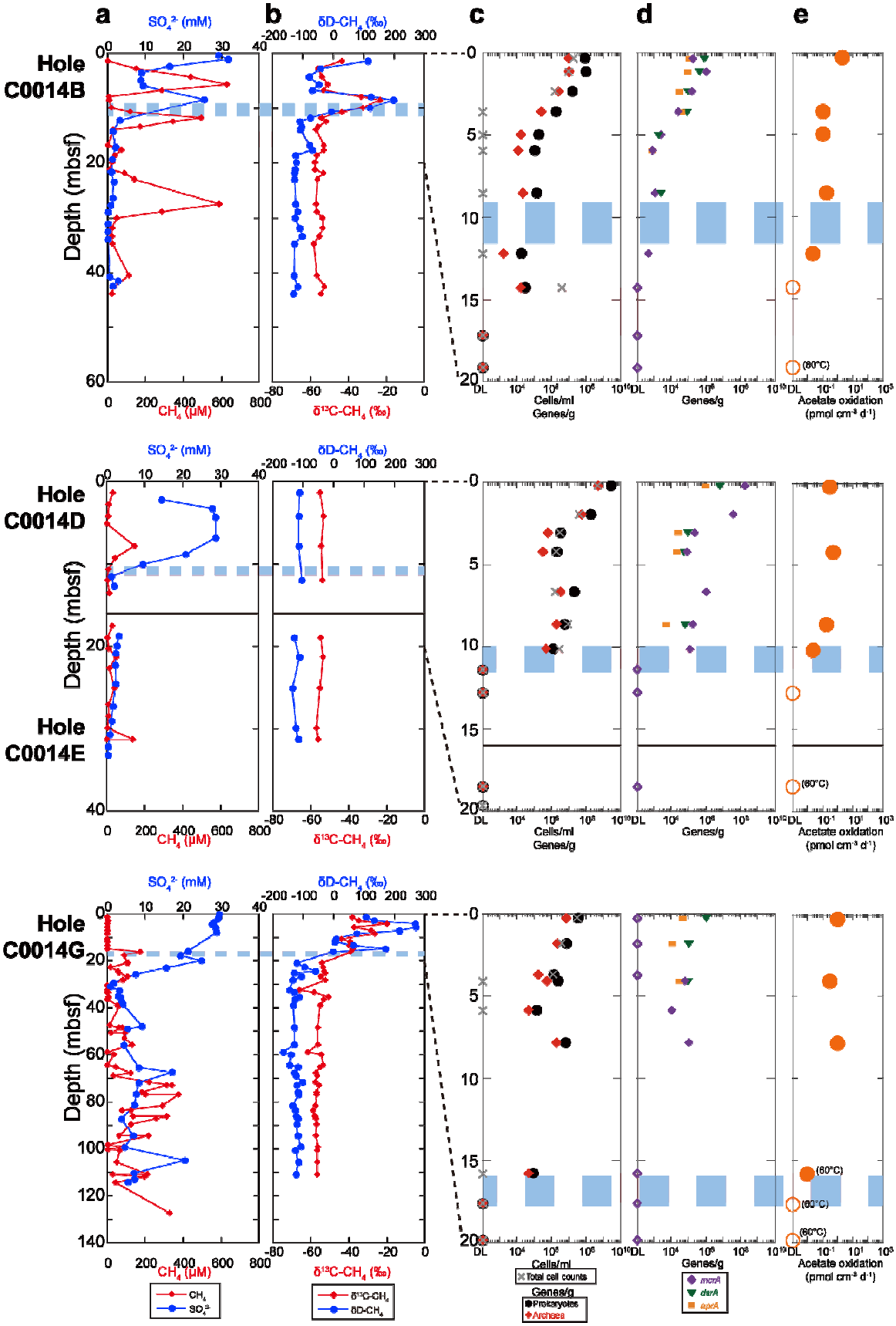
a
b
c
d
e
Hole C0014B
Hole C0014D
Hole C0014E
Hole C0014G
Depth (mbsf)
SO4 2- (mM)
δD-CH4 (‰)
CH4 (μM)
δ13C-CH4 (‰)
Cells/ml
Genes/g
Acetate oxidation (pmol cm-3 d-1)
(60°C)
Total cell counts
Prokaryotes
Archaea
mcrA
dsrA
apsA

Fig. 1

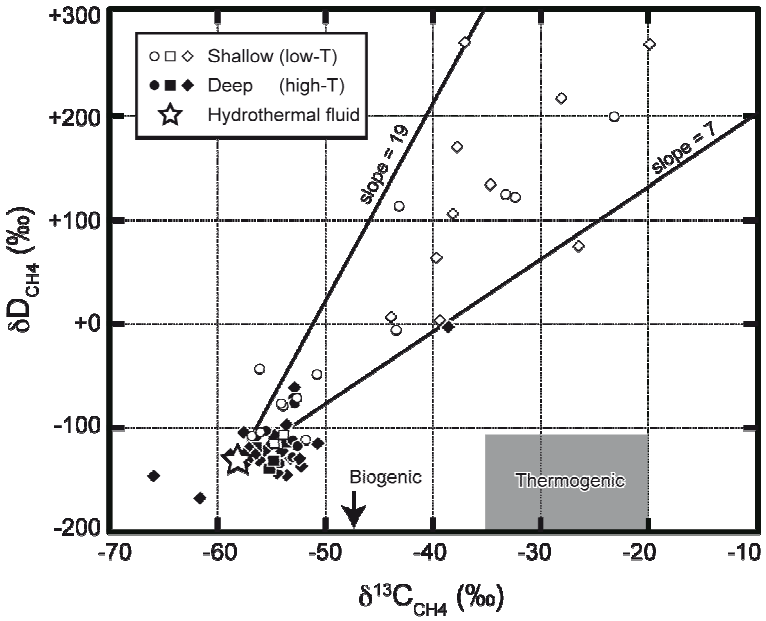

Fig. 2

Cross plots of $\delta^{13}C_{CH4}$ and δD_{CH4} values. The open star represents the previously reported high-temperature hydrothermal fluid value (Kawagucci *et al.*, 2011). Open and filled symbols represent samples obtained from shallow (low-temperature) and deep (high-temperature) zones in each hole, respectively (criteria of the depth are shown in Figure 1). Circles, squares and diamonds represent Holes C0014B, C0014DE and C0014G, respectively. Representative values for thermogenic and biogenic methane (Kawagucci *et al.*, 2013b) are shown by a shaded area and an arrow, respectively. The diagonal lines, with slopes of 7 and 19, represent the lowest and highest values of the co-variation exhibited in microbial methane oxidation.

