Molecular Ecology of Free-Living Chemoautotrophic Microbial Communities at a Shallow-sea Hydrothermal Vent

(Molekulare Ökologie von freilebenden chemoautotrophen mikrobiellen Gemeinschaften an einer Flachwasserhydrothermalquelle)

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Summary

Deep-sea hydrothermal systems are unique habitats for microbial life with primary production based on chemosynthesis. They are considered to be windows to the subsurface biosphere. Their far more accessible shallow-sea counterparts are valuable targets to study the effects of hydrothermal activity on geology, seawater chemistry and microorganisms. Such an area of shallow-sea hydrothermal venting is observed approximately 2.5 km east off Panarea Island (Sicily, Italy). This system is characterized by fluid temperatures of up to 135°C, gas emissions dominated by CO_2 and precipitation of elemental sulfur on the seafloor. It is quite well studied, yet, only very few studies exist on its microbial ecology. This thesis is therefore targeting the microbiology of sediment cores as part of an interdisciplinary project which combines geological, geochemical, biomarker and molecular biological investigations. It was intended to correlate the environmental parameters with the taxonomic composition and the metagenomes of the microbial community thereby gaining insights into the interaction of geosphere and biosphere.

All samples were taken at Hot Lake, an oval-shaped (~10 by 6 meters) shallow (~2.5 m deep) depression at 18 m below sea level. The sediments in this depression are strongly affected by hydrothermal activity. *In situ* temperatures at 10 cm below sea floor of 36°C and 74°C were measured at two different sites within Hot Lake. Based on the physico-chemical parameters, a thermodynamic modeling was performed which revealed sulfur oxidation and sulfur reduction to be exergonic at Hot Lake.

Microbial community structures of different sediment layers were first screened by automated rRNA intergenic spacer analysis (ARISA). Based on the ARISA fingerprints, a total of eight bacterial and archaeal 16S rRNA gene libraries were constructed from surface to bottom layers of sediments to gain more insights into microbial diversity. Comparative sequence analyses revealed a dominance of sequences affiliated with *Epsilonproteobacteria*, *Deltaproteobacteria* and *Bacteroidetes*. In the surface sediments, sequences close to anoxygenic phototrophic *Chlorobi* were also detected. In the bottom sediments, thermophilic bacteria such as *Thermodesulfobacteria* spp. were found. Hyperthermophilic *Archaea*

sequences related to *Desulfurococcaceae* and *Korarchaeota* were retrieved from 74°C hot sediment. Based on the most closely related cultured representatives, it could be deduced that the majority of microorganisms in Hot Lake sediments have a sulfur-dependent metabolism, including sulfide oxidation, sulfur reduction or sulfate reduction.

Fluorescence *in situ* hybridization showed the dominance of *Bacteria* in all depths of sediments. With increasing depth and temperature, the abundance of *Archaea* increased relatively to that of *Bacteria*. Metagenomic analyses revealed that *Epsilonproteobacteria* were dominating surface sediments of Hot Lake where they gain energy from sulfur metabolism to fix CO_2 by the reductive tricarboxylic acid (rTCA) cycle. This is consistent with findings reported from deep-sea hydrothermal vent systems.

The results have led to the conclusion that mixing between hydrothermal fluids and seawater results in distinctly different temperature gradients and ecological niches in Hot Lake sediments. Overall, the correlation of geochemical profiles, IPL analyses, characterization of the microbiological community and metagenomic analyses provided strong evidence for a sulfur-dominated metabolism in the surface sediments of Hot Lake.

Zusammenfassung

Tiefseehydrothermalquellen sind einzigartige Lebensräume für mikrobielle Lebensgemeinschaften, deren Primärproduktion auf Chemosynthese beruht. Sie sind Fenster in die Biosphäre des Untergrunds. Die leichter zugänglichere Flachwasserhydrothermalgebiete sind wertvolle Ziele, um die Auswirkungen hydrothermaler Aktivitäten auf die Geologie, die Meerwasserchemie und die Mikroorganismen zu untersuchen. Ein solches Gebiet befindet sich ungefähr 2,5 km östlich der Insel Panarea (Sizilien, Italien). Die Temperatur der Hydrothermalfluide steigt hier bis auf 135°C an. Die emittierte Gase enthalten überwiegend CO_2. Auf dem Meeresboden kommt es zur Präzipitation von Elementarschwefel. Obwohl das Gebiet recht gut untersucht ist, gibt es bisher nur sehr wenige Untersuchungen zur mikrobiellen Ökologie der Hydrothermalquellen von Panarea. Diese Dissertation ist Teil eines interdisziplinären Projekts, das geologische, geochemische, Biomarker- und molekularbiologische Untersuchungen von Sedimentkernen kombiniert. Es war beabsichtigt, durch die Korrelation der Umweltparameter mit der taxonomischen Zusammensetzung und dem Metagenom der mikrobiellen Gemeinschaft Einblicke zu gewinnen, wie die Geosphäre mit der Biosphäre wechselwirkt.

Alle hier untersuchten Proben stammen vom „Hot Lake“, einer ovalen, flachen Vertiefung, die in 18 m Wassertiefe liegt. Die Sedimente im Becken werden stark von hydrothermaler Aktivität beeinflusst. In 10 cm Tiefe herrschen hier an zwei Messpunkten Temperaturen von 36°C und 74°C. Basiert auf den gemessenen physikochemischen Parametern zeigten thermodynamische Berechnungen, dass sowohl die Schwefeloxidation als auch die Schwefelreduktion exergonisch sind.

Die Zusammensetzung der mikrobiellen Gemeinschaften wurde zuerst mittels ARISA verglichen, wobei in unterschiedlichen Sedimenttiefen deutliche Unterschiede vorhanden waren. Vergleichende 16S rRNA-Genanalysen zeigten eine Dominanz von Sequenzen der *Epsilonproteobacteria*, *Deltaproteobacteria* und *Bacteroidetes*. In der Oberflächenschicht wurden auch Sequenzen von anoxygenen phototrophen Chlorobien entdeckt. In tieferen

Sedimentschichten wurden Sequenzen von thermophilien Bakterien (z.B. *Thermodesulfobacteria*) gefunden. Sequenzen von hyperthermophilien *Archaea* (z.B. *Desulfurococcaceae* und *Korarchaeota*) wurden nur in 74°C heißem Sediment gefunden. Von den nächsten kultivierten Verwandten ist bekannt, dass sie die Energie meist durch Schwefel-basierte Stoffwechselwege gewinnen (z.B. Sulfidoxidation, Schwefelreduktion oder Sulfatreduktion).

Die Fluoreszenz *in situ* Hybridisierung zeigte, dass *Bacteria* in allen Tiefen dominierten. Mit zunehmender Tiefe und Temperatur stieg der Anteil von *Archaea* im Verhältnis zu *Bacteria* an. Eine metagenomische Analyse zeigte, dass *Epsilonproteobacteria* in der Oberflächenschicht dominierten, wo sie Energie aus dem Schwefelstoffwechsel für die CO_2-Fixierung durch den reversen Tricarbonsäurezyklus (rTCA) nutzten. Dies bestätigte Befunde von Tiefseehydrothermalquellen.

Die Ergebnisse zeigen, dass durch die Mischung von Hydrothermalfluiden und Meerwasser bei verschiedenen Temperaturen verschiedene ökologische Nischen im Sediment von Hot Lake entstehen. Zusammenfassend wurden durch die Korrelation von geochemischen Profilen und IPL-Analysen mit der Charakterisierung der mikrobiologischen Lebensgemeinschaften einschließlich der metagenomischen Analysen starke Hinweise für eine dominierende Rolle des Schwefelstoffwechsels in den Oberflächensedimenten von Hot Lake gefunden.

List of abbreviations

ANOSIM	analysis of similarity
ARISA	automated rRNA intergenic spacer analysis
ATP	adenosine triphosphate
bsf	below seafloor
bsl	below sea level
DNA	deoxyribonucleic acid
DGGE	denaturing gradient gel electrophoresis
et al.	and others
FISH	Fluorescence *in situ* hybridization
IPLs	Intact polar lipids
KEGG	Kyoto Encyclopedia of Genes and Genomes
kya	thousand years ago
nano-SIMS	nanometer-scale secondary ion mass spectrometry
nMDS	non-metric multidimensional scaling
ORF	open reading frame
OTU	operational taxonomic units
PCR	polymerase chain reaction
RNA	ribonucleic acid
rTCA	reductive tricarboxylic acid
spp.	Species
Sox	sulfur oxidation

I Introduction

1. Hydrothermal vents

Hydrothermal vents appear commonly at tectonically active sites where plates are moving apart or at volcanic hotspots both in shallow regions close to the water surface and in deeper waters (Figure 1) (Martin et al., 2008). They are characterized by the emission of thermal fluids from the subsurface, often accompanied by the formation of hydrothermal mineral deposits in the form of chimney structures surrounding advecting vent fluids and/or the deposition of mineral particles following mixing of vent fluids with seawater (Jannasch and Mottl, 1985). Through water-rock interaction these hydrothermal fluids are highly reduced compared to sea water (Tivey, 2007). Hydrothermal vents are often considered "oases" for endemic species that depend on chemosynthesis-based food webs (Beaulieu et al., 2011). Because the vents are so discrete and may be ephemeral on both short (ecological) and long (evolutionary) time scales, it is an intriguing question for biologists how the populations were established and maintained at these specific environments.

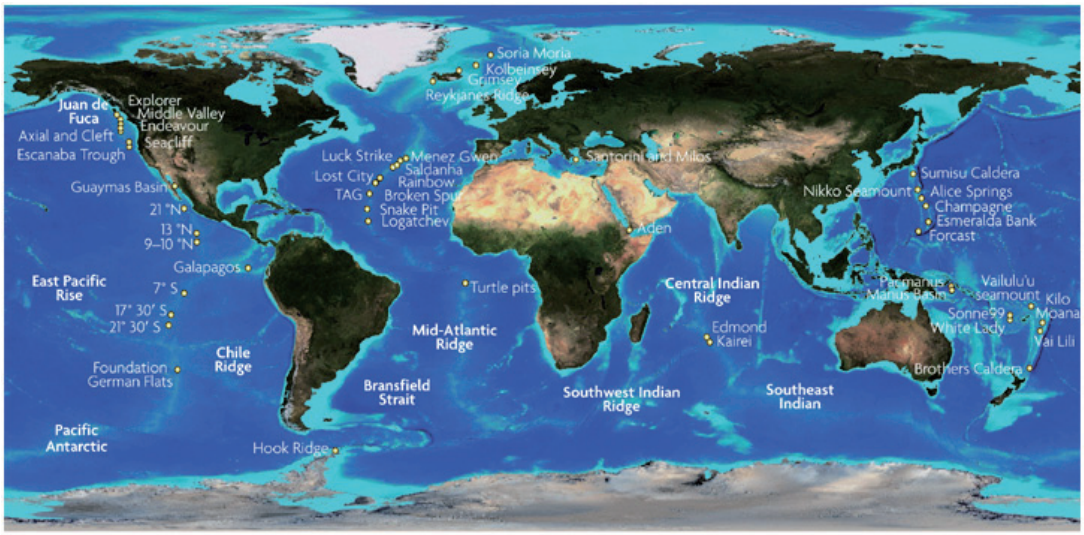


Figure 1. Global distribution of known hydrothermal vents (Martin et al., 2008)

1.1 Deep-sea hydrothermal vents

The first deep-sea hydrothermal vent was discovered in 1977 at the Galápagos Rift, a part of sea floor spreading axes (Corliss et al., 1979). This finding initiated a new era of scientific investigations on deep-sea hydrothermal vents. Starting from the East Pacific Rise, warm (5°C-23°C) and hot vent fields (270°C-380°C) were found (Jannasch and Mottl, 1985). It was shown that chemosynthesis instead of photosynthesis is at the basis of the food chain (Jannasch and Mottl, 1985). Chemosynthesis was proposed in 1890 by Sergey Nikolayevich Winogradsky in contrast to photosynthesis. The process involves biosynthesis of organic carbon compounds from CO_2 based on the energy gained by the oxidation of reduced inorganic compounds. A variety of different deep sea hydrothermal niches have been investigated (Figure 2) (Orcutt et al., 2011). Free-living microbial communities and symbioses using different strategies to adapt to the environments have been broadly studied (Baker et al., 2010).

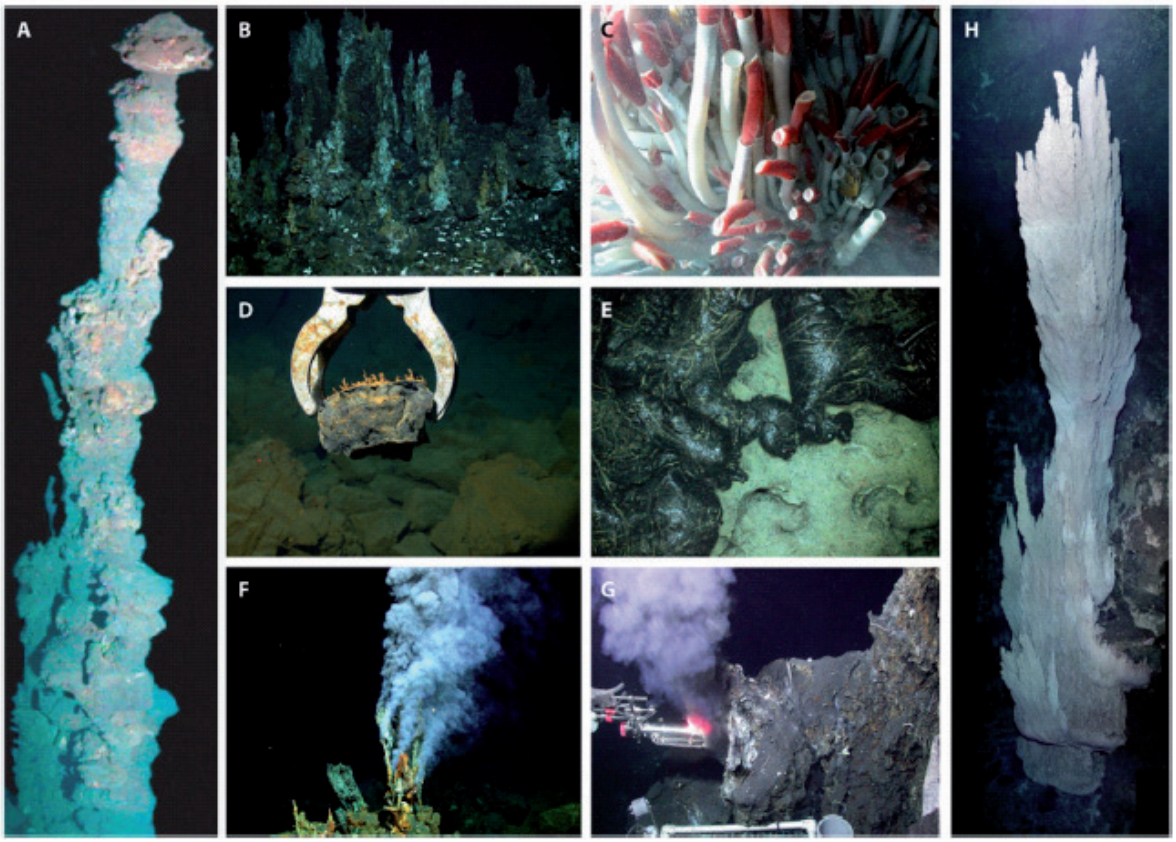


Figure 2. Photographs of several ocean crusts and hydrothermal vents in the dark ocean (Orcutt et al., 2011). (A) Sulfide chimney. (B) Active and inactive hydrothermal chimneys. (C) *Riftia pachyptila* tube worms at East Pacific Rise. (D) Piece of altered basaltic oceanic crust. (E) Young basalt flows. (F) White smoker hydrothermal chimney. (G) Black smoker hydrothermal chimney. (H) Sixty-meter-tall carbonate chimney.

1.2 Shallow-sea hydrothermal vents

The cut-off between “shallow” and “deep” hydrothermal vent fields was defined by Tarasov and colleagues (Tarasov et al., 2005) at a depth of approximately 200 m, based on faunal differences. Shallow hydrothermal vents are present all over the world and usually occur near active coastal or submarine volcanoes (Figure 3) (Gamo and Glasby, 2003). Deep-sea hydrothermal fluids are mainly derived from the circulation of seawater beneath the seafloor while coastal hydrothermal fluids may consist of a more complex mixture of seawater, meteoric water (groundwater) and magmatic fluids. Tidal forcing, sea level change and earthquake activity may as well affect the rates of fluid venting and dispersion of hydrothermal plumes. The chemical composition of coastal hydrothermal fluids is variable because it depends not only on water-rock interaction at high temperatures but also on the rate of subduction of the slab material at the convergent plate margin and the decomposition of organic matter within the coastal sediments. The penetration of light might allow for photosynthesis at shallow vent systems. At shallower depths the sedimentation of organic matter formed by photosynthesis is more pronounced and must be considered as an additional source of nutrition.

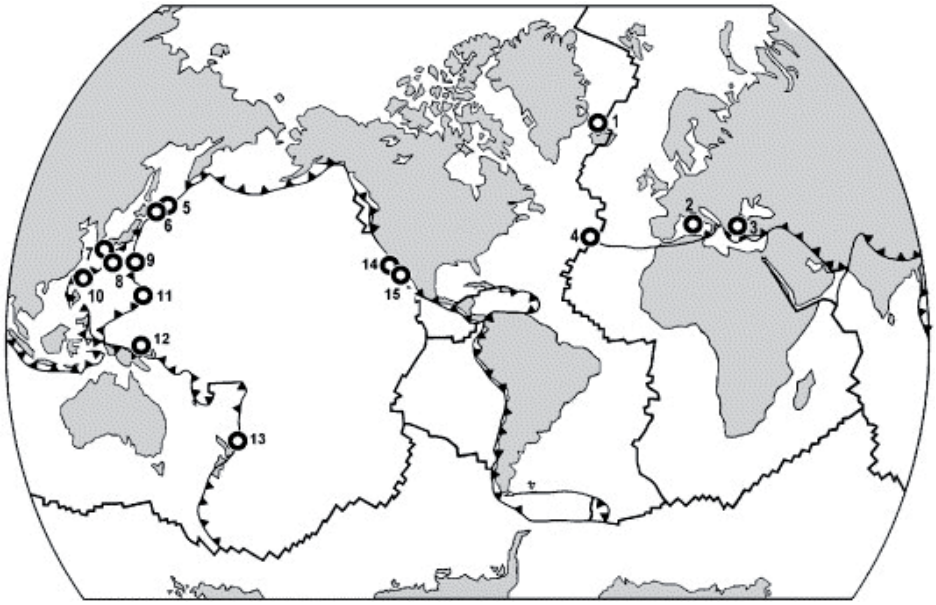


Figure 3. Areas of shallow-water (< 200 m) hydrothermal venting. (1) Kolbeinsey. (2) Tyrrhenian Sea (Capes Palinuro and Messino, Bahia Pozzuoli and Panarea Island). (3) Aegean Sea (Islands Santorini and Milos). (4) Azores. (5) Kraterbright. (6) Kunashir Island. (7) Kagoshima Bay. (8) Tokora and Iwo Islands. (9) Ogasawara Islands. (10) Kueishan Ialand. (11) Mariana Islands. (12) Papua New Guinea. (13) New Zealand. (14) California. (15) Baja California. Modified from Tarasov et al. (Tarasov et al., 2005).

1.2.1 Physical and chemical characteristics of shallow-sea hydrothermal vents

The existence of venting in shallow waters is often observed by the presence of streams of gas bubbles. This is caused by the reduced solubility of gases at lower pressures and leads to bubble formation as gas-saturated water rises through the sediments (Fitzsimons et al., 1997; Duan et al., 1992; Dando et al., 2000). Phase separation can occur in shallow-sea hydrothermal vents and lead to the discharge of both low and high salinity fluids (Dando et al., 2000). When the fluids boil in the subsurface, it results in phase separation and leaves residual hydrothermal brine. In the subduction zone off Milos, Greece, for example (Figure 3 (3)), anoxic brine was observed which resulted in the growth of bacterial mats dominated by sulfur bacteria (Fitzsimons et al., 1997). The temperature of fluids at shallow depths is normally between 10°C to 119°C (Figure 4) (Dando et al., 1995; Tarasov et al., 1999; Tarasov et al., 2005). Main gas compositions observed at shallow hydrothermal vents are usually dominated by CO_2 with different concentration of CH_4, H_2S and H_2 (Dando et al., 1995; Hoaki et al., 1995; Tarasov et al., 1999; Dando et al., 2000; Ishibashi et al., 2008).

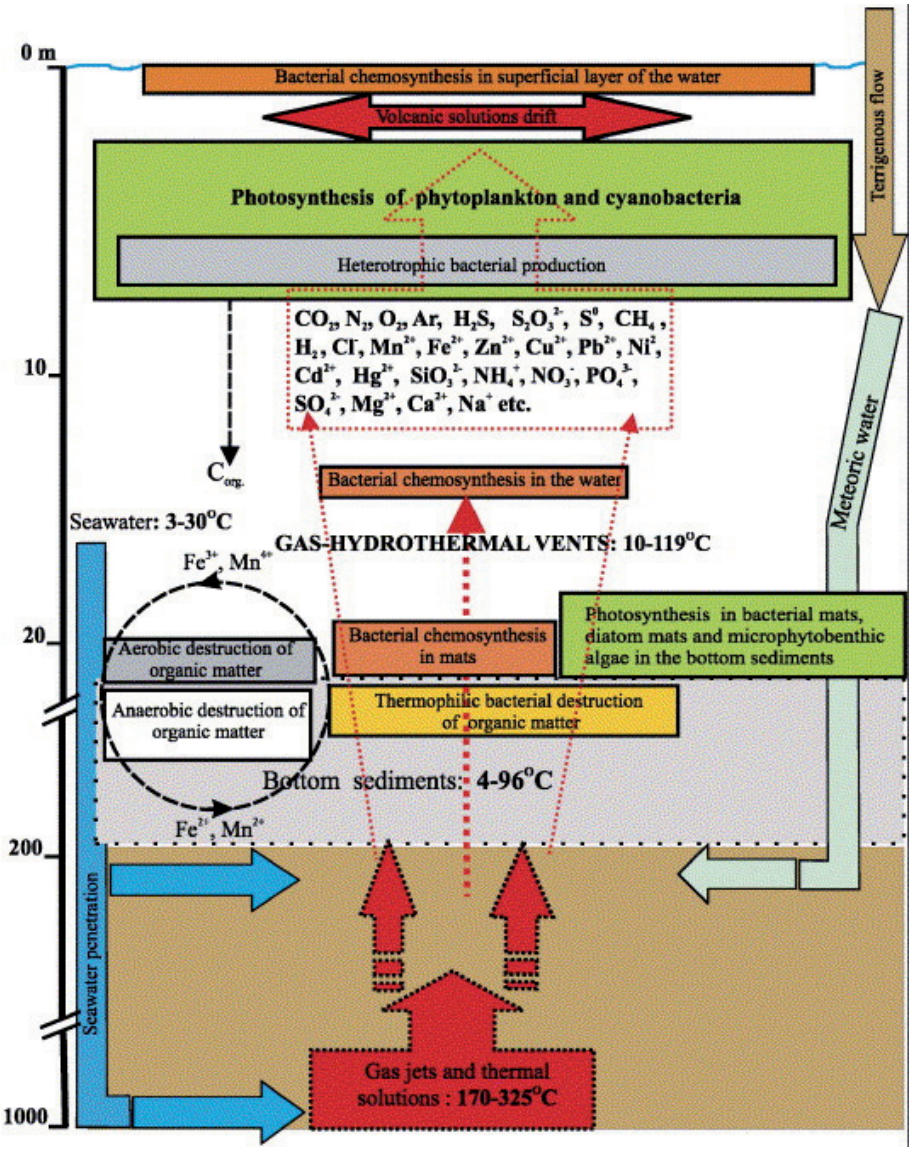


Figure 4. Major biological and geochemical processes in coastal shallow-sea hydrothermal vent systems (Tarasov et al., 2005).

As seawater percolates through the seafloor, currents are generated by high heat flow. The chemical composition is altered and the dissolved sulfate is depleted. At the same time, there is a loading of fluids with reduced metals (Bischoff and Dickson, 1975). The leaching of the crust and the subsequent mixing of the generated fluids with seawater results in mineral precipitates (Edmond et al., 1979). Vent fluids at shallow-sea hydrothermal vents are usually enriched in H_2S, H_2, CH_4, Fe (II) and different trace elements and depleted in Mg^{2+} and SO_4^{2-} compared to standard sea water concentration (Table 1).

Table 1. Chemical composition of shallow-water vent fluids compared to seawater. Modified from Tarasov et al. (Tarasov et al., 2005).

Element or compound; free gas	Hydrothermal fluid at vent opening (min–max)	Coastal sea water (min–max)
Venting fluid:		
H_2S	0.1–2400	0
H_2	0.001–0.220	0
CH_4	0.007–0.200	0
Mn	0.2–675	0.004–0.025
Fe	0.03–875	0.004–0.5
Zn	0.4–12.5	0.002–0.01
Cu	0.4–2.3	0.0–0.006
Pb	14.5–4350	0.05–3.4
Cd	up to 90	0.20–5.4
Ni	up to 220	1.4
B	455–1000	270
Ca	5–62	8–11
Na	45–1254	499
K	2.4–13.0	10.5
Cl	34–592	530–580
Mg	00.0–68.0	45–57
SO_4	2–38.5	27
Si	5–7800	0.1–110
PO_4^{3-}	0.2–12.4	0.1–2.2
NH_4^+	1.2–1000	0–3
NO_2^-	up to 0.1	0–0.05
NO_3^-	0.1–5.1	1.1–29.0
HCO_3^-	0.0–13.0	2.3
Alk	2.1–2.4	2.4–2.5
Salinity	19.5–34.54	35.40
pH	1.6–8.2	7.9–8.1

1.2.2 Hydrothermal systems around the Aeolian Islands

The hydrothermalism in the Mediterranean Sea originates from the collision of the African and the European plate, with the subduction of the oceanic African plate beneath the European plate. This subduction gives rise to active volcanic arcs in the Tyrrhenian and Aegean Seas.

Well known examples of volcanism are Etna, Vulcano, Stromboli and Vesuvius in Italy and Santorini and Nisiros in Greece (Dando et al., 2000). Bubble plumes have been detected and bacterial mats with high content of minerals are often observed (Dando et al., 1995). Sulfide deposits of hydrothermal origin, consisting of pyrite, hematite, sphalerite, galena and barite have been found at the Aeolian Island Arc off Panarea (Marani et al., 1997) and at the Palinuro seamount (Eckhardt et al., 1997). Fumarolic activity as well as sulfide deposits have been observed on the submerged beach sand on Baia di Levante on the Vulcano Island (Honnorez, 1969).