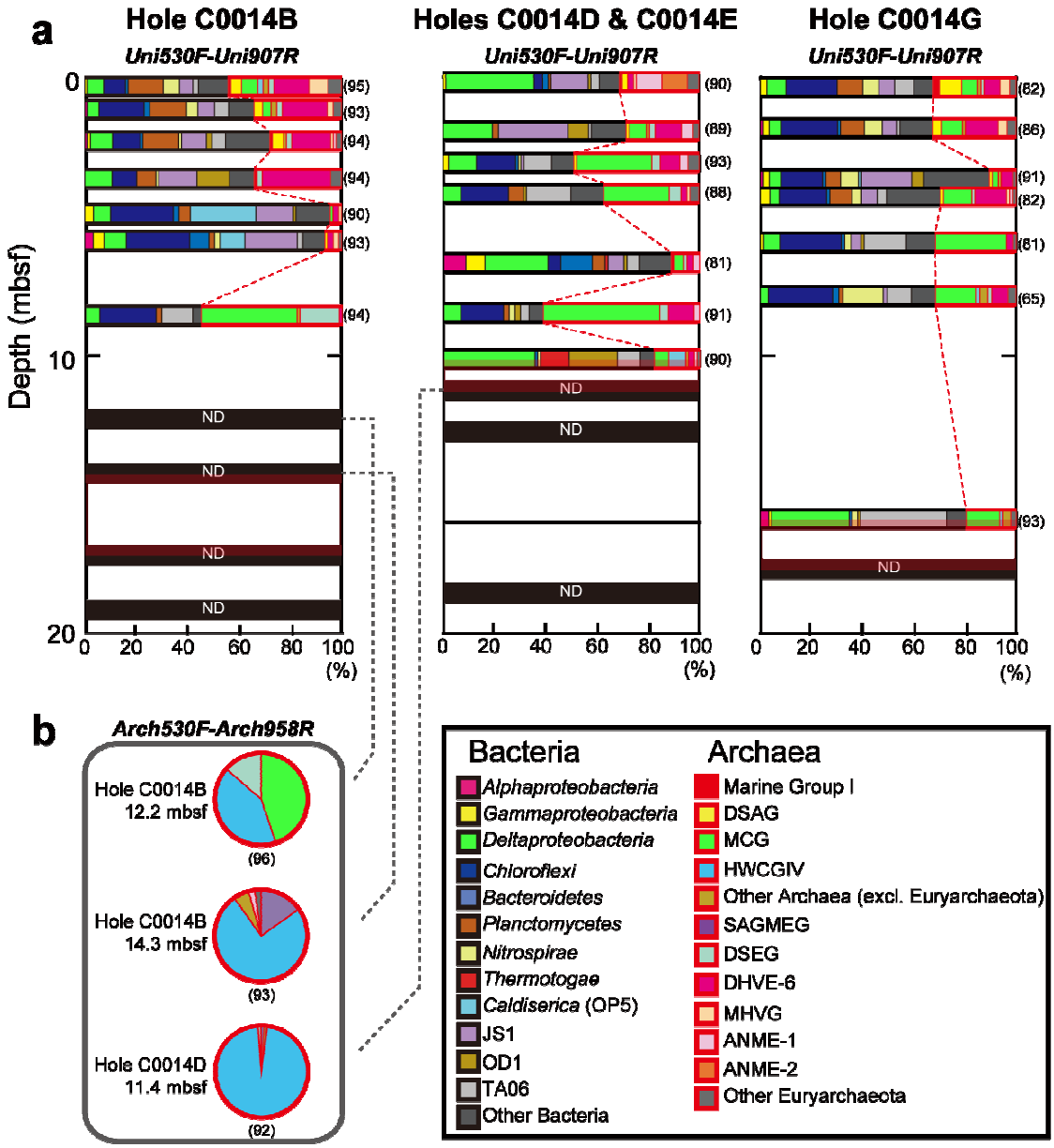

Fig. 3

16S rRNA gene phylotype compositions in the sediments from Site C0014, displayed (**a**) with respect to sediment depth using a universal primer set (Uni530F-907R) and (**b**) as pie diagrams at specific depths using an archaea-specific primer set (Arc530F-Arc958R). Archaeal 16S rRNA gene amplicons were obtained from three samples, which were not amplified with Uni530F-907R (shown as "ND" in the black column). The red dotted lines denote the cutoff between Bacteria and Archaea. The numbers in parentheses indicate the number of clones. The red-shaded layers represent the possible depth limit of active subseafloor microbes, as determined in Figure 1

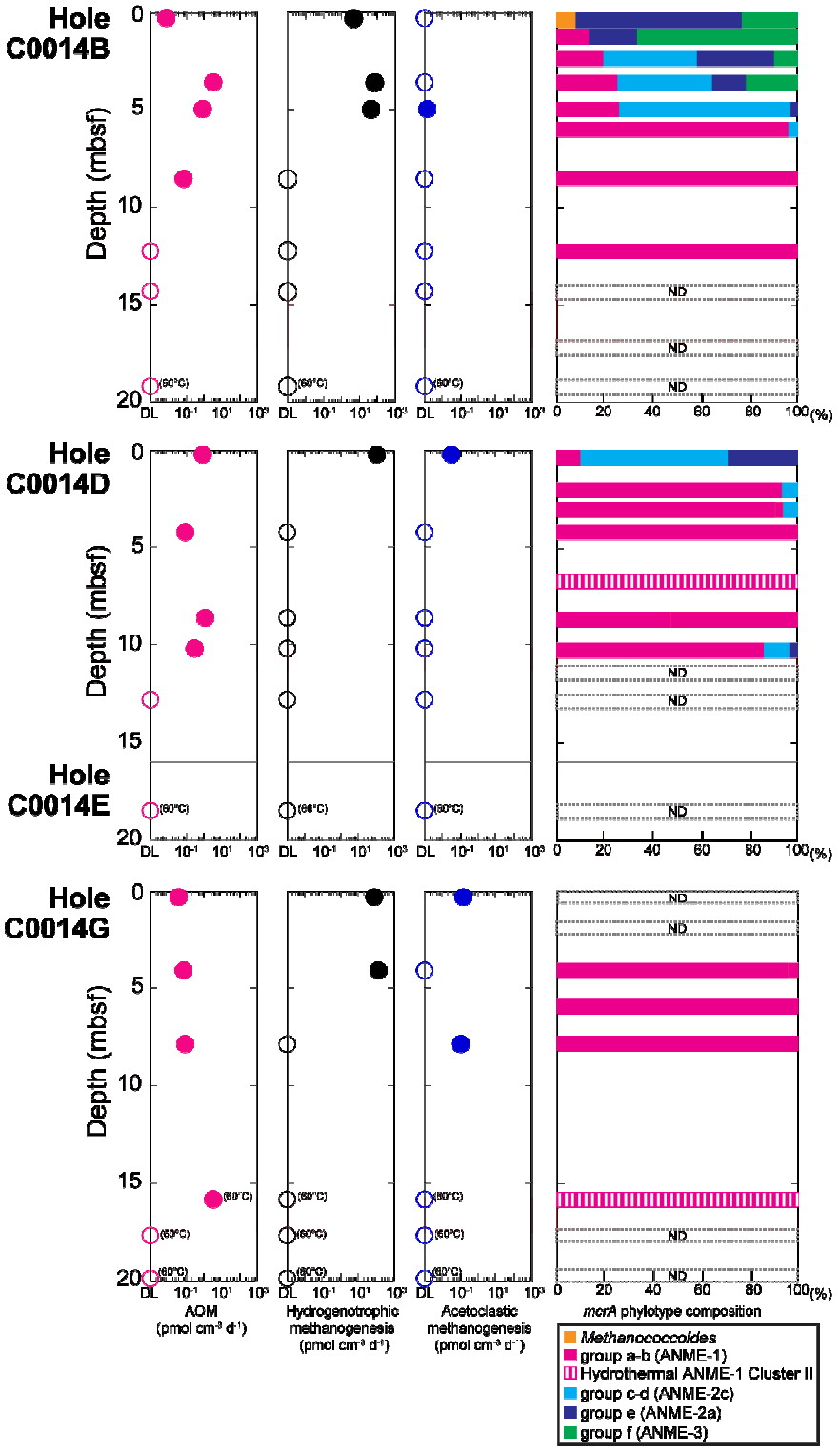

Fig. 4

Site C0014 depth profiles of the potential activity of AOM, hydrogenotrophic methanogenesis and acetoclastic methanogenesis (from left to right). The phylogenetic affiliation of mcrA genes is shown in the rightmost column. Open circles on the y-axes denote analyses below the detection limit. The red-shaded layers represent the possible depth limit of active subseafloor microbes, as determined in Figure 1.

Supplementary Information

Supplementary Information Materials and Methods

Sampling Sites and Sample Collections.

IODP Expedition 331 was conducted at the Iheya North hydrothermal field in the Mid-Okinawa Trough using the D/V *Chikyu* in September 2010 (Takai *et al.*, 2011). IODP Sites C0013 and C0014 were located 100 and 450 m east, respectively, of a hydrothermal activity center that hosts several hydrothermal mounds and high-temperature fluid vents. The study area was covered with hemipelagic sediments and pumiceous volacaniclastic deposits (Takai *et al.*, 2011). A detailed geological and lithological description is available elsewhere (Takai *et al.*, 2011; Miyoshi, 2013). The drilling sites were surrounded by colonies of chemosynthetic animals, such as *Shinkaia crosnieri*, *Bathymodilous* spp. and *Calyptogena okutanii*. *In situ* temperatures were measured using an advanced piston corer temperature tool (APCT-3) and thermoseal strips (Nichiyu Giken Co., Ltd., Kawagoe, Japan) during the drilling and coring operations (Supplementary Figure S1).