The Aeolian Islands are composed of seven major islands - Alicudi, Flicudi, Salina, Vulcano, Lipari, Panarea and Stromboli and several associated seamounts (Figure 5). They belong to the Aeolian archipelago, representing a ring-shaped volcanic arc in the south-eastern Tyrrhenian Sea. The arc has a diameter of approximately 200 km. It extends to the Preloritanian-Calabrian orogenic belt and the abyssal Marsili basin (Gabbianelli et al., 1990; Esposito et al., 2006; Gugliandolo et al., 2006; Capaccioni et al., 2007). The volcanic activity lasted during the entire Quaternary, starting about 400 kya and is still existent (Calanchi et al., 2002; Gugliandolo et al., 2006). The Aeolian volcanic arc can be divided into three sections. Panarea and Stromboli constitute the eastern sector. Both Islands are arranged along NE – SW trending extensional faults (Esposito et al., 2006). The Panarea volcanic complex consists of the main island Panarea as well as several small islets to its east (Basiluzzo, Bottaro, Lisca Bianca, Lisca Nera, Panarelli, Formiche and Dattilo). Underwater gas discharges have been observed off Panarea among these small islets. The emissions are usually adjacent to white sulfur deposits associated with hydrothermal fluids (Italiano and Nuccio, 1991).

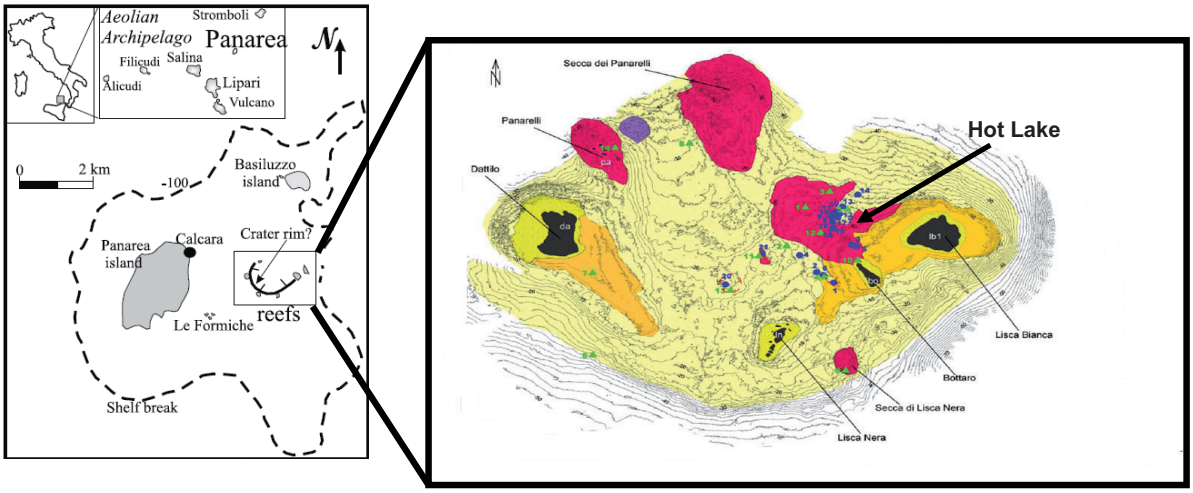


Figure 5. Map of Panarea Island and surrounded islets. Sampling site of this thesis: Hot Lake (Esposito et al., 2006; Capaccioni et al., 2007).

The venting gases at Panarea are dominated by CO_2 with more than 95% of the total emitted gases. Variable concentrations of reactive gases, such as H_2S, O_2, CH_4, CO and H_2 as well as inert gases (N_2, Ar, He) have been observed. Thermal fluids samples have been as well collected and analyzed. The enrichment of salts in the thermal fluids indicates high-temperature water-rock interaction (Italiano and Nuccio, 1991; Caracausi et al., 2005; Tassi et al., 2009). The temperature detected at the emission points were in the range of 30°C to 130°C (Maugeri et al., 2011). The thermal fluids escape from fractures in the rocks or diffuse through the sandy sediments. At the venting areas, Fe-mineralization and sulfide deposits have been often observed (Gabbianelli et al., 1990; Gamberi et al., 1997).

2. Microbial diversity and community structure at shallow-sea hydrothermal vents

Shallow-sea hydrothermal vents as well as their deep-sea counterparts supply energy at niches for diversified microorganisms (Figure 4). Bacterial mats are common features. They reach a thickness of up to 30 cm. Those at shallow sites often have a more complex nature than those at deep sea sites (Tarasov et al., 2005). Some mats also contain algae like diatoms (Ryabushko and Tarasov, 1989; Starynin et al., 1989; Hoaki et al., 1995). The bacterial mats are generally composed of sulfur bacteria of the genera *Thiobacillus*, *Thiomicrospira* and *Thiosphaera*. Also filamentous sulfur bacteria such as *Thiothrix* or *Beggiatoa* can be found. Important biogeochemical processes in these mats are the oxidation of reduced sulfur compounds and autotrophy (Tarasov et al., 2005). Cyanobacteria have been extensively studied at terrestrial sites in Greece and around vent outlets at Vulcano Island (Giaccone, 1969; Anagnostidis and Pantazidou, 1988).

Many volcanic areas serve as habitats for a wide variety of high temperature (thermophilic) microorganisms. They thrive in subsurface parts of hydrothermal systems and in the sediment near thermal emissions. To date, more than 200 species of thermophiles are known and over 35 species of thermophiles and hyperthermophiles have been isolated from west Pacific and Mediterranean vents (Table 2) (Dando et al., 1999; Kostyukova et al., 1999; Amend et al., 2003). Among these groups of thermophiles and hyperthermophiles, most of the isolates from the Tyrrhenian Sea have been isolated from Vulcano Island. Of the more than two dozen known hyperthermophilic genera from continental and marine systems worldwide, at least ten of them are present at Vulcano Island.

Table 2. Thermophilic and hyperthermophilic *Archaea* and *Bacteria* isolated from Mediterranean Sea (Dando et al., 1999).

	T_{max} (°C)	T_{opt} (°C)	metabolism	form
Methanococcus thermolithotrophicus[a]	70	65	methanogen, H_2/CO_2 and on formate	irregular cocci
Archaeglobus fulgidus[b]		83	facultative autotroph, reduces SO_4= and S_2O_3=, with H_2	
Ferroglobus placidus[c]	95	85	oxidises Fe^{2+}, H_2 and H_2S in the presence of nitrate	coccus
Sulpholobus solfatarius[d]		87	heterotroph	irregular cocci
Pyrobaculum aerophilum[e]		100	nitrate reducing heterotroph	rods
Desulfurococcus mobilis[f]				
Stetteria hydrogenophila[g]	102	95	heterotroph requiring S°	
Pyrococcus furiosus[h]	103	100	fermentative heterotroph	cocci
Pyrococcus abyssi[f]	(102)	(96)	heterotroph	
Pyrococcus horikoshii[f]	(102)	(98)	heterotroph	
Pyrococcus P3 sp. nov.[i]	100	90	anaerobic heterotroph	irregular cocci
Staphylothermus marinus[f,j]	98	92	S°-dependent anaerobic heterotroph	cocci, forming aggregates
Staphylothermus P8 sp.nov.[i]	90	85	anaerobic heterotroph	irregular cocci
Thermosphaera aggregans[f]				
Pyrodictium occultum[k]		105	reduces sulphur with H_2	disk with hyphae
Pyrodictium brockii[k]		105	sulphur reducer requiring H_2 and CO_2	disk-shaped
"Thermodiscus" sp.[f]				
Sulfophobococcus zilligii[f]				
Thermococccus celer[l]		80	anaerobic heterotroph	motile coccus
Thermococcus chitonophagus[f]				
Thermococcus acidaminovorans[m]	93	85	anaerobic heterotroph, utilising amino acids, reduces S°	motile coccus
Thermococcus alcaliphiluss[n]	90	85	anaerobic heterotroph, reduces sulphur	cocci
Thermococcus P5 sp. nov.[i]	95	90	anaerobic heterotroph	irregular cocci
Thermococcales P6 gen. nov.[i]	90	85	anaerobic heterotroph requiring S°	irregular cocci
Pyrobacterium aerophilum[o]		100	microaerophile, dissimilatory nitrate reducer	motile rods
Thermotoga neapolitana[p]	90	80	carbohydrate fermentor, reducing S°	non-motile rods
Thermotoga maritima[q]	90	80	carbohydrate fermentor, reducing S°	motile rods
Thermonema rosianum[r]	65	60	aerobic heterotroph	non-motile filaments

The microbial diversity in hydrothermal vents off Panarea has also been studied. Mesophilic chemolithotrophic sulfur oxidizing bacteria resembling *Thiobacillus* spp. have been isolated from vent fluids (Gugliandolo et al., 1999). Moreover, several thermophilic microbial strains (*Thermococcus stetteri, T. peptonophilus, T. celer, Paleococcus profundus* and *P. barossii*) have been isolated off Panarea. These organisms were isolated from a Panarea vent system at 20 m bsl with fluid temperature of 80°C.

Submarine hydrothermal vents are known for extremes in geochemical conditions and sharp physical and chemical gradients. They offer a variety of habitats or microniches to metabolically diverse microorganisms (Jannasch and Mottl, 1985; Baross and Deming, 1995; Karl, 1995). In order to comprehend the spatial distribution and changes in community structure along these gradients, cultivation independent methods were applied for the study of microbial diversity. The microbial abundance in thermal fluids has been investigated by direct cell staining of 4',6-diamidino-2-phenylindole (DAPI) at several vent sites off Lipari, Vulcano and Panarea in the Aeolian arc (Gugliandolo et al., 1999). Picophytoplankton as well as picoplankton has been quantified indicating the importance of photosynthesis in these ecosystems (Gugliandolo et al., 1999). Besides enumeration of general microbial abundances, fluorescence *in situ* hybridization (FISH) has been applied to samples from Vulcano. It has been shown that *Archaea* were more abundant than *Bacteria* in the hot sediments at Vulcano Island. New probes for hyperthermophiles have been designed to investigate the community structure (Rusch and Amend, 2004; Rusch et al., 2005; Rusch and Amend, 2008).

The biodiversity of both *Bacteria* and *Archaea* thriving at vent systems off Panarea has been studied with the fingerprinting method, denaturing gradient gel electrophoresis (DGGE). Microorganisms will be detected only if their proportion is greater than 1% of the community (Muyzer et al., 1993). Samples including hydrothermal fluid, thermal water and sediment samples were taken at three different vent sites. These sites have been characterized by different physico-chemical parameters. The biggest difference was in temperatures and pH values. DGGE results revealed the dominance of different groups of *Bacteria* and *Archaea*. Bacterial 16S rRNA sequences affiliated mostly with thermophilic *Firmicutes*, *Gammaproteobacteria*,

Epsilonproteobacteria, *Alphaproteobacteria* and *Chlorobi* whereas archaeal sequences were mostly related to clusters of sequences originating from other hydrothermal vents and without any cultivated representative (Maugeri et al., 2009; Maugeri et al., 2010; Maugeri et al., 2011).

3. Microbial metabolism at shallow-sea hydrothermal vents

3.1 Metabolic diversity

Geochemistry of shallow hydrothermal vents is not only strongly influenced by the temperature and chemical composition of the hydrothermal fluids but also by the activity of microorganisms. The presence of gas phase and enrichment of O_2 compared to deep sea vents is as well a profound feature of shallow hydrothermal systems. In addition, the entrainment of meteoric water mixing with thermal fluids and the input of organic material results in multiple ecological niches (Rusch et al., 2005; Pichler, 2005; Tarasov et al., 2005). Many of the isolated thermophilic and hyperthermophilic *Archaea* and *Bacteria* are able to obtain their energy through the oxidation of reduced sulfur compounds. *Halothiobacillus kellyi* isolated from the vent systems at the Aegean Sea has been shown to be a sulfur oxidizer (Sievert et al., 2000b). *Archaeoglobus fulgidus* isolated from Vulcano Island is known as thermophilic sulfate-reducing archaeon which can oxidize H_2 (Stetter, 1988). Methanogens such as *Methanococcus thermolithotrophicus* grow on H_2 and CO_2 (Huber et al., 1982). With the input of enriched metal species from the thermal fluids, additional redox pairs can serve as energy sources for microorganisms. An anaerobic, Fe^{2+}-oxidizing archaeon was isolated from a shallow submarine hydrothermal system at Vulcano Island. In addition to ferrous iron this species can also use H_2 and sulfide as electron donors while NO_3^- can serve as electron acceptor. In the presence of H_2, also $S_2O_3^{2-}$ can serve as electron acceptor for this archaeon (Hafenbradl et al., 1996).

Photoautotrophs utilize solar energy and dissolved inorganic carbon as their carbon source. In the water column of shallow submarine systems, photosynthesis has been described and contributes to carbon assimilation (Sorokin et al., 1998). Direct counting of autofluorescent picophytoplankton and the presence of 16S rRNA sequences affiliated to *Chlorobi* off Panarea supported the importance of photosynthesis (Maugeri et al., 2009). *Chlorobi* are also known as green sulfur bacteria. They obtain energy through anoxygenic photosynthesis. Reduced sulfur compounds serve as electron donors. CO_2 is assimilated and fixed by the reductive tricarboxylic acid cycle (Evans et al., 1966; Fuchs et al., 1980). Sulfide is oxidized to sulfate with the

intermediate accumulation of elemental sulfur globules outside of the cells. Some strains also use thiosulfate and H_2 as photosynthetic electron donors.