The collected cores were immediately cut into 1.5-m-long sections on deck. Then, whole round cores (WRCs), approximately 10 to 20 cm in length, were taken from each core section for subsequent microbiological and geochemical analyses. Microbiological subsamples were obtained from the inner parts of the WRCs with a sterilized spatula. For the radiotracer incubation and culture experiments, a portion of the sediment was anaerobically stored at 4°C in glass vials with argon as the headspace gas. The subsamples for DNA analysis were immediately stored at -80 °C in heat-sealed laminated foil bags containing an oxygen scavenger (Lin *et al.*, 2010). The WRCs for pore-water chemistry were collected from the sections adjacent to the microbiology WRCs.

Geochemical Analyses.

Methane concentrations in the pore-water were derived from the headspace concentrations measured using the GC-FID on board the research vessel (Takai *et al.*, 2011) and the following mass balance approach:

$$CH_4 = [\chi_M \times P_{atm} \times V_H]/[R \times T \times V_{pw}],$$

where

V_H = volume of headspace in the sample vial,

V_{pw} = volume of pore-water in the sediment sample,

χ_M = molar fraction of methane in the headspace gas (obtained from GC analysis),

P_{atm} = pressure in the vial headspace (assumed to be the measured atmospheric pressure when the vials were sealed),

R = universal gas constant, and

T = temperature of the vial headspace in degrees Kelvin.

The volume of the interstitial water in the sediment sample was determined based on the bulk mass of the wet sample, the sediment's porosity (which was extrapolated from shipboard moisture and density [MAD] measurements in adjacent samples), shipboard grain density data and the density of the pore-water adjusted for salinity based on shipboard data.

Stable carbon and hydrogen isotope ratios for CH_4 were determined at the Japan Agency for Marine-Earth Science and Technology (JAMSTEC) with a MAT253 (Thermo Fisher Scientific, Bremen, Germany) by continuous-flow isotope ratio mass spectrometry (CF-IRMS) as described previously (Umezawa *et al.*, 2009), with some modifications. A helium-purged purification line made of stainless-steel tubing including several 2-position valves (VICI Precision Sampling, Inc., Louisiana, USA) with chemical and cold traps was used. Ultra-pure helium (purity > 99.9999%: Iwatani Gasnetwork Corporation, Osaka, Japan) was used with further purification by a Molecular Sieve 5A column at -196 ºC (LqN_2 bath). The sample gas is introduced into a 30 ml/min (approximately +0.2 MPa) helium stream, the 'precon stream', in the purification line via a gas tight syringe (PRESSURE-LOK® series, VICI Precision Sampling, Inc., Louisiana, USA). Methane in the sample gas was first separated from CO_2, H_2O and most non-methane volatile organic carbons by a stainless-steel coil held at -110 ºC (ethanol/LqN_2 bath) and a chemical trap filled with magnesium perchlorate ($Mg(ClO_4)_2$; Merck KGaA, Darmstadt, Germany) and Ascarite II (sodium-hydroxide-coated silica; Thomas Scientific, Swedesboro, New Jersey, USA) and then condensed on a stainless-steel tubing trap filled with Hayesep-D porous polymer (60/80 mesh, Hayes Separations Inc., Texas, USA) held at -130 ºC (ethanol/LqN_2 bath). After transfer of the condensed CH_4 by turning the valve position to another helium stream, the 'GC stream', set at 1.0 ml/min, the CH_4 condensed was released at >80 ºC (hot water bath), again condensed in a capillary trap made of PoraPLOT Q (length: 20 cm; ID: 0.32 mm) held at -196 ºC (LqN_2 bath), and finally released at room temperature. After complete separation in the 25-m-long PoraPLOT Q column

(ID: 0.32 mm), the effluent CH_4 was delivered to combustion or pyrolysis units (Thermo Fisher Scientific, Massachusetts, USA) to be converted into CO_2 or H_2, respectively. In this study, the combustion and pyrolysis units were kept at 960 ºC and 1,440 ºC, respectively. Prior to the sample injection, the pyrolysis unit was conditioned by ≥5 injections of 0.2 ml of pure CH_4 to form a graphite coat on the inner walls of the tubing for quantitative conversion of the sample CH_4. The CH_4-derived CO_2 and H_2 were finally introduced into the open split interface of the MAT253 for carbon or hydrogen isotope ratio analyses. Mass-2 and -3 signals were processed using the ISODAT software package (Thermo Fisher Scientific), on which H_3^+ factor was corrected. This study presents stable isotope ratios with conventional delta notation in permil. The analytical precisions for the $\delta^{13}C_{CH4}$ and δD_{CH4} values were estimated via repeated analyses of a standard gas to be within 0.5‰ and 5‰, respectively. The determined δ values were calibrated with commercial and/or in-house standard gases with the following values: –74.01‰ and –39.03‰ for $\delta^{13}C_{CH4}$ and –185.9 ‰ for δD_{CH4}.

The DIC concentration analysis was conducted by the CO_2 acid extraction and coulometer detection method onboard the *Chikyu* during IODP Expedition 331. The procedure is described in detail elsewhere (Noguchi *et al.*, 2013). The detection limit and precision of this procedure were 66.6 μmol/kg and ±4.9%, respectively.

The $\delta^{13}C_{DIC}$ values of the pore-water samples were measured with a ThermoFinnigan Delta Plus XP IRMS instrument connected to a Flash EA 1112 Automatic Elemental Analyzer via a ConFlo III interface in a similar manner as described previously (Miyajima *et al.*, 1995; Toki *et al.*, 2004). The standard deviation of the repeated carbon isotope analysis of the laboratory standard ($NaHCO_3$ solution) was <0.2‰.

Oxygen Isotopic Composition and Formation Temperature Estimates of Hydrothermally Altered Mud.

The sediment samples collected from shallow depths at Sites C0013 and C0014 (C0013B 1T-1 80-82, C0013D 1H-2 68-80, C0014B 2H-7 40-50 and C0014B 2H-10 20-30) were used in this study. The samples were disaggregated in distilled water and rinsed several times to remove dissolved salts. Clay fraction samples (< 2 μm) were obtained by suspending bulk sediment samples in distilled water for 5 h according to Stokes' law. Clay minerals in the clay fraction samples were identified by X-ray diffraction (XRD) at an onshore laboratory and were reported elsewhere

(Miyoshi, 2013). The oxygen and hydrogen isotope values of the clay fraction samples were determined and reported by Miyoshi (2013). The clay mineral assemblage and oxygen isotope values of the samples are provided in Supplementary Table S1.

The clay-fraction sample C0013B 1T-1 80-82 from 0.8 mbsf consisted primarily of kaolinite and illite. The clay-fraction samples C0013D 1H-2 68-80, C0014B 2H-7 40-50 and C0014B 2H-10 20-30 consisted primarily of kaolinite and smectite. The obtained oxygen isotope value is that of the mixture of these clay minerals. We calculated two types of oxygen isotope equilibrium temperatures to estimate the formation temperatures of the clay minerals at these depths. One is based on the oxygen isotope equilibrium temperature of kaolinite, and the other is based on that of illite (C0013B 1T-1 80-82) or smectite (C0013D 1H-2 68-80, C0014B 2H-7 40-50 and C0014B 2H-10 20-30).

The oxygen isotope equilibration temperatures between the clay minerals and water were calculated according to the temperature dependence of the equilibrium (Sheppard and Gilg, 1996), which can be described as follows:

$$1000 \ln \alpha \text{ kaolinite-water} = 2.76 \times 10^6 \times T^{-2} - 6.75$$

$$1000 \ln \alpha \text{ illite-water} = 2.39 \times 10^6 \times T^{-2} - 3.76$$

$$1000 \ln \alpha \text{ smectite-water} = 2.55 \times 10^6 \times T^{-2} - 4.05$$

where T is temperature in Kelvin and “α” is the fractionation factor.

Water equilibrated with clay minerals should have oxygen isotope values ranging between those of the bottom seawater and the hydrothermal fluid. Indeed, high-temperature hydrothermal fluid started to discharge from certain holes at Sites C0013, C0014 and C0016 during the drilling (Takai *et al.*, 2011). The newly discharged hydrothermal fluids sampled 5 months after the drilling operation had an average oxygen isotope value of +1.22‰ (Kawagucci *et al.*, 2013). Although the oxygen isotope value of the local bottom seawater in the Iheya North Knoll was not reported, the value is considered to have a typical value (0.0‰). In this study, these two values (1.22‰ and 0.0‰) were applied in the estimation.

DNA Extraction, Amplification, Sequencing and Phylogenetic Analysis of the 16S rRNA and McrA Genes.

DNA was extracted from 2 g of sampled sediment using the PowerMAX Soil DNA isolation kit (MoBio Laboratories, Carlsbad, CA) according to the manufacturer’s protocol, with minor modifications. Before the physical cell disruption, the samples

were incubated at 65 °C for 5 min. Then, mechanical shaking was performed for 10 min with a ShakeMaster (BioMedical Science, Tokyo, Japan). A simultaneous blank sample was also used as a negative control during the DNA extraction process. The extracted DNA was concentrated by ethanol precipitation and stored at -80°C.

The 16S rRNA gene fragments were amplified by PCR using a universal primer set, Uni530F-Uni907R (Nunoura *et al.*, 2012), for all samples. An archaea specific primer set, composed of Arch_530F, Arch2_530F, Nano_530F (Nunoura *et al.*, 2012) and Arc958R (DeLong, 1992), was used for the samples obtained from the deepest part of the microbially habitable terrain. PCR amplification of the negative control for DNA extraction was used to assess experimental contamination. The gene fragments of *mcrA* were also amplified using primers ME3MF, ME3MF-e and ME2r' (Nunoura *et al.*, 2008). The amplification conditions and primer sequences for each of the PCR amplifications are summarized in Supplementary Table S2.

The PCR products were purified after gel electrophoresis, cloned into vector pCR2.1-TOPO (Invitrogen, Carlsbad, CA) and then transformed into chemically competent *Escherichia coli* DH5α (Invitrogen, Carlsbad, CA, USA). The inserted DNA was sequenced with the M13 forward primer using an ABI3730xl DNA analyzer.

Partial 16S rRNA and *mcrA* gene sequences with >97% and >95% sequence identity, respectively, were assigned to the same phylotype. Representative 16S rRNA gene sequences were aligned using the SINA aligner (Pruesse *et al.*, 2012). Phylogenetic affiliations were identified by the maximum-parsimony method using the SILVA SSU Ref 111 Database in the ARB software program (Ludwig *et al.*, 2004). The 16S rRNA gene sequences from the WRCs with a >97% similarity to those of the drilling fluids reported previously (Yanagawa *et al.*, 2013) were defined as potential contaminants. Representative *mcrA* gene sequences were aligned with closely related *mcrA* gene sequences from public databases using the CLUSTALW program, and the ambiguous nucleotide positions were corrected manually. Phylogenetic affiliations were assigned based on the phylogenetic trees constructed by the neighbor-joining method in the ARB software. Bootstrap analysis was performed with 1,000 replicates.

Quantitative Fluorescence PCR.

Q-PCR for 16S rRNA genes was performed as previously described (Nunoura *et al.*, 2008; Blazejak and Schippers, 2011; Breuker *et al.*, 2013). Copy numbers of the 16S rRNA genes were determined using a universal primer-probe set (Takai and

Horikoshi, 2000) and an archaea-specific primer-probe set (Takai and Horikoshi, 2000). The functional genes *dsrA*, *aprA* and *mcrA*, which encode dissimilatory sulfite reductase alpha subunit, adenosine 5'-phosphosulfate reductase alpha subunit and methyl coenzyme M reductase alpha subunit, respectively, were quantified using each specific primer set (Hales *et al.*, 1996; Kondo *et al.*, 2004; Schippers and Neretin, 2006; Meyer and Kuever, 2007; Nunoura *et al.*, 2008; Blazejak and Schippers, 2011). The primers, probes and Q-PCR conditions used in the present study are summarized in Supplementary Table S2. After each Q-PCR, melting curves were measured for SYBR green I assays. All Q-PCR assays were performed in triplicate.

Cultivation Tests.

To estimate the abundance of culturable microorganisms represented by a variety of physiological and metabolic characteristics, a series of serial dilution cultures were performed using sediment slurries from a variety of depths. For anaerobic heterotrophs, methanogens and sulfate reducers, basal culture media were prepared using the modified MJ seawater (Takai *et al.*, 1999), which was composed of 0.14 g of K_2HPO_4, 4.5 g of $MgCl_2 \cdot 6H_2O$, 0.125 g of NH_4Cl, 20 g of NaCl, 0.8 g of $CaCl_2 \cdot 2H_2O$, 0.33 g of KCl, 0.5 mg of $NiCl_2 \cdot 6H_2O$, 2 g of $NaHCO_3$, 1 µg of sodium selenite, 0.5 µg of sodium tungstate, 1 mg of resazurin, 10 ml of a trace mineral solution containing chloride-based reagents as a substitute for sulfate-based reagents, and 1 ml of a vitamin solution (Balch *et al.*, 1979). The pH of the medium was adjusted to approximately 7.0-7.5 with HCl or NaOH before autoclaving. Anaerobic heterotrophs were cultured using basal medium containing yeast extract (0.1% [w/v]), tryptone (0.1% [w/v]) and glucose (0.01% [w/v]) under a headspace gas of N_2/CO_2 (80:20 [v/v]). For the cultivation of methanogens, the basal medium containing acetate (5 mM) and formate (5 mM) were used, whereas the basal medium for sulfate reducers contained pyruvate (10 mM), lactate (5 mM) and Na_2SO_4 (30 mM) under a gas phase of 80% H_2 and 20% CO_2. The cultivated abundance of aerobic heterotrophs was evaluated through colony-forming units on marine agar 2216 (BD). The incubations were conducted at 30 °C and 60 °C based on the *in situ* temperatures in Supplementary Figure S1. An axenic culture was obtained after the serial dilution analysis. The culture purity was determined from cell morphology by microscopy (Olympus BX51F). For phylogenetic analysis of the isolates, DNA extraction, PCR amplification and sequencing were performed as described

previously (Miyashita *et al*., 2009). The 16S rRNA gene fragment was amplified by PCR using EUB338F (Amann *et al*., 1990; Daims *et al*., 1999) and 1492R (Weisburd *et al*., 1991). The obtained sequences were subjected to similarity analysis with the BLAST program (Altschul *et al*., 1997).

Microbial Activity Measurements.

The potential rates of methane production and oxidation and acetate consumption (i.e., acetoclastic methanogenesis and oxidation of the methyl group of acetate to CO_2) were determined through radioisotope tracer incubation experiments. Sediment samples (3 cm^3) were added to glass vials, amended with 9 ml of anoxic artificial seawater (MJ medium) and sealed with butyl-rubber stoppers in an anoxic glove chamber. To estimate the anaerobic methane oxidation (AOM) rate, the headspace of the vials was filled with 200 kPa of methane, and this was followed by the injection of ^{14}C-labeled methane (0.3 MBq) into the slurry samples. For hydrogenotrophic methanogenesis rates, 1% H_2 was added to the headspace, and ^{14}C-bicarbonate (0.5 MBq) was injected into the samples. The designated concentrations of methane and hydrogen were determined based on the results of headspace gas analyses, described previously (Takai *et al.*, 2011). For acetoclastic methanogenesis and acetate oxidation rates, [2-^{14}C] acetate (0.6 MBq) was injected into the samples along with 200 kPa of nitrogen gas in the headspace. The samples were incubated for 1 month at 30°C for sediments shallower than 15 mbsf and 60°C for sediments deeper than 15 mbsf. After the incubation, microbial reactions were stopped by alkalization with 0.5 ml of 10 N NaOH, and the slurry samples were stored at -20°C until the radioactivity measurements. To determine the rates of hydrogenotrophic and acetoclastic methanogenesis, the headspace gas was injected into a gas chromatograph (Shimadzu GC-2014, Shimadzu, Kyoto, Japan) with a stainless packed column Shincarbon ST (Shinwa Chemical Industries, Kyoto, Japan) for gas separation. The radioactivity of a portion of the reaction products (i.e., $^{14}CH_4$) in the headspace was measured using the high-sensitivity radioactivity detector RAGA Star (Raytest, Straubenhart, Germany). For AOM and acetate oxidation rates, the samples were acidified with 1 ml of 6 N HCl to release CO_2 into the headspace before the radioactivity measurement of $^{14}CO_2$. The potential activity was calculated by the following equations:

$$\text{Potential rate} = k \times \Phi \times C,$$

$$\text{Turn over } (k) = F \times (\alpha_p/\alpha_s) / t,$$

where Φ is the porosity of the sediments, *C* is the in situ substrate concentration, *F* is the isotope fraction factor, α_p is the radioactivity of the product pool, α_s is the radioactivity of the substrate pool and *t* is the incubation time (Tasumi *et al.*, 2015). The lower detection limit of the potential activity is on the order of nmol cm^{-3} d^{-1}.