In addition of autotrophy, a vast majority of known thermophiles and hyperthermophiles are facultative or obligate heterotrophs. They catalyze a tremendous array of varying metabolic processes. Electron donors in redox reactions include H_2, Fe^{2+}, H_2S, S^0, $S_2O_3^{2-}$, $S_4O_6^{2-}$, sulfide minerals, CH_4, various mono-, di-, and hydroxy-carboxylic acids, alcohols, amino acids, and complex organic substrates. Electron acceptors include O_2, Fe^{3+}, CO_2, CO, NO_3^-, NO_2^-, NO, N_2O, SO_4^{2-}, SO_3^{2-}, $S_2O_3^{2-}$ and S^0 (Amend and Shock, 2001). Members of *Thermococcales*, *Archaeoglobus*, *Thermosphaera* and *Thermotoga* are known to gain energy by oxidizing or fermenting aldoses (Stetter, 1988).

3.2 Biological thermodynamics

Cultivation independent methods applied to study the microbial diversity at shallow-sea hydrothermal vent systems revealed a similarity of community structures between deep- and shallow-sea vent systems (Sievert et al., 1999; Maugeri et al., 2009; Sievert et al., 2000a). To understand the microbiology and ecology of microbial habitats, it is important to consider how microorganisms utilize substrates and gain energy. Beyond the metabolisms observed from isolated *Bacteria* and *Archaea*, potential energetic reactions are not fully discovered or understood at shallow-sea hydrothermal vents.

Mixing of reduced hydrothermal fluids and oxidized seawater yields a variety of redox couples. Through geochemical modeling of the mixing of hydrothermal fluids and seawater, without direct observation, available metabolic energy can be calculated (McCollom and Shock, 1997; McCollom, 2000). The amount of potential energy for biosynthesis depends on the availability and speciation of electron donors and acceptors. The potential for primary biomass production could be estimated by considering the amount of chemical energy available from redox disequilibria. The familiar equation being used is

$$\Delta G = \Delta G^\circ + RT \ln Q$$

Where ΔG is the free energy of the reaction, ΔG° is the standard free energy, R is the universal gas constant, T the temperature, and Q the activity quotient of the compounds involved in the reaction. Common redox reactions have been described and characterized for deep sea hydrothermal vent systems (Table 3).

Table 3. Common redox reactions and associated standard free energies of reactions that occur at deep sea hydrothermal vents (Orcutt et al., 2011).

Pathway	Reaction	$\Delta G°$ (kJ/mol)[a]
Oxic respiration	$CH_2O + O_2 \rightarrow CO_2 + H_2O$	−770
Denitrification	$CH_2O + 4/5NO_3^- \rightarrow 1/5CO_2 + 2/5N_2 + 4/5HCO_3^- + 3/5H_2O$	−463
MnO_2 reduction	$CH_2O + 3CO_2 + H_2O + 2MnO_2 \rightarrow 2Mn^{2+} + 4HCO_3^-$	−557
Fe(III) oxide reduction	$CH_2O + 7CO_2 + 4Fe(OH)_3 \rightarrow 4Fe^{3+} + 8HCO_3^- + 3H_2O$	−697
Sulfate reduction	$CH_2O + 1/2SO_4^{2-} \rightarrow HCO_3^- + 1/2H_2S$	−98
Sulfate reduction (from methane)	$CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O$	−33
Methanogenesis (from acetate)	$CH_3COOH \rightarrow CH_4 + CO_2$	−24
Methanogenesis (from H_2/CO_2)	$H_2 + 1/4HCO_3^- + 1/4H^+ \rightarrow 1/4CH_4 + 3/4H_2O$	−57
Fermentation (from ethanol)	$CH_3CH_2OH + H_2O \rightarrow CH_3COOH + 2H_2$	−181
Fermentation (from lactate)	$CH_3CH_2COO^- + 3H_2O \rightarrow CH_3COOH + HCO_3^- + 3H_2$	−1,075
Acetogenesis	$H_2 + 1/2CO_3^- + 1/4H^+ \rightarrow 1/4CH_3COO^- + H_2O$	−90
Hydrogen oxidation	$H_2 + 1/2O_2 \rightarrow H_2O$	−263
Methane oxidation	$CH_4 + 2O_2 \rightarrow CO_2 + 2H_2O$	−859
Sulfide oxidation	$H_2S + 2O_2 \rightarrow SO_4^{2-} + 2H^+$	−750
	$H_2S + 8/5NO_3^- \rightarrow SO_4^{2-} + 4/5N_2 + 4/5H_2O + 2/5H^+$	−714
Fe(II) oxidation	$Fe^{2+} + 1/4O_2 + H^+ \rightarrow Fe^{3+} + 1/2H_2O$	−48
	$Fe^{2+} + 1/5NO_3^- + 6/5H^+ \rightarrow Fe^{3+} + 3/5H_2O + 1/10N_2$	−44
	$Fe^{2+} + MnO_2 + 2H^+ \rightarrow Fe^{3+} + MnO + H_2O$	ND
Mn(II) oxidation	$Mn^{2+} + O_2 \rightarrow MnO_2$	−149
	$Mn^{2+} + 2/5NO_3^- + 4/5H_2O \rightarrow MnO_2 + 1/5N_2 + 8/5H^+$	−79
Nitrification	$NH_4^+ + 2O_2 \rightarrow NO_3^- + 2H^+ + H_2O$	−302
Anammox	$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O$	−345

Thermodynamic modeling has been used to evaluate possible metabolisms in submarine vents, sediment seeps and geothermal wells in the hydrothermal system of Vulcano Island (Amend et al., 2003; Rogers and Amend, 2006). Several possible metabolisms such as aceticlastic methanogenesis (Jones et al., 1983), sulfate, sulfite and S^0 reduction (Stetter, 1988; Huber et al., 1997), aerobic and anaerobic sulfide oxidation (Brannan and Caldwell, 1980; Hirayama et al., 2005), nitrate reduction (Huber et al., 2002), Fe(III) reduction and Fe(II) oxidation (Johnson et al., 2009) as well as aerobic H_2 oxidation (Arai et al., 2010) have been calculated and detected in Vulcano Island. Combining the methods applied to deep sea research and well documented gas and fluid investigation at shallow sea hydrothermal systems, thermodynamic modeling can be a direct and quantitative approach to determine which of a plethora of possible catabolic strategies are exergonic or endergonic.

4. Genomic and metagenomic studies of hydrothermal vents

Genomic and metagenomic studies have provided useful insights in the function of microbial groups at extreme environments. Through the decoding of genomic information, links between biosphere and lithosphere could be elucidated (Figure 6) (Reysenbach and Shock, 2002).

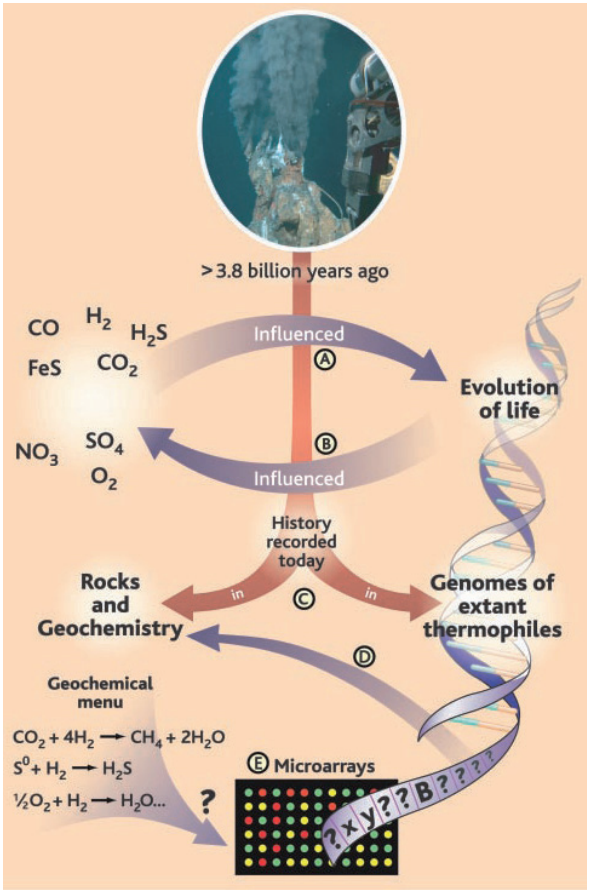


Figure 6. Biological processes and geochemistry interact with each other. Genome sequences provide genetic information pertaining to their geochemical and ecological history and their metabolic potential (Reysenbach and Shock, 2002).

Microbial diversity studies for example have shown the prevalence and versatility of *Epsilonproteobacteria* at the deep sea hydrothermal vents (Campbell et al., 2006). These groups of *Bacteria* are known to be phylogenetically related to important pathogens, like *Helicobacter*

pylori. Genomes of two deep sea vent *Epsilonproteobacteria* strains have been analyzed (Nakagawa et al., 2007). Both genomes lacked certain orthologs of virulence genes of pathogenic *Epsilonproteobacteria*, such as type IV secretion pathway and *cag* pathogenicity island genes. However, some common virulence genes do exist such as the *N*-linked glycosylation (NLG) gene cluster. It leads to the speculation that bacterial NLG might have a role in deep-sea *Epsilonproteobacteria* for maintaining a symbiotic relationship with hydrothermal vent invertebrates (Hooper and Gordon, 2001; Nakagawa et al., 2007).

Comparative phylogenetic analyses based on the small subunit ribosomal RNA gene of environmental microbial communities have indicated that the microbial diversity is much greater than those assessed by standard cultivation and isolation techniques (Amann et al., 1995; Takai and Horikoshi, 1999; Tringe and Rubin, 2005). Direct sequencing of environmental DNA – referred to as metagenomics, has brought the research in microbial ecology to a higher and broader level. Random shotgun sequencing of DNA from a natural acidophilic biofilm has initiated the first large scale environmental shotgun sequencing project (Tyson et al., 2004). To address the physiology of the uncultivated microorganisms and decipher how they thrive under these seemly hostile conditions, short-insert plasmid libraries were constructed, sequenced and the obtained sequence information was assembled. Almost complete genomes of *Leptospirillum* group II and *Ferroplasma* type II could be reconstructed as wall as three partial genomes. Analysis of the gene complement for each organism revealed the pathways for carbon and nitrogen fixation and energy generation, and provided insights into survival strategies in an extreme environment (Tyson et al., 2004). Another study of whole genome shotgun sequencing method has been applied to study the microbial community of the Sargasso Sea. This technique circumvents the PCR bias because not all rRNA genes can be amplified by the universal primers. Abundant previously unknown phylotypes were discovered and archaeal-associated genes coding for nitrification were detected (Venter et al., 2004).

Through the invention and development of new technologies, the so called next generation sequencing techniques, the associated timelines and costs of genome and metagenome sequencing have changed and widened the scope of biological research (Mardis,

2008). One of the platforms is Roche/454 FLX which uses pyrosequencing technology (Margulies et al., 2005). This approach has already been applied to comparative metagenomic studies. An example was shown in the study of a hydrothermal vent field at the Juan de Fuca Ridge. A fosmid library was constructed and genes for mismatch repair and homologous recombination were found and clustered closely with those from Lost City vent site. It suggests that the microorganisms have evolved extensive DNA repair systems to cope with the potential deleterious effects on the genomes. Reconstruction of the metabolic pathways revealed the presence of sulfur oxidation putatively coupled to nitrate reduction (Xie et al., 2011).

II Aims of this study

The deep-sea biosphere has been considered to be one of the most barren habitats on Earth and yet it has been shown to host dense microbial communities (Corre et al., 2001; Takai et al., 2003; Crépeau et al., 2011). Studies of deep-sea hydrothermal systems have yielded important information on the evolution as well as the chemical and physical limits to life. Their counterparts – shallow-sea hydrothermal systems are much easier to access and exhibit similar geochemical characteristics. Nevertheless, they are still poorly investigated. The shallow-sea hydrothermal vent systems located off Panarea has been discribed at 1890 (Italiano and Nuccio, 1991). Geological investigation has been going on for decades and the sites were revisited annually. However, micro-biological studies at this 4 km^2 hydrothermal vent systems are still limited to cultured thermophilic sulfur oxidizers and relatively simple diversity studies of surface sediments and hydrothermal fluids (Gugliandolo et al., 1999; Gugliandolo et al., 2006; Maugeri et al., 2011). In this thesis, the microbial community was investigated in sediments of Hot Lake, a hydrothermal site off Panarea. It is a depression located at 18 m below sea level. The area is covered with white mats of elemental sulfur and microorganisms. This study was part of an interdisciplinary study and paralleled by the investigation of physico-chemical characteristics of pore waters, geological analyses of sediments and the analysis of intact polar lipids (IPLs), aiming to resolve the key metabolisms driving this ecosystem.

Mixing of reduced hydrothermal fluids from the subsurface with oxidized seawater generates chemical disequilibria. Chemolithotrophs can take the advantage using these disequilibria to obtain energy through the coupling of redox reactions. The first objective was to understand the chemical composition and the temperature profiles in the depression of Hot Lake. Based on the information of physical and chemical parameters, thermodynamic modeling of redox pairs could be assessed. It supplied us with a hypothesis on potential metabolisms fueling this ecosystem. The second goal was to investigate the microbial diversity and community structure applying the full cycle rRNA approach. From the 16S rRNA clone library, phylogenic information on members of microbial communities was gained. Subsequently, oligonucleotide probes targeted 16S rRNA were applied to quantify main clusters of *Bacteria* and *Archaea* using the method fluorescence *in situ* hybridization (FISH). The third objective

was to gain insights into relevant chemosynthetic pathways. Metagenomic analysis was applied to analyze key genes in total environmental DNA and to reveal more information on the genetic capabilities of the key microbial groups. The focus was on genes indicative of carbon fixation, sulfur transformations and cycles.