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Supplementary Table S1. Oxygen isotopic compositions and formation temperatures of clay minerals from Sites C0013 and C0014

Sample ID	Depth (mbsf)	Dominant clay mineral	$\delta^{18}O_{(clay\ fraction)}$ (‰)	Formation temperature of kaolinite*		Formation temperature of smectite*		Formation temperature of illite*		Reference
				$T_{seawater}$ (°C)†	$T_{hydrothermal}$ (°C)‡	$T_{seawater}$ (°C)†	$T_{hydrothermal}$ (°C)‡	$T_{seawater}$ (°C)†	$T_{hydrothermal}$ (°C)‡	
C0013B 1T-1 80-82	0.8	kaolinite, illite	+9.6	138	155			151	171	Miyoshi 2013; Miyoshi *et al*., 2015
C0013D 1H-2 68-80	4.5	kaolinite, smectite	+8.0	160	179	187	213			
C0014B 2H-7 40-50	12.8	kaolinite, smectite	+12.5	106	119	120	136			
C0014B 2H-10 20-30	15.1	kaolinite, smectite	+8.7	150	168	175	198			

*Formation temperatures of clay minerals were estimated based on an assumption that the δ18O values of kaolinite, illite and smectite were the same as the δ18O values of the clay fraction
†Oxygen isotope equilibrium temperature between seawater and clay mineral
‡Oxygen isotope equilibrium temperature between hydrothermal fluid and clay mineral

Supplementary Table S2. Primers, probes and amplification conditions for PCR and Q-PCR assays

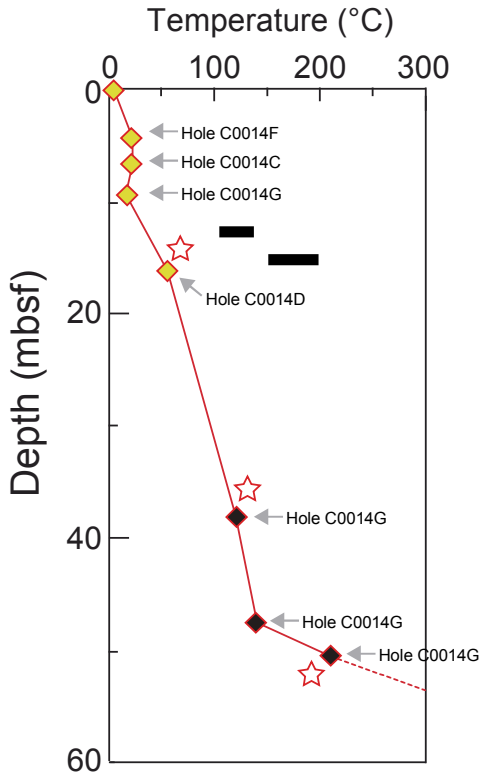
Target gene	Assay	Amplification condition	Primer/Probe	Sequence (5'-3')	Reference
Prokaryotic universal 16S rRNA gene	PCR	40 cycles of 96°C for 25s, 50°C for 45s and 72°C for 60s	Uni530F mix	-	Nunoura *et al.,* 2012
			Uni907R mix	-	Nunoura *et al.,* 2012
Archaeal 16S rRNA gene	PCR	40 cycles of 96°C for 25s, 50°C for 45s and 72°C for 60s	Arch_530F	GTGBCAGCCGCCGCGG	Nunoura *et al.,* 2012
			Arch2_530F	YTGCCAGCCGCCGCGG	Nunoura *et al.,* 2012
			Nano_530F	GTGGCAGTCGCCACGG	Nunoura *et al.,* 2012
			Arc958R	YCCGGCGTTGAMTCCAATT	Delong, 1992
Methyl coenzyme M reductase alpha subunit (*mcrA*)	PCR	40 cycles of 94°C for 40s, 52°C for 30s and 72°C for 60s	ME3MF	ATGTCNGGTGGHGTMGGSTTYAC	Nunoura *et al.,* 2008
			ME3MF-e	ATGAGCGGTGGTGTCGGTTTCAC	Nunoura *et al.,* 2008
			ME2r'	TCATBGCRTAGTTDGGRTAGT	Nunoura *et al.,* 2008
Prokaryotic universal 16S rRNA gene	Q-PCR	96°C for 2 min and 50 cycles of 96°C for 25s and 57°C for 6 min	Uni340F	CCTACGGGRBGCASCAG	Takai and Horikoshi, 2000
			Uni806R	GGACTACNNGGGTATCTAAT	Takai and Horikoshi, 2000
			Uni516F (Taqman probe)	TGYCAGCMGCCGCGGTAAHACVNRS	Takai and Horikoshi, 2000
Archaeal 16S rRNA gene	Q-PCR	96°C for 2 min and 50 cycles of 96°C for 25s and 59°C for 6 min	Arch349F	GYGCASCAGKCGMGAAW	Takai and Horikoshi, 2000
			Arch806R	GGACTACVSGGGTATCTAAT	Takai and Horikoshi, 2000
			Arch516F (Taqman probe)	TGYCAGCCGCCGCGGTAAHACCVGC	Takai and Horikoshi, 2000
Methyl coenzyme M reductase alpha subunit (*mcrA*)	Q-PCR	40 cycles of 94°C for 40s, 52°C for 30s and 72°C for 60s	ME3MF	ATGTCNGGTGGHGTMGGSTTYAC	Nunoura *et al.,* 2008
			ME3MF-e	ATGAGCGGTGGTGTCGGTTTCAC	Nunoura *et al.,* 2008
			ME2r'	TCATBGCRTAGTTDGGRTAGT	Hales *et al.,* 1996
Dissimilatory sulfite reductase alpha subunit (*dsrA*)	Q-PCR	95°C for 10 min and 40 cycles of 95°C for 15s and 60°C for 1 min	DSR-1F+	ACSCACTGGAAGCACGGCGG	Kondo *et al.,* 2004
			DSR-R	GGTTRKACGTGCCRMGGTG	Kondo *et al.,* 2004
Adenosine 5'-phosphosulfate reductase alpha subunit (*aprA*)	Q-PCR	95°C for 10 min and 40 cycles of 95°C for 15s and 60°C for 1 min	AprA-1-FW	TGGCAGATCATGATYMAYGG	Meyer *et al.,* 2007
			AprA-5-RV	GCGCCAACYGGRCCRTA	Meyer *et al.,* 2007

Supplementary Table S3. Results of quantitative cultivation analyses using Hole C0014B core samples