III Materials and methods

1. Site description

Hot lake (also called *Lago Caldo*), is an oval-shaped (~10 by 6 meters) shallow (~2.5 m deep) depression in the seafloor at ~18 m water depth, located approximately 2 km east of the main island of Panarea (38°38.432'N, 15°6.602'E). During the time of sampling, the bottom of the depression contained sediments and detritus of sea grass. Microbial mats embedded in elemental sulfur precipitates (Figure 7) covered rocks and were hanging from the underside of the walls of the depression (Figure 8). When the depression has not been disturbed, there was an obvious halocline (Steinbrückner, 2009). Temperatures in the sediments of this brine pool were typically in the range of ~35 to 45ºC, but could reach as high as 94ºC (Sieland, 2009).

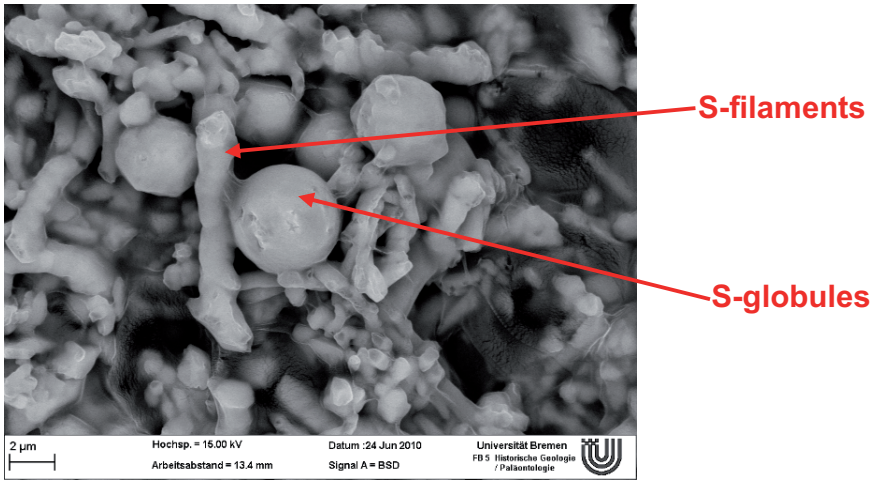


Figure 7. Sulfur morphology from white mat at Hot Lake (Viola Krukenberg and Wolfgang Bach, unpublished data).

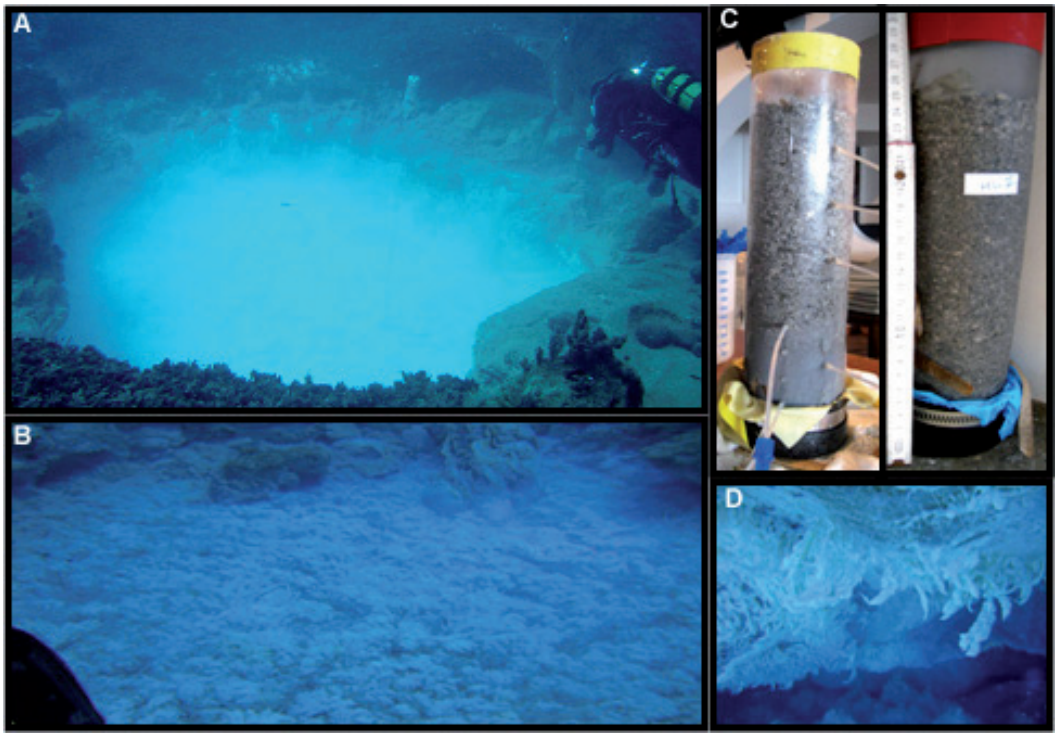


Figure 8. (A) Overview of Hot Lake. (B) Inside of the depression containing sediments, detritus of sea grass and ubiquitous mats containing elemental sulfur and microbes. (C) Sediment cores taken from 2009 at two locations (Hot Lake I and Hot Lake II) inside of Hot Lake. (D) Mats hanging from the walls of the depression.

2. Sample collection

Pore fluids, sediment cores and sulfur/microbial mats were collected at Hot Lake by SCUBA diving during field expeditions in July 2008, May 2009, and June 2010. For further molecular analyses, samples of 2009 were characterized thoroughly whereas samples from 2008 and 2010 were investigated with automated rRNA intergenic spacer analysis for diversity study.

Prior to sampling the sediment cores, the *in situ* temperature was determined approximately every square meter with a temperature probe. In 2009, a set of sediment cores of about 20 cm length was collected at a medium temperature (Hot Lake I, 36°C at 10 cm) and a high temperature (Hot Lake II, 74°C at 10 cm) site, respectively, within the depression. Samples were stored at room temperature and processed within 2 hours. Pore fluid retrieval with rhizones and the subsequent analyses were carried out by Roy Price (University of South California, USA) following methods outlined in Kölling et al. (Kölling et al., 2005). As pore water was generally lost during core slicing, cores for pore water analyses were subsequently also used for sample preparation for molecular and intact polar lipid analyses. Sediment cores were sliced in 1~2 cm intervals from top to bottom. Samples for DNA extraction were frozen at -20°C.

3. Thermodynamic modeling of potential reactions

To evaluate the amount of energy available at a given temperature, pressure, and chemical compositions at Hot Lake, thermodynamic modeling was preformed. The Gibbs energy ΔG can be calculated with the equation

$$\Delta G = \Delta G^0 + RT \ln Q$$

where ΔG^0 represents the standard Gibbs energy of reaction, R is the universal gas constant, T is the temperature in Kelvin, and $\ln Q$ denotes the reaction activity quotient. Values of ΔG were calculated at the temperatures and pressures of interest with the computer software package SUPCRT92 (Johnson et al., 1992).Values of Q can be calculated from the equation

$$Q = \Pi ai^{\,vi}$$

where ai is the activity and were calculated from the measured pore water compositions from the sediment core of Hot Lake II (22 cm, by Roy price, USC, USA. unpublished data) and the venting gas concentrations from the venting sites (Francesco Italiano, INGV, Palermo. unpublished data). Activities were calculated using the REACT speciation module in THE GEOCHEMIST'S WORKBENCH software package (v.7.0, Rockware, University of Illinois, Bethke & Yeakel, 2008). Values of ΔG for all redox reactions were normalized per mole of electrons transferred and all reactions were written in the direction in which they are exergonic.

4. DNA extraction

Genomic DNA was extracted from 10 g of homogenized sediment. Sediment samples from 2008 and those of layer 0-1 cm, 1-2 cm, 5-7 cm, and 17-20 cm sampled in 2009 at Hot Lake II were extracted with the SDS based DNA extraction method published by Zhou et al. (Zhou et al., 1996) including three times freeze (liquid nitrogen) and thaw (42°C water bath) cycles. The DNA was dissolved in 200 µl 1x TE buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA).

DNA from Hot Lake 2010, Hot Lake I (2009) sediment layers 0-1 cm, 1-2 cm, 7-9 cm, 13-15 cm, and 15-17 cm as well as from Hot Lake II (2009) sediment layers 7-9 cm, 14-17 cm and 17-20 cm was extracted using the UltraClean® Mega Soil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. The DNA was precipitated with 5 M NaCl and 96% ice cold ethanol and centrifuged at 2500g for 30 minutes. The DNA was then dissolved in 1x TE buffer and quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

5. Automated rRNA intergenic spacer analysis (ARISA)

DNA quantities were standardized to 10 ng per 25 µl master mix for the PCR amplification. Primers used for the amplification of the intergenic spacer (ITS) region were previously described by (Cardinale et al., 2004): ITSF (5′-GTCGTAACAAGGTAGCCGTA-3′) and ITSReub (5′-GCCAAGGCATCCACC-3′) labeled with the phosphoramidite dye HEX (6-carboxy-1, 4 dichloro-20, 40, 50, 70-tetra-chlorofluorescein). The primers were complementary to position 1423–1443 of the 16S rRNA gene (ITSF) and position 38–23 of the 23S rRNA gene (ITSReub) of *Escherichia coli*. PCR products were visualized on a 1.5% agarose gel prior to purification by gel filtration on a Sephadex G-50 Superfine column (Sigma Aldrich, Munich, Germany). The separation of fragments by capillary electrophoresis, evaluation of electrophoretic signals and subsequent binning into operational taxonomic units (OTUs) was done as reported elsewhere (Ramette, 2009). An OTU was considered to be present if it appeared in at least two of the three PCR replicates, and fingerprint profiles were standardized by dividing each individual peak area by the total area of peaks in a given profile using Gen Mapper. The consensus ARISA table sampled by operational taxonomic unit (OTUs) was used to calculate pair wise similarities among samples based on the Bray–Curtis similarity index. The resulting matrix was examined for patterns in bacterial community structure by using non-metric multidimensional scaling as implemented in the data analysis package – PAST. Analysis of similarity (ANOSIM) was further carried out to test for significant differences among sample groupings.

6. 16S rRNA gene clone library construction

Oligonucleotide primers GM3F (5′-AGA GTT TGA TCM TGG C-3′) (Muyzer et al., 1995) and GM4R (5′-TAC CTT GTT ACG ACT T-3′) (Muyzer et al., 1995) were used to amplify almost complete 16S rRNA genes from *Bacteria*. Archaeal 16S rRNA genes were amplified with the universal archaeal primers ARCH20F (5´-TTC CGG TTG ATC CYG CCR G-3´) (DeLong, 1992) and Uni1392R (5′-ACG GGC GGT GTG TRC-3′) (Stahl et al., 1988). The 20 µl reaction contained 10-100 ng DNA as template, 0.5 µM of each primer (Biomers.net GmbH), 10 mM of dNTPs (Roche Deutschland Holding GmbH), 1 x amplification buffer and 5 U of Eppendorf-Taq DNA Polymerase (Eppendorf, Hamburg, Germany).

PCRs were performed in ten replicates with 26-28 cycles (*Bacteria*) and 35 cycles (*Archaea*) to minimize PCR bias. After 5 min at 94°C each cycle consisted of 1 min at 94°C, 1 min at 48°C (*Bacteria*) or 58°C (*Archaea*), and 3 min at 72°C. The amplicons were pooled, purified using a PCR purification kit (QIAGEN, Hilden, Germany). Afterwards the purified PCR products were ligated using the pGEM®-T Easy Vector Systems (Promega, Madison, WI) according to the manufacturers recommendations and transformed into chemically competent *E. coli* TOP 10 cells (Invitrogen). Clones with a correct insert size of ~1500 bp were sequenced using ABI BigDye Terminator chemistry and an ABI377 sequencer (Applied Biosystems).

Sequencing was conducted using the vector primers M13F (5′-GGA AAC AGC TAT GAC CAT G-3′) and M13R (5′-GTT GTA AAA CGA CGG CCA GT-3′) for full length sequences. Sequencing for the bacterial partial sequences was conducted using internal bacterial primer GM1F (5´-CCA GCA GCC GCG GTA AT-3´) (Muyzer et al., 1993). As for the archaeal partial sequences the internal archaeal primer ARCH958R (5´-TCC GGC GTT GAM TCC AAT T- 3´) (DeLong, 1992) was utilized.

7. Phylogenetic analysis and probe design

The phylogenetic affiliation of 16S rRNA gene sequences was inferred with the ARB software package (Ludwig et al., 2004) based on release SILVA 104 of the SILVA database (Pruesse et al., 2007). OTUs were generated based on a minimal alignment of 500 bp using the *Mothur* software package (Schloss and Handelsman, 2006; Schloss et al., 2009). For a single representative of each OTU, the complete 16S rRNA gene sequence was determined. Phylogenetic trees were calculated by parsimony, neighbor-joining, and maximum-likelihood (RAxML and PhyML) algorithms applying different base frequency filters of 30%, 50% and 60%. For tree calculation, only almost full length sequences (> 1400 bp) were considered and partial sequences were added to the reconstructed tree by maximum parsimony criteria without allowing changes in the overall tree topology. Relevant long and short sequences available in public databases were included in all phylogenetic analyses.