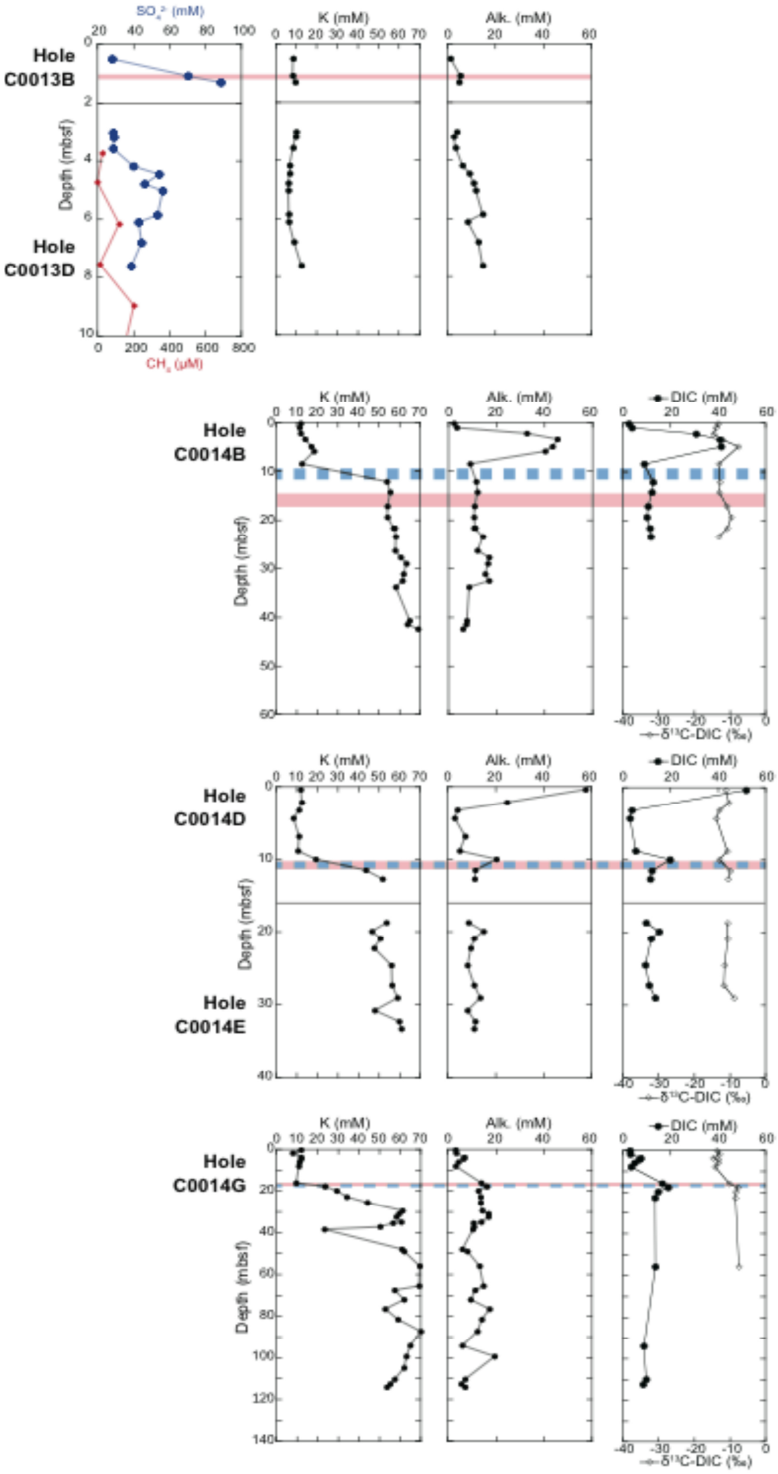
Depth (mbsf)	Cultivation conditions (medium, added compounds, gases in headspace)*											
	Marine agar, Air			MJ medium with 0.1% yeast extract, 0.1% Tryptone and 0.01% Glucose, N_2/CO_2			MJ medium with 10 mM pyruvate, 5 mM lactate and 30 mM Na_2SO_4, H_2/CO_2			MJ medium with 5 mM acetate and 5 mM formate, H_2/CO_2		
	Colony Formation Unit (CFU/g)	Closest phylogenetic relative [accession number]	Sequence similarity (%)	Population density (cells/g)	Closest phylogenetic relative [accession number]	Sequence similarity (%)	Population density (cells/g)	Closest phylogenetic relative [accession number]	Sequence similarity (%)	Population density (cells/g)	Closest phylogenetic relative [accession number]	Sequence similarity (%)
0.3	3.9E+04	*Geofilum rubicundum* [AB362265]	100	5.5.E+07	Clostridia bacterium S710(0)-1 [GU136592]	98	5.5.E+02	*Desulfomicrobium norvegicum* [NR_025407]	100		ND	
1.1	2.1E+05	*Geofilum rubicundum* [AB362265]	100	4.6.E+06	Clostridia bacterium S710(0)-1 [GU136592]	98	4.6.E+02	*Desulfomicrobium norvegicum* [NR_025407]	100		ND	
2.4	1.4E+05	*Geofilum rubicundum* [AB362265]	100	4.6.E+06	Clostridia bacterium S710(0)-1 [GU136592]	98	4.6.E+02	*Desulfomicrobium norvegicum* [NR_025407]	100		ND	
3.6	3.9E+04	*Geofilum rubicundum* [AB362265]	100	5.2.E+06	Clostridia bacterium S710(0)-1 [GU136592]	98	5.2.E+02	*Desulfomicrobium norvegicum* [NR_025407]	100		ND	
5.0	1.5E+02	*Geofilum rubicundum* [AB362265]	100	5.5.E+06	Clostridia bacterium S710(0)-1 [GU136592]	98		ND			ND	
6.0	2.5E+03	*Geofilum rubicundum* [AB362265]	100	5.0.E+04	Clostridia bacterium S710(0)-1 [GU136592]	98		ND			ND	
8.5	9.6E+05	*Geofilum rubicundum* [AB362265]	100	6.0.E+05	Clostridia bacterium S710(0)-1 [GU136592]	98		ND			ND	
12.2		ND		5.9.E+03	Clostridia bacterium S710(0)-1 [GU136592]	98		ND			ND	
23.2		ND			ND			ND			ND	
26.2		ND			ND			ND			ND	
27.5		ND			ND			ND			ND	

*All cultivations were performed at 30˚C.

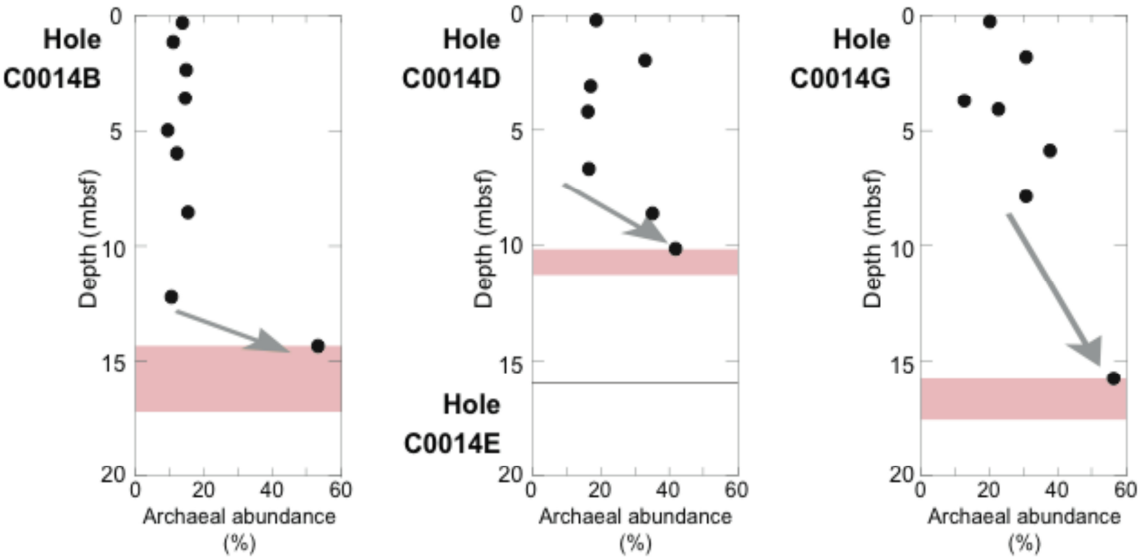
ND; not detected



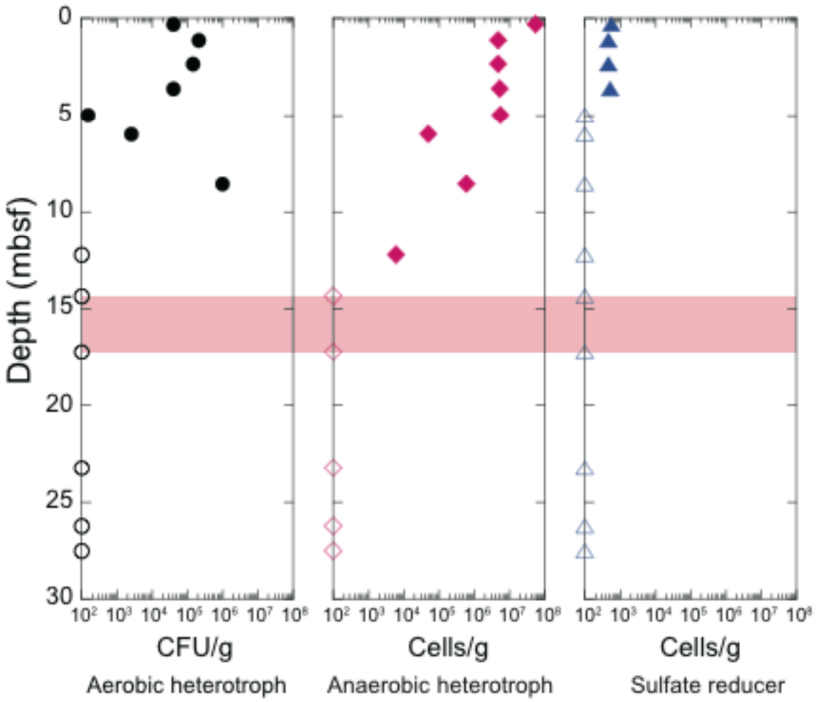
Supplementary Figure S1. Temperature profile at IODP Site C0014, originally published elsewhere (Takai *et al.*, 2011), integrating temperature information from adjacent holes. Yellow diamonds indicate *in situ* temperatures determined with the APCT-3 temperature shoe. Black diamonds indicate the minimum temperature determined by a thermoseal strip taped to the outer surface of the core liner. Stars denote minimum estimates of the temperature. Black bars indicate the estimated formation temperatures of the clay minerals in Supplementary Table S1.



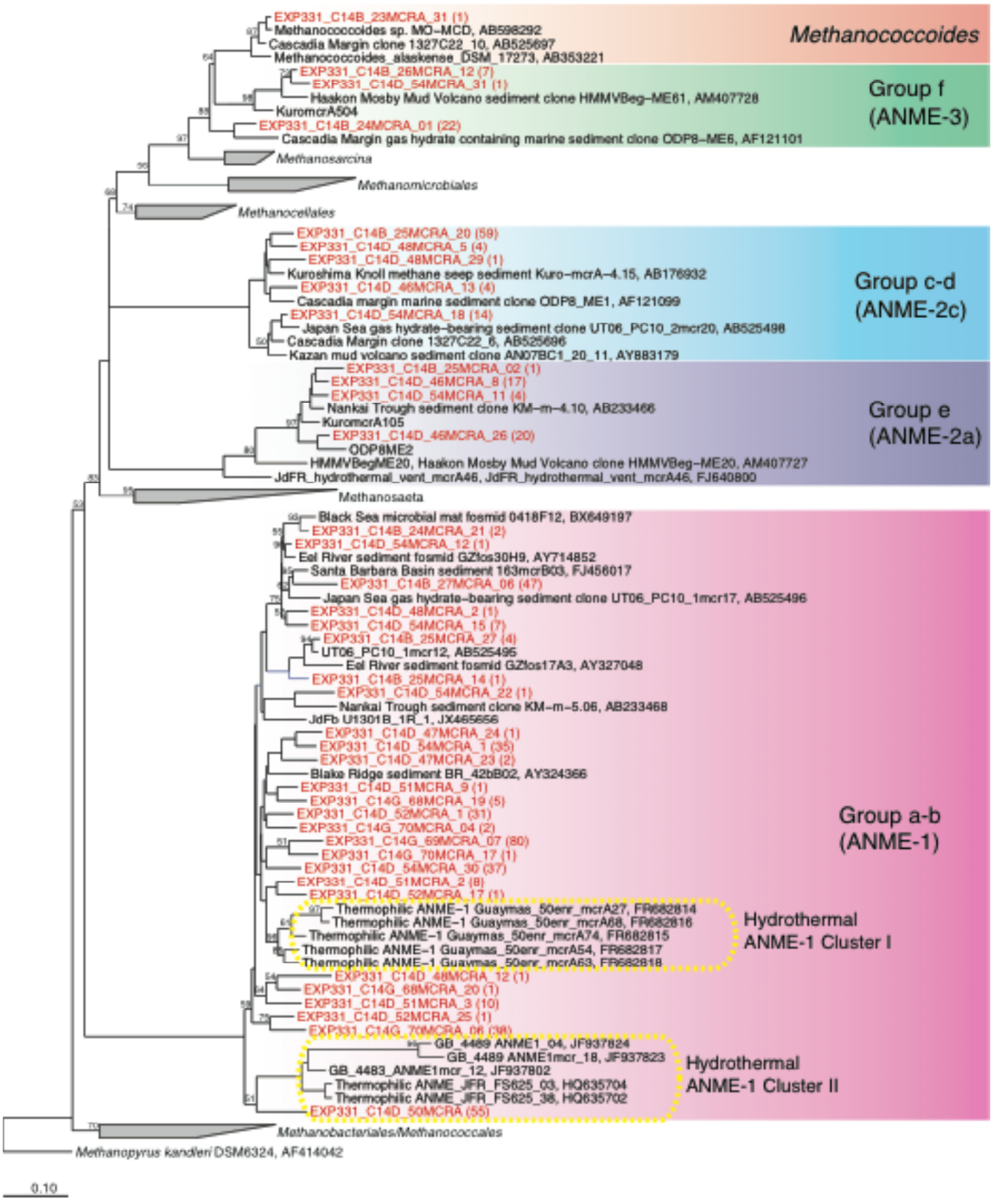
Supplementary Figure S2. Depth profiles of pore-water sulfate, methane, potassium and total alkalinity at Sites C0013, and profiles of potassium, alkalinity and DIC at Site C0014. A portion of the data were originally reported by Takai *et al*. (2011). The red-shaded layers represent the possible depth limit of active subseafloor microbes, as determined in Figure 1. The possible impermeable layers are indicated by the blue dashed lines.



Supplementary Figure S3. Depth profiles of the relative abundance of archaea in Hole C0014B. The relative archaeal abundances were determined by the ratio of the number of archaeal 16S rRNA gene copies to that of total prokaryotic 16S rRNA genes. The red-shaded layers represent the possible depth limit of the active subseafloor biosphere, as determined in Figure 1.



Supplementary Figure S4. Culturability of aerobic heterotrophs, anaerobic heterotrophs and sulfate reducers in Hole C0014B core samples. Open symbols on the y-axes denote values below the detection limit. The red-shaded layers represent the possible depth limit of active subseafloor microbes, as determined in Figure 1.



Supplementary Figure S5. Phylogenetic tree of the *mcrA* gene sequences from the subseafloor sediments at Site C0014. The sequences in red were obtained in this study. The numbers in parentheses show the total number of phylotypes. Bootstrap values are expressed as percentages of 1000 trials. The values at the nodes represent scores greater than 50%. The scale bar represents a 10% estimated sequence divergence.

List of publications

Breuker, A., Blazejak, A., Bosecker, K. and Schippers, A. (2009) Diversity of Iron Oxidizing Bacteria from Various Sulfidic Mine Waste Dumps. *Advanced Materials Research*, **71-73**, 47–50.

Breuker, A., Köweker, G., Blazejak, A. and Schippers, A. (2011) The deep biosphere in terrestrial sediments in the Chesapeake Bay area, Virginia, USA. *Frontiers in Microbiology*, **2,** 2011.

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Breuker, A., Stadler, S. and Schippers, A. (2013) Microbial community analysis of deeply buried marine sediments of the New Jersey shallow shelf (IODP Expedition 313). *FEMS Microbiology Ecology*, **85**, 578–592.

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Persönliche Erklärung zur Dissertation

Gemäß §6(1) der Promotionsordnung der Naturwissenschaftlichen Fakultät der Gottfried-Wilhelm-Leibniz-Universität Hannover

für die Promotion zum Dr. rer. nat

Hierdurch erkläre ich, dass ich meine Dissertation mit dem Titel

„Analysis of the microbial composition of the deep biosphere in four different sediments “

selbstständig verfasst habe und die benutzen Hilfsmittel und Quellen sowie gegebenenfalls die zu Hilfeleistungen herangezogenen Institutionen vollständig angegeben habe.

Die Dissertation wurde nicht schon als Masterarbeit, Diplomarbeit oder andere Prüfungsarbeit verwendet.