8. Cell staining and catalyzed-reporter deposition fluorescence in situ hybridization (CARD-FISH)

The cell fixation for total cell counts and FISH was carried out directly after sampling. 0.5 ml sediment was fixed with 4% formaldehyde in 1x phosphate buffered saline (PBS; 10 mM sodium phosphate, 130 mM sodium chloride, pH 8.0) for 2-4 hours at 4°C. Afterward, samples were centrifuged for 5 min at 10000 rpm, washed twice with 1x PBS and finally stored in 1.5 ml 50% 1x PBS/ethanol at −20°C until further processing. To dislodge cells from sediment grains fixed samples were treated by mild sonication for 7x 30 s with a MS73 probe (Sonopuls HD70, Bandelin, Germany) (cycle 20, 30% power). One ml supernatant was exchanged for 1 ml fresh 50% 1xPBS/ethanol, followed by an additional sonication step. This procedure was repeated seven times and supernatants were combined.

Catalyzed-reporter deposition FISH (CARD-FISH) was performed following the protocol by Pernthaler et al. (Pernthaler et al., 2002). The sediment samples were filtered on GTTP filters with 0.2 μm pore size (Millipore, Germany). For permeabilization of rigid archaeal cell walls, cells were treated with Proteinase K solution (15 μg/ml) for 3 min at room temperature. Oligonucleotides were purchased from Biomers (Ulm, Germany). Oligonucleotide probes used in this study are listed in Table 4. For reference cell visualization, samples were stained with 4'6'-diamidino-2-phenlyindole (DAPI) for 10 min (1 μg/ml) and washed with sterile filtered water and 80% ethanol for seconds. Air-dried filters were embedded in Citifluor (Citifluor Ltd., Leicester, UK). The given CARD-FISH counts are means calculated from 100 randomly chosen microscopic fields with at least two separate CARD-FISH procedures. Cells were counted using an epifluorescence microscope (Axioplan, Zeiss, Germany). Parallel to every hybridization, total cell counts were enumerated separately from the rest parts of filters stained by SybrGreen I (Invitrogen). The staining procedure included mounting and fixing the sample by a solution of polyvinylalcohol (moviol) (Lunau et al., 2005).

9. Probe design and optimization of hybridization conditions

Oligonucleotide probes were designed using the probe tool in the ARB software package (Ludwig et al., 2004). The probes were tested for coverage (target group hits) and specificity (outgroup hits) *in silico* with the ARB probe match tool (Ludwig et al., 2004). For evaluation of probe coverage, only sequences that possessed sequence information at the probe binding site were considered. Probe specificity was based on 512037 prokaryotic sequences of the SILVA SSU Ref dataset Release 104 (Pruesse et al., 2007). At least up to two mismatches per sequence were reviewed manually. Specific hybridization conditions were determined by applying different formamide concentrations (0%, 10%, 20%, 30%, 40% and 50%) directly on the environmental samples. The consistent morphology was taken as the primary criteria for verification of the probes.

Table 4. Oligonucleotide probes and hybridization conditions used in this study

Target	Probe	Probes sequences (5'-->3')	FA%	Reference
Most Bacteria	EUB 338	GCT GCC TCC CGT AGG AGT	35	Amann et al., 1990
	EUB 338-II	GCA GCC ACC CGT AGG TGT	35	Daims et al., 1999
	EUB 338-III	GCT GCC ACC CGT AGG TGT	35	Daims et al., 1999
Most Archaea	ARCH915	GTG CTC CCC CGC CAA TTC CT	35	Stahl and Amann, 1991
Control probe complementary to EUB 338	NON338	ACT CCT ACG GGA GGC AGC	35	Wallner et al., 1993
Epsilonproteobacteria	EPSY914	GGT CCC CGT CTA TTC CTT	45	Grote et al., 2007
Arcobacter sp.	ARC94	TGC GCC ACT TAG CTG ACA	20	Snaidr et al., 1997
Deltaproteobacteria	Delta495a	AGT TAG CCG GTG CTT CCT	30	Loy et al., 2002
	Delta495b	AGT TAG CCG GCG CTT CCT	30	Loy et al., 2002
	Delta495c	AAT TAG CCG GTG CTT CCT	30	Loy et al., 2002
competitor for *Deltaproteobacteria*	cDelta495a	AGT TAG CCG GTG CTT CTT	30	Loy et al., 2002
	cDelta495b	AGT TAG CCG GCG CTT CKT	30	Loy et al., 2002
	cDelta495c	AAT TAG CCG GTG CTT CTT	30	Loy et al., 2002
Gammaproteobacteria	GAM42a	GCC TTC CCA CAT CGT TT	35	Manz et al., 1992
competitor for GAM42a	BET42a	GCC TTC CCA CTT CGT TT	35	Manz et al., 1992
most *Flavobacteria* , some *Bacteroidetes* , some *sphingobacteria*	CF319a	TGG TCC GTG TCT CAG TAC	35	Manz et al., 1996
Aquificae (except *Desulfurobacteriaceae*)	AQI338	GCT GCC CCC CGT AGG GGT	40	Kubo et al., 2011
Euryarchaeaota /Korarchaeota	EURY514	GCG GCG GCT GGC ACC	20	Jurgens et al., 2000
Crenarchaeota	CREN512	CGG CGG CTG ACA CCA G	10	Jurgens et al., 2000
Green sulfur bacterium	BSV26	TTC CGA ACT CAA GGC TTG	30	this study
Green sulfur bacterium	OPB56	CCC AGG TGG TAT GCT TAA	30	this study
uncultured Bacteroidetes	VC2.1 BAC22_upper	ATC GGC TTG GTA AGC TGC	35	this study
uncultured Bacteroidetes	VC2.1 BAC22_lower	TTG CGT AGG CAG TTC CCT	40	this study

10. Pyrosequencing of genomic DNA

Genomic DNA was extracted from the surface sediment layer (0-2 cm) of Hot Lake I and II with the SDS based DNA extraction method published by Zhou et al. (Zhou et al., 1996) including three times freeze (liquid nitrogen) and thaw (42°C water bath) cycles. The DNA was dissolved in 200 µl 0.5x TE buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA). A total of ~ 0.2 mg DNA was used for direct sequencing using the GS DNA Library Preparation Kit, following the instructions of the GS FLX Shotgun DNA Library Preparation Manual (Roche Diagnostics). Pyrosequencing resulted in 515,111 reads for Hot Lake I and 476,604 reads for Hot Lake II. The reads were assembled using the Newbler Assembly software (version 2.5.3, Roche Diagnostics). Moreover, the unassembled reads were de-replicated with a CD-Hit-based 454 replicate filter (Gomez-Alvarez et al., 2009), allowing 1% mismatches in the overlapping regions and up to three base pairs difference in the start position.

11. ORF prediction, annotation, phylogenetic and metabolic analyses of pyrosequencing derived data

Gene prediction was carried out by using a combination of the Metagene (Noguchi et al., 2006) and Glimmer3 (Delcher et al., 2007) softwares. Ribosomal RNA genes were detected by using the rRNA prediction algorithm by Huang et al. (Huang et al., 2009) and transfer RNAs by tRNAscan-SE (Lowe and Eddy, 1997). The annotation of the metagenome sequence was performed with a modified GenDB v2.2.1 system (Meyer et al., 2003), supplemented by the tool JCoast, version 1.6 (Richter et al., 2008). The predicted ORFs were compared against public sequence databases (nr, SWISSPROT, KEGG) and protein family databases (Pfam, InterPro, and COG). Signal peptides were predicted with SignalP v3.0 (Nielsen et al., 1999; Emanuelsson et al., 2007) and transmembrane helices with TMHMM v2.0 (Krogh et al., 2001). Predicted protein coding sequences were automatically annotated by MicHanThi (Quast, 2006). The MicHanThi software predicts gene functions based on similarity searches using the NCBI-nr (including Swiss-Prot) and InterPro database. Metabolic analyses were performed using JCoast against KEGG database and Pfam database (E-value of 10^{-5}).

12. Taxonomic classification of metagenome sequences

Taxonomic classification was performed through MG-RAST, a pipeline for 16S rRNA tags (Peplies, J. in prep.) and the so-called Taxometer pipeline (Waldmann, J. in prep.). Assembled sequence reads were annotated automatically to assign to a putative gene function. Every sequence submitted to MG-RAST by running BLAT (Kent, 2002) was then compared to the GenBank database for taxonomic classification.

Unassembled sequence reads from metagenome sequencing were preprocessed (quality control and alignment) through the bioinformatics pipeline of the SILVA project (Pruesse et al., 2007). Pyrosequencing reads shorter than 200 nt and more than 2% of ambiguities or 2% of homopolymers were removed. The remaining reads were aligned against the SSU rRNA seed of the SILVA database release 106 (Pruesse et al., 2007) and used for downstream analysis. Through this method, putative partial SSU rRNA gene reads within the data set could be extracted. Subsequently, remaining reads wer dereplicated, clustered and classified on a sample by sample basis. Dereplication (identification of identical reads ignoring overhangs) was carried out by cd-hit-est of the cd-hit package 3.1.2 (http://www.bioinformatics.org/cd-hit) using an identity criterion of 1.00 and a wordsize of 8. Remaining sequences were clustered again with cd-hit-est using an identity criterion of 0.98 (same wordsize). The longest read of each cluster was used as a reference for taxonomic classification with a local BLAST search against the SILVA SSURef 106 NR dataset (http://www.arb-silva.de/projects/ssu-ref-nr/) using blast-2.2.22+ (http://blast.ncbi.nlm.nih.gov/Blast.cgi) with standard settings. The full SILVA taxonomic path of the best blast hit has been assigned to the reads of the value for [(% sequence identity + % alignment coverage)/2] at least 93.0. In the final step, the taxonomic path of each cluster reference read was mapped to the additional reads within the corresponding cluster plus the corresponding replicates, identified in the previous analysis step, to finally obtain (semi-) quantitative information (number of individual reads representing a taxonomic path).

Through the Taxometer pipeline, a consensus from four individual taxonomic prediction tools was used to infer the taxonomic affiliation of the metagenome sequences: (a) CARMA

(Krause et al., 2008) infers taxonomy of sequences by post-processing genes with HMMER hits to the Pfam database. (b) KIRSTEN (Kinship Relationship Reestablishment, unpublished) infers taxonomy of sequences by post-processing BLAST hits by means of rank-based statistical evaluations on all 27 levels of the NCBI taxonomy with an increasing stringency from the superkingdom down to the species level. (c) SU tag analysis (Waldmann *et al.*, in preparation) extracts all full and partial 16S ribosomal RNA genes from the de-replicated reads, maps them to a well-curated reference tree provided by the SILVA rRNA database project (Pruesse et al., 2007) and then uses this information to infer the taxonomy of the contigs into which the reads were assembled. (d) SSAHA2 (Ning et al., 2001) was used to map the de-replicated pyrosequencing reads on a well-chosen set of marine reference genomes taken from EnvO-lite environmental ontology. For each sequence, the combined mapping information was used to infer its taxonomic affiliation.

IV Results

1. Prediction of possible energy gaining processes at Hot Lake from thermodynamic modeling

Through premilinary mineralogy investigation of the sediments of Hot Lake, metal sulfide, such as FeS_2 or Fe-monosulfide were found. These compounds could be formed during microbially mediated sulfate reduction or iron reduction (Rioux, 2004). In this thesis, fourteen reactions which can be exploited for primary production by chemolithoautotrophic microorganisms have been evaluated using geochemical models. The energetic evaluations performed in this study were calculated with a vent fluid-to-seawater mixing ratio of 50:1. From the thermodynamic models, all chemolithoautotrophic reactions were shown to be exergonic under microaerophilic condition (Figure 9). Among the reactions, it appeared that hydrogen oxidation (knallgas reaction), sulfide oxidation, Manganese (IV) oxide reduction and Fe (II) oxidation bear the most negative ΔG, and are therefore the favorable redox pairs for microorganisms at Hot Lake. However, the concentration of common electron acceptors such as O_2 and nitrate was below the detection limit (Frank Wenzhöfer, MPI Bremen/AWI, Roy Price, USC, USA, personal communication) showing these acceptors would be utilized immediately by the microbes.

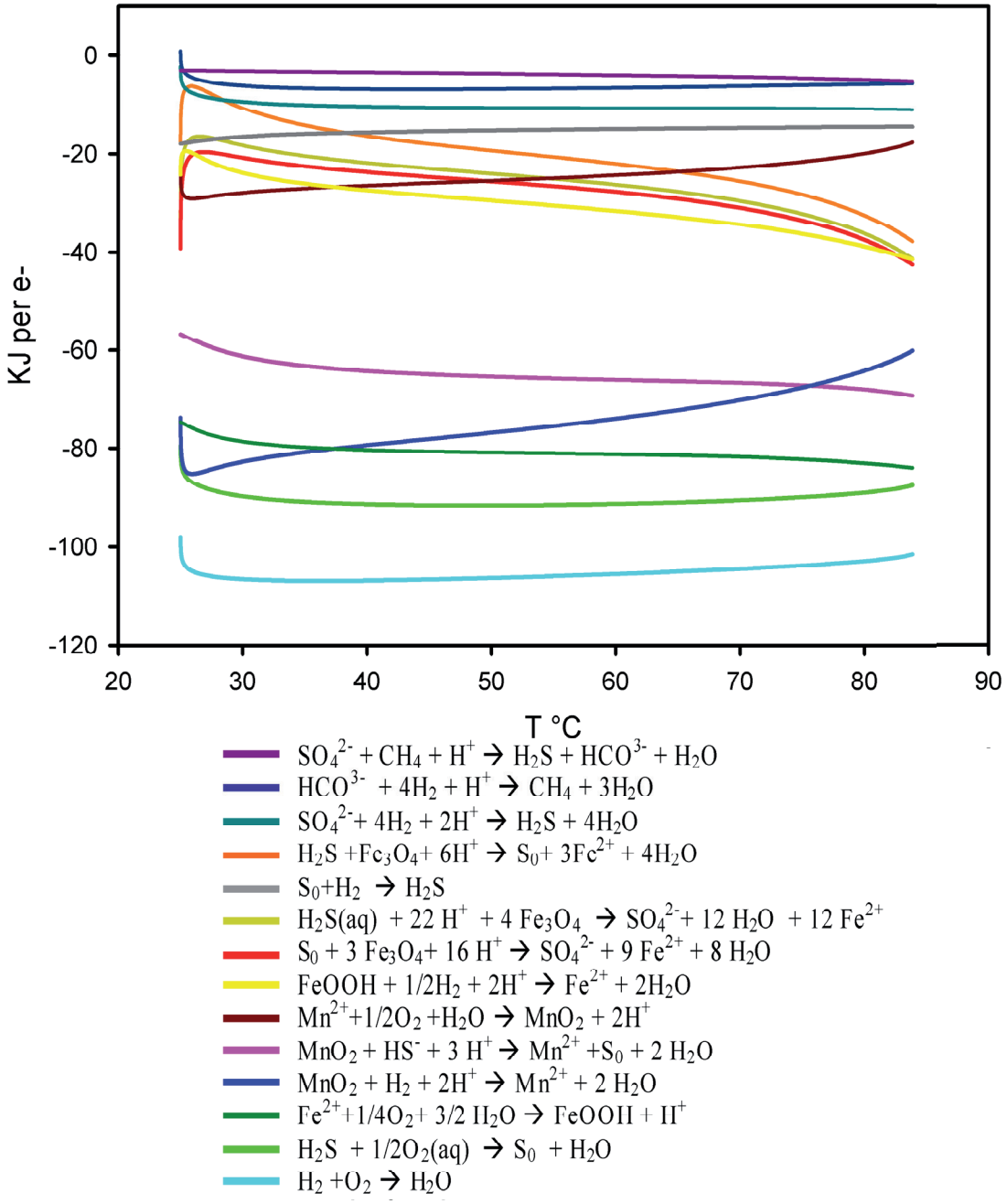


Figure 9. Free energy yields of reactions (in kJ/mol e^-) calculated from a thermal fluids-seawater reaction model at temperatures ranging from 20-90°C.

2. Community structure analysis by automated rRNA intergenic spacer analysis

A molecular fingerprinting technique such as automated rRNA intergenic spacer analysis (ARISA) is an alternative culture independent method to study the microbial diversity. This method has been shown to be highly reproducible, robust and time-efficient in previous comparative analyses of microbial community structures (Ramette, 2009). Sediment samples from different layers in three subsequent years at Hot Lake (Table 5) were compared.

Table 5. List of samples of automated rRNA intergenic spacer analysis

Samples	Year	Depth (cm)	T°C at 10 cm
STV1	2008	26-33	n.a.
STV2	2008	19-26	n.a.
STV3	2008	12-19	n.a.
HLI_0-1	2009	0-1	36.8
HLI_1-2	2009	1-2	36.8
HLI_2-3	2009	2-3	36.8
HLI_5-7	2009	5-7	36.8
HLI_7-9	2009	7-9	36.8
HLI_9-11	2009	9-11	36.8
HLI_11-13	2009	11-13	36.8
HLI_13-15	2009	13-15	36.8
HLII_0-1	2009	0-1	73.7
HLII_1-2	2009	1-2	73.7
HLII_2-3	2009	2-3	73.7
HLII_5-7	2009	5-7	73.7
HLII_7-9	2009	7-9	73.7
HLII_9-11	2009	9-11	73.7
HLII_11-14	2009	11-14	73.7
HLII_14-17	2009	14-17	73.7
HL06_0-1	2010	0-1	54.7
HL06_1-2	2010	1-2	54.7
HL06_4-6	2010	4-6	54.7
HL06_6-8	2010	6-8	54.7
HL06_8-10	2010	8-10	54.7
HL06_12-15	2010	12-15	54.7

n.a.,not available

DNA was extracted from different sediment layers for automated rRNA intergenic spacer analysis (ARISA). Different patterns among each sample were visualized in a nonmetric multidimensional scaling (NMDS) plot (Figure 10) after statistic analysis. To test for significant differences among different years and the two sites from 2009, analysis of similarities (ANOSIM) was applied. According to the classification from Clarke and Gorley (Clarke and Gorley, 2006), a value $R = 1$ indicates that the groups are separated and $R = 0$ denotes no separation between groups. To define the differences more adequately, $R > 0.75$ is commonly interpreted as well separated, $R > 0.5$ as separated but overlapping and $R < 0.25$ as barely separable. Table 6 shows the relationship among groups. The bacterial community structure in 2008 was well separated from those in 2009 and 2010. Diversity at Hot Lake I in 2009 showed significant difference compared to Hot Lake II in 2009 and the samples in 2010. By the ordination of NMDS, four groupings could be visualized: the samples from 2008, the samples from Hot Lake I, the upper layers of Hot Lake II (2009) and samples from 2010, and the deeper layers of Hot Lake II (2009). In order to test whether different DNA extraction method result in different ordination, samples from 17-20 cm of Hot Lake II were tested and they appeared closely on the plot (data not shown).

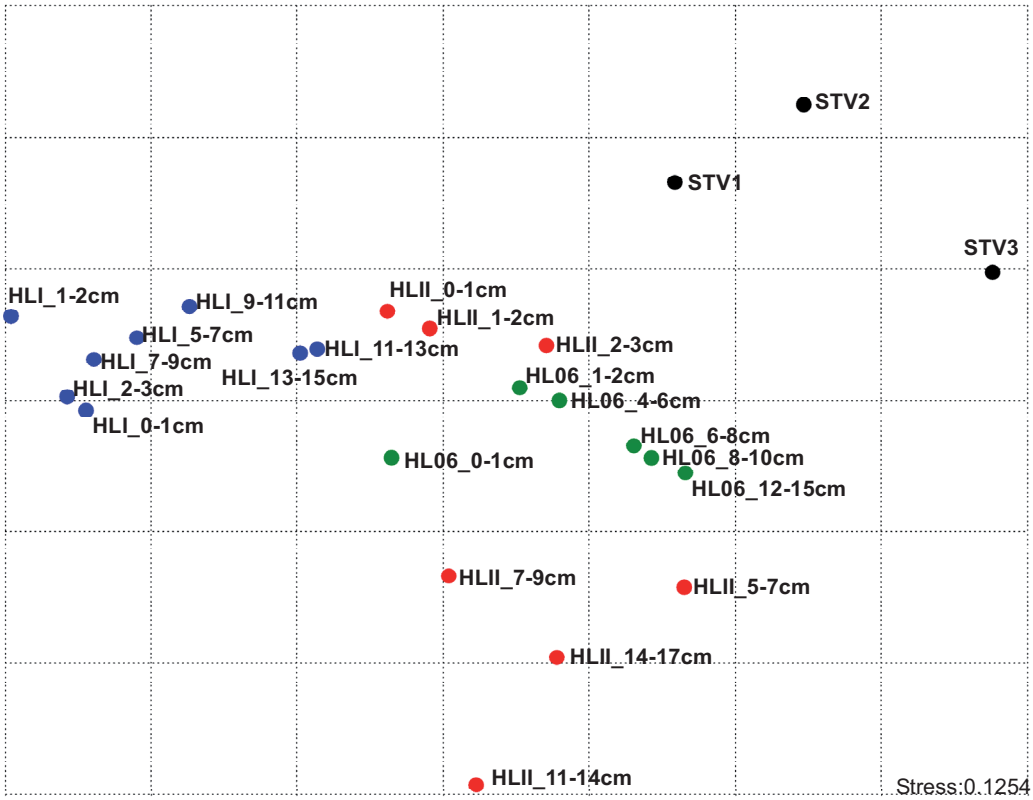


Figure 10. Nonmetric multidimentional scaling (NMDS) plot presenting the differences seen in the automated ribosomal intergenic spacer analysis (ARISA) profiles of samples from different layers of Hot Lake from 2008 to 2010 in a reduced two-dimensional space (stress value= 0.1254). Black: Hot Lake 2008. Blue: Hot Lake I 2009. Red: Hot Lake II 2009. Green: Hot Lake 2010.

Table 6. One way-ANOSIM significance testing; Distance measure: Bray-curtis

	R value			
	Hot Lake 2010	**Hot Lake I 2009**	**Hot Lake II 2009**	**Hot Lake 2008**
Hot Lake 2010		0.9603**	0.2407*	0.9568*
Hot Lake I 2009	0.9603**		0.7085**	1**
Hot Lake II 2009	0.2407*	0.7085**		0.7381**
Hot Lake 2008	0.9568*	1**	0.7381**	

*:$P \leq 0.05$; **:$P \leq 0.01$.

3. Diversity of bacterial 16S ribosomal RNA genes

Molecular fingerprinting methods gave us insights into changes of the microbial community structure. To assess diversity of *Bacteria* and *Archaea* in more detail, 16S rRNA clone libraries were constructed from the same sediment samples used for ARISA. The focus was on the samples from 2009. Pore water analysis and correlated intact polar lipids (IPLs) analysis were as well carried out from this year and will be discussed together later on with the community compositions.

From these two sites of Hot Lake, four clone libraries were constructed. At Hot Lake I, the ordination of the NMDS from the results of ARISA showed high similarity. Therefore DNAs from different layers of sediments (0-11cm) were pooled to gain an overview of microbial community structure. At Hot Lake II, the ordination was scattered and widely distributed. The temperature profile and chemical parameters from pore water analysis of Hot Lake II revealed a stronger influence of hydrothermal fluids (Figure 28, Figure 29). To gain better insight into how microorganisms adapt to and survive at the harsh environment, three different layers – 0-1 cm, 5-7 cm and 14-17 cm of Hot Lake II sediments were chosen for the 16S rRNA gene analyses. The bacterial 16S rRNA gene diversity for each sample is shown in Figure 11. A total of 133 bacterial SSU rRNA gene clones were sequenced from the pooled Hot Lake I sample (Figure 11). From each of the Hot Lake II depth samples, nearly 170 bacterial SSU rRNA gene clones were sequenced (Figure 11). Overall, the dominant groups in the four clone libraries belonged *to Epsilonproteobacteria*, *Deltaproteobacteria* and *Bacteroidetes* (Figure 11).

Sequences related to *Sulfurospirillum* within the class *Epsilonproteobacteria* were found to be most abundant at Hot Lake I as well as in 0-1 cm and 5-7 cm at Hot Lake II. Other epsilonproteobacterial sequences were closely related to *Sulfurimonas*, *Campylobacter*, *Nitratiruptor, Sulfurovum* and *Arcobacter*. Sequences related to *Desulfobacteraceae* and cluster SVA0485 were the dominant groups within the class *Deltaproteobacteria* at Hot Lake I and Hot Lake II. Uncultured bacteria of the VC2.1 Bac22 group, belonging to the class

Bacteroidetes, first discovered at a deep Mid-Atlantic Ridge hydrothermal vent (Reysenbach et al., 2000) was found to be dominant at Hot Lake I and Hot Lake II.

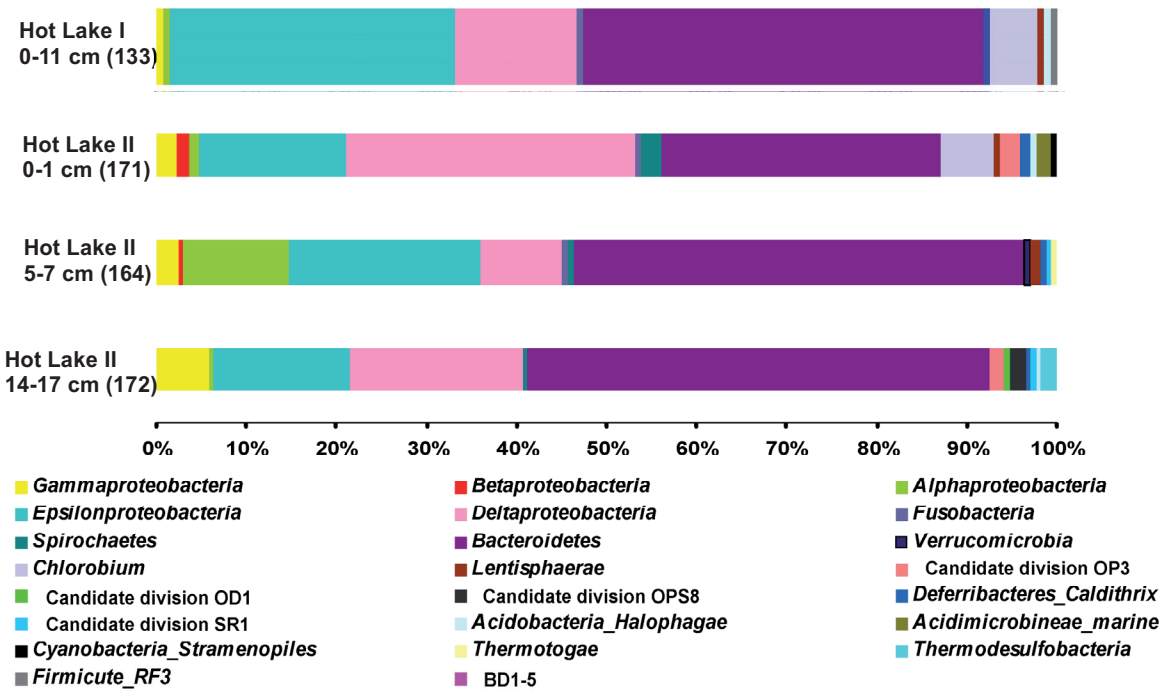


Figure 11. Clone affiliations and frequencies in bacterial 16S rRNA libraries from sediment at Hot Lake I and Hot Lake II. The number of sequenced clones in each clone library is shown in parentheses.