Anja Breuker, 20.10.2016

Curriculum Vitae

Date of birth:	28. January 1962, Göttingen, Germany
Family status:	Married, three children
1981	Abitur Stadtgymnasium Detmold

Studies:

1981 - 1988	Study of medicine at Georg August Universität Göttingen
2003	Study of biology at Leibniz University of Hannover

Degrees:

1990	Ärztliche Approbation
1998	Fachärztin für Frauenheilkunde
2009	Diplom Biologin

Professional Experience:

1989 - 2016	Working as a physician at different hospitals
2008	Diploma thesis: Phylogenie Eisenoxidierender Mikroorganismen in der Stammsammlung der Bundesanstalt für Geowissenschaften und Rohstoffe (BGR), Hannover
Since 2009	PhD student at BGR Hannover: Analysis of the microbial composition of the deep biosphere in four different sediments

Appendix

Oligotrophy

Figure: Definitions used to characterize oligotrophic *Bacteria* (Schut Frits, 1997)

Definition	Source
'Oligotrophic bacteria are heterotrophic bacteria capable of growth in the presence of organic nutrients equivalent to 16.8 mg C l^{-1}'	Akagi et al. (1977)
'Bacteria capable of growth on unamended BWA (agar-solidified Chesapeake Bay water)'	Mallory et al. (1977)
'Oligocarbophilic bacteria are capable to grow on media containing only minerals, and they meet their carbon and energy requirements from trace amounts of organic substances [...] found in the air.'	Moaledj (1978)
'A trophic group of bacteria that can grow only in the presence of a minor amount of nutrilites and not in the presence of a large amount.'	Yanagita et al. (1978)
'Those [bacteria] that develop at the first cultivation on media with the minimal content of organic matter of about 1–15 mg C/l and that grow on such media at subsequent recultivation though they can grow on richer media.'	Kuznetsov et al. (1979)
'Organisms that grow in media containing organic matter at a concentration of 1 mg C per litre. [...]. Obligate oligotrophs may decrease in number or disappear with the onset of man-made eutrophication, facultative oligotrophs can tolerate or rapidly adapt to the higher concentrations of organic substances.'	Ishida & Kadota (1981)
'Oligotrophic bacteria can [...] be conceived of as those whose survival in nature depends on their ability to multiply in habitats of low nutrient fluxes (approaching zero to a fraction of a mg C/litre/day).'	Poindexter (1981)
'Bacteria which grow at substrate concentrations of less than 1 mg carbon l^{-1}.'	Ishida et al. (1982)
'Bacteria that can be isolated on a low-nutrient medium (unsupplemented Bushnell Haas agar) and that are restricted to growth at low nutrient concentrations.'	Horowitz et al. (1983)
'Oligotrophs are defined as those organisms known to be able not only to survive but particularly to multiply under conditions of extremely low and often discontinuous supply of nutrients. In other words, organisms adapted to low and irregular fluxes of substrates.'	Van Gemerden & Kuenen (1984)
'Obligately oligotrophic bacteria are capable of growth in $SF10^{-4}$ (0.2 mg C l^{-1}) but not in $SF10^{-1}$ (200 mg C l^{-1})'	Ishida et al. (1986)
'Among oligotrophs we tentatively define the obligate oligotroph as an organism which does not grow in rich (200 mg C/l) media, and the facultative oligotroph as an organism which grows in not only poor (0.2 mg C/l) but also rich media.'	Ishida et al. (1989)
'Oligotrophic isolates are defined as bacteria capable of growth on OEMS agar (0.4 mg C l^{-1}).'	Upton & Nedwell (1989)
'Oligotrophic bacteria can broadly be defined as organisms that grow on low concentrations of organic substrates' Obligate oligotrophs cannot grow at substrate concentrations above 6 g C l^{-1}	Fry (1990)
'... oligotrophic micro-organisms are prokaryotic and eukaryotic organisms that are evolutionarily adapted to exploit ecological niches characterised by low substrate concentrations and low energy fluxes. Oligotrophs, [...] may develop in rich as well as in poor environments [....].'	Semenov (1991)

Table: *'Bathyarchaeota'*

Authors	Name of MCG or MCG subgroups	Title	Remark
Vetriani *et al.* 1999	Subgroup of MCG: Marine Benthic Group C	Population structure and phylogenetic characterization of marine benthic *Archaea* in deep-sea sediments	
Takai *et al.* 2001	Terrestrial Miscellaneus Crenarcheaotic Group (TMCG)	Archaeal diversity in waters from deep South African gold mines	
Reed *et al.* 2002	NT-A3, NT-A4	Microbial communities from methane hydrate-bearing deep marine sediments in a forearc basin	
Inagaki *et al.* 2003	'MCG'	Microbial communities associated with geological horizons in coastal subseafloor sediments from the Sea of Okhotsk	Proposal to rename the Terrestrial Miscellaneus Crenarchaeotic Group (TMCG) to Miscellaneus Crenarchaeotic Group (MCG)
Sorensen and Teske 2006	MCG-1 - MCG-4	Stratified Communities of active *Archaea* in deep marine subsurface sediments	MCG-1 = NT-A4
Webster *et al.* 2006	PM-1 - PM-8	Prokaryotic community composition and biogeochemical processes in deep subseafloor sediments from the Peru Margin	
Jiang *et al.* 2011	MCG-A - MCG-F	Stratification of archaeal communities in shallow sediments of the Pearl River Estuary, Southern China	
Kubo *et al.* 2012	MCG-1 - MCG-17	*Archaea* of the Miscellaneous Crenarchaeotal Group are abundant, diverse and widespread in marine sediments	
Meng *et al.* 2012	MCG-A - MCG-F (after Jiang *et al.*)	Genetic and functional properties of uncultivated MCG *Archaea* assessed by metagenome and gene expression analyses	metagenomics analysis, proposed candidate phylum *'Bathyarchaeota'* for MCG

Table: *'Aigarchaeota'*

Author	Name	Title	Remark
Nunoura *et al.* 2005	Hot Water Crenarchaeotic Group I (HWCG I)	Genetic and functional properties of uncultivated thermophilic crenarchaeotes from a subsurface gold mine as revealed by analysis of genome fragments	
Nunoura *et al.* 2011	Hot Water Crenarchaeotic Group I (HWCG I)	Insights into the evolution of *Archaea* and eukaryotic protein modifier systems revealed by the genome of a novel archaeal group.	Ca. *'Caldiarchaeum subterraneum'*, proposed candidate phylum *'Aigarchaeota'*

Table: DSAG/MBG-B and *'Lokiarchaeota'* partly from (Jørgensen and Zhao, 2016)

Author	Name	Title	Remark
Takai and Horikoshi 1999	Deep Sea Hydrothermal Vent Crenarchaeotic Group1	Genetic Diversity of *Archaea* in Deep-Sea Hydrothermal Vent Environments	
Vetriani *et al.* 1999	Marine Benthic Group B (MBG-B)	Population structure and phylogenetic characterization of marine benthic *Archaea* in deep-sea sediments	
Takai *et al.* 2001	DSAG	Distribution of *Archaea* in a Black Smoker Chimney Structure	
Jorgensen *et al.* 2013	DSAG: α-, β-, γ- clade	Quantitative and phylogenetic study of the Deep Sea Archaeal Group in sediments of the Arctic mid-ocean spreading ridge	monophyly of the α-, β- and γ- clade according 16S rRNA phylogeny
Spang *et al*. 2015	*'Lokiarchaeota'*	Complex archaea that bridge the gap between prokaryotes and eukaryotes	*'Lokiarchaeota'* are affiliated to the γ- clade of the DSAG

Phylogenetic tree constructions

The phylogenetic trees were constructed with the arb software (www.arb-home.de, database SSURef_108_SILVA_09_09_11_opt.arb and SILVA_123.1_SSURef_Nr99). 16S rRNA gene sequences and sequences from whole genome/whole shotgun projects were downloaded from NCBI or the SILVA online site (www.arb-silva.de). Identification of 16S rRNA gene sequences was carried out with with rnammer (Lagesen *et al.*, 2007). Sequences were aligned with the SILVA online aligner (Quast *et al.*, 2013) or the Integrated Aligner in arb and manually curated after importing to arb. Next neighbors of found 16S rRNA sequences in the New Jersey sediment and the CBIS sediment were identified with BLAST (Altschul *et al.*, 1990). 16S rRNA gene sequences with more than 1000 bp belonging to major subgroups were selected. The archaeal trees were constructed with RAxML (filter "archaea") with Aquifex as outgroup. 16S rRNA gene sequences < 1000 bp were added after tree construction. The tree of the dissimilatory iron reducers was constructed with filter "bacteria" and iron reducing *Archaea* were chosen as outgroup. The tree of the bacterial 16s rRNA gene sequences was constructed with 16S rRNA sequences < 1400 bp without filter with RAxML with *Archaea* as outgroup. MCG trees: Subgroups of *'Bathyarchaeota'* and the THSCG group are collapsed if no 16S rRNA gene sequences of this study could be allocated. Adjustment was made using the itol online service (Letunic and Bork, 2016).

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