Sequences affiliated to *Gammaproteobacteria* and *Alphaproteobacteria* were detected both at Hot Lake I and Hot Lake II (Figure 11). Betaproteobacterial sequences were detected only at 0-1 cm and 5-7 cm at Hot Lake II. Sequences affiliated to *Fusobacteria* were detected at Hot Lake I and in the 0-1 cm and 5-7 cm layers at Hot Lake II. Sequences related to *Spirochaetes* only appeared at Hot Lake II. Verrucomicrobial sequences were detected at Hot Lake I and at 5-7 cm depth at Hot Lake II. Sequences affiliated to *Chlorobium*, known as green sulfur bacteria, were found at Hot Lake I and at Hot Lake II at 0-1 cm. Sequences affiliated to

Lentisphaerae were detected at Hot Lake I and in the 0-1 cm and 5-7 cm layer at Hot Lake II. Sequences affiliated to several candidate divisions such as OP3, OD1, OPS8 and sequences related to *Caldithrix* were found exclusively at Hot Lake II. Sequences related to *Halophagae* were detected at Hot Lake I and at 0-1 cm, 14-17 cm at Hot Lake II. Acidimicrobineal and Cyanobacterial sequences were detected in 0-1 cm at Hot Lake II. Sequences affiliated to *Thermodesulfobacteria* were detected only in 14-17 cm at Hot Lake II. Sequences affiliated to *Thermotogae* were found at 5-7 cm at Hot Lake II. The two groups are known as hyperthermophiles (Huber et al., 1991).

Maximum Likelihood phylogenetic trees of *Epsilonproteobacteria, Deltaproteobacteria* and *Bacteroidetes* were built for a better understanding the evolutionary relationship of the microorganisms dwelling at Hot Lake (Figure 12, Figure 13, and Figure 14). Epsilonproteobacterial clones were affiliated to the genus *Sulfurovum*, *Sulfurimonas*, *Arcobacter*, *Sulfurospirillum*, *Campylobacter* and *Nitratiruptor* (Figure 12). Most of the sequences were closely related to clones from uncultured bacteria detected at other hydrothermal vents. Within the the genus *Sulfurovum*, sequences from Hot Lake I were found to be closely related to the SUP01 group from the hydrothermal plume inside the Suiyo Seamount caldera (Sunamura et al., 2004). Other sequences within *Sulfurovum* were affiliated with clones from uncultured bacteria at basaltic flanks of the East Pacific Rise and *Riftia pachyptila* associated symbionts.

In the genus *Sulfurimonas*, sequences were closely related to those from shallow submarine hydrothermal system off Taketomi Island, Japan (Hirayama et al., 2007) and from iron oxidizing *Bacteria* at the seafloor of volcanoes on the South Tonga Arc (Forget et al., 2010). Abundant *Sulfurimonas* sequences were shown to be related to sequences of gill symbionts and hydrothermal vent microbial mat clones from Loihi Seamount, Hawaii (Moyer et al., 1995). Within the genus *Arcobacter*, sequences were found close to cold seep clones and *Osedax* symbionts. As for *Sulfurospirillum*, sequences were found to be close to the bacterial clones from the Lost City Hydrothermal Field. Furthermore, sequences of *Campylobacter* from Hot Lake affiliated with those from Dudley site in the Main Endeavour vent Field of Juan de

Fuca Ridge (Zhou et al., 2009). Only sequences from Hot Lake II were found affiliated with the genus *Nitratiruptor*. The closest relative was a sequence form the Iheya North field in the Mid-Okinawa Trough (Nakagawa et al., 2005).

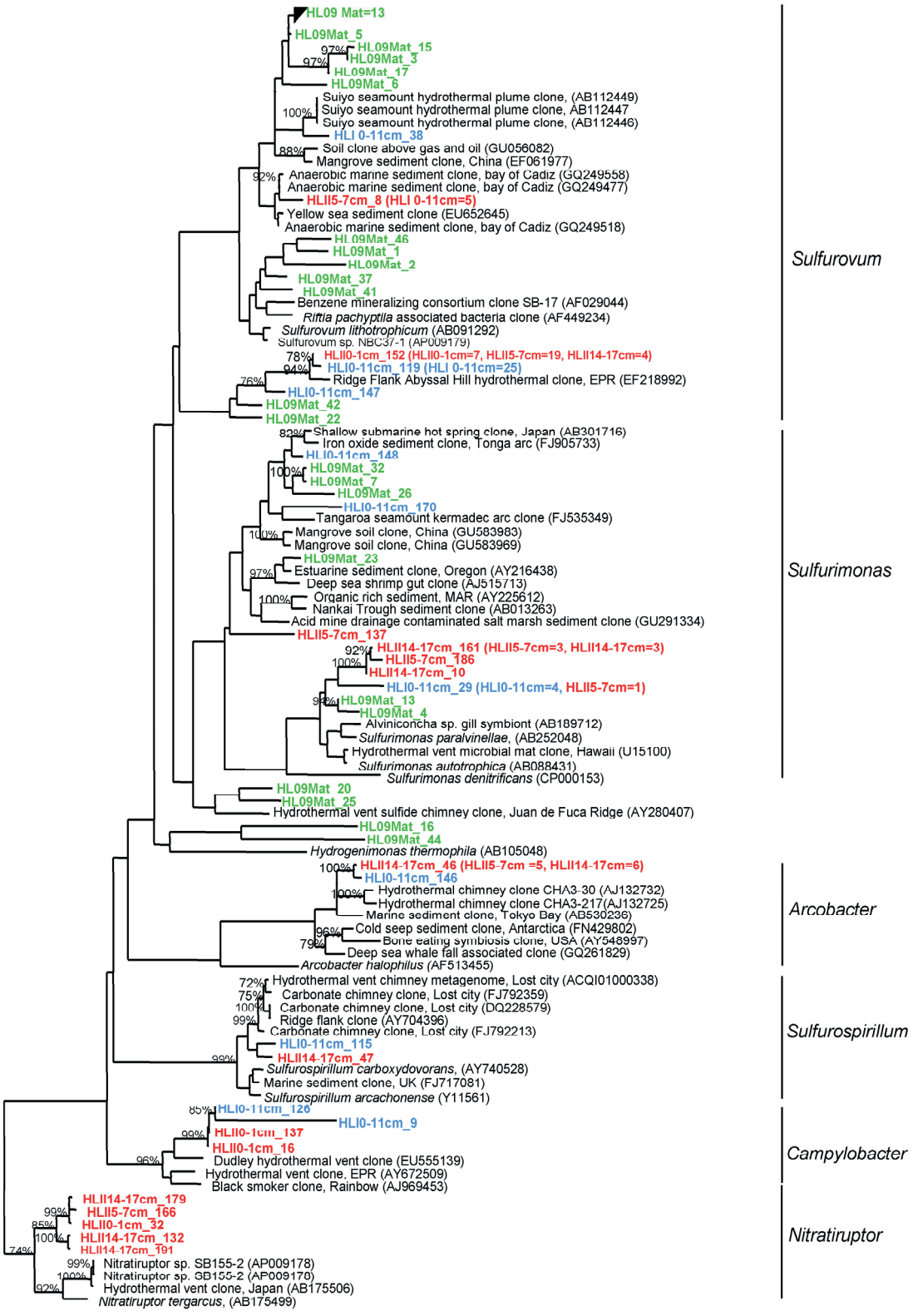

HL09 Mat=13
HL09Mat_5
HL09Mat_15
HL09Mat_3
HL09Mat_17
HL09Mat_6
Suiyo seamount hydrothermal plume clone, (AB112449)
Suiyo seamount hydrothermal plume clone, AB112447
Suiyo seamount hydrothermal plume clone, (AB112446)
HLI 0-11cm_38
Soil clone above gas and oil (GU056082)
Mangrove sediment clone, China (EF061977)
Anaerobic marine sediment clone, bay of Cadiz (GQ249558)
Anaerobic marine sediment clone, bay of Cadiz (GQ249477)
HLII5-7cm_8 (HLI 0-11cm=5)
Yellow sea sediment clone (EU652645)
Anaerobic marine sediment clone, bay of Cadiz (GQ249518)
HL09Mat_46
HL09Mat_1
HL09Mat_2
HL09Mat_37
HL09Mat_41
Benzene mineralizing consortium clone SB-17 (AF029044)
Riftia pachyptila associated bacteria clone (AF449234)
Sulfurovum lithotrophicum (AB091292)
Sulfurovum sp. NBC37-1 (AP009179)
HLII0-1cm_152 (HLII0-1cm=7, HLII5-7cm=19, HLII14-17cm=4)
HLI0-11cm_119 (HLI 0-11cm=25)
Ridge Flank Abyssal Hill hydrothermal clone, EPR (EF218992)
HLI0-11cm_147
HL09Mat_42
HL09Mat_22
Shallow submarine hot spring clone, Japan (AB301716)
Iron oxide sediment clone, Tonga arc (FJ905733)
HLI0-11cm_148
HL09Mat_32
HL09Mat_7
HL09Mat_26
HLI0-11cm_170
Tangaroa seamount kermadec arc clone (FJ535349)
Mangrove soil clone, China (GU583983)
Mangrove soil clone, China (GU583969)
HL09Mat_23
Estuarine sediment clone, Oregon (AY216438)
Deep sea shrimp gut clone (AJ515713)
Organic rich sediment, MAR (AY225612)
Nankai Trough sediment clone (AB013263)
Acid mine drainage contaminated salt marsh sediment clone (GU291334)
HLII5-7cm_137
HLII14-17cm_161 (HLII5-7cm=3, HLII14-17cm=3)
HLII5-7cm_186
HLII14-17cm_10
HLI0-11cm_29 (HLI0-11cm=4, HLII5-7cm=1)
HL09Mat_13
HL09Mat_4
Alviniconcha sp. gill symbiont (AB189712)
Sulfurimonas paralvinellae, (AB252048)
Hydrothermal vent microbial mat clone, Hawaii (U15100)
Sulfurimonas autotrophica (AB088431)
Sulfurimonas denitrificans (CP000153)
HL09Mat_20
HL09Mat_25
Hydrothermal vent sulfide chimney clone, Juan de Fuca Ridge (AY280407)
HL09Mat_16
HL09Mat_44
Hydrogenimonas thermophila (AB105048)
HLII14-17cm_46 (HLII5-7cm =5, HLII14-17cm=6)
HLI0-11cm_146
Hydrothermal chimney clone CHA3-30 (AJ132732)
Hydrothermal chimney clone CHA3-217(AJ132725)
Marine sediment clone, Tokyo Bay (AB530236)
Cold seep sediment clone, Antarctica (FN429802)
Bone eating symbiosis clone, USA (AY548997)
Deep sea whale fall associated clone (GQ261829)
Arcobacter halophilus (AF513455)
Hydrothermal vent chimney metagenome, Lost city (ACQI01000338)
Carbonate chimney clone, Lost city (FJ792359)
Carbonate chimney clone, Lost city (DQ228579)
Ridge flank clone (AY704396)
Carbonate chimney clone, Lost city (FJ792213)
HLI0-11cm_115
HLII14-17cm_47
Sulfurospirillum carboxydovorans, (AY740528)
Marine sediment clone, UK (FJ717081)
Sulfurospirillum arcachonense (Y11561)
HLI0-11cm_126
HLI0-11cm_9
HLII0-1cm_137
HLII0-1cm_16
Dudley hydrothermal vent clone (EU555139)
Hydrothermal vent clone, EPR (AY672509)
Black smoker clone, Rainbow (AJ969453)
HLII14-17cm_179
HLII5-7cm_166
HLII0-1cm_32
HLII14-17cm_132
HLII14-17cm_191
Nitratiruptor sp. SB155-2 (AP009178)
Nitratiruptor sp. SB155-2 (AP009170)
Hydrothermal vent clone, Japan (AB175506)
Nitratiruptor tergarcus, (AB175499)
Sulfurovum
Sulfurimonas
Arcobacter
Sulfurospirillum
Campylobacter
Nitratiruptor
0.10

Figure 12. Phylogenetic tree showing the affiliation of 16S rRNA gene sequences from *Epsilonproteobacteria*. The tree was calculated by maximum-likelihood analyses applying a 50% sequence conservation filter. Bootstrap values based on 100 replicates are given at the nodes (only that > 70%). The number of sequences with 97% identity is shown in brackets. The bar represents 10% estimated sequence changes. Sequences obtained in this study from Hot Lake I are indicated in blue and Hot Lake II in red.

Sequences affiliated with *Deltaproteobacteria* were found to be related to *Desulfobacteraceae*, *Desulfobulbaceae*, *Desulfovibrio*, SVA0485, *Desulfurellaceae* and *Desulfuromusa*. Within the cluster *Desulfobacteraceae*, sequences from Hot Lake affiliated with those from hypersaline sediments (Lloyd et al., 2006; López-López et al., 2010). Sequences related to *Desulfobulbaceae* were found to be closely related to those from Rainbow vent field. Abundant sequences were affiliated with cluster SVA0485. They share close relationship with sequences detected at many other hydrothermal vents such as the Brothers volcano at the Kermadec arc (New Zealand) (Stott et al., 2008) and the Guaymas Basin (Teske et al., 2002). Cultured relatives of the genus *Desulfuromusa* to which some of the sequences from Hot Lake affiliated have been describe to gain energy from sulfur reduction (Liesack and Finster, 1994).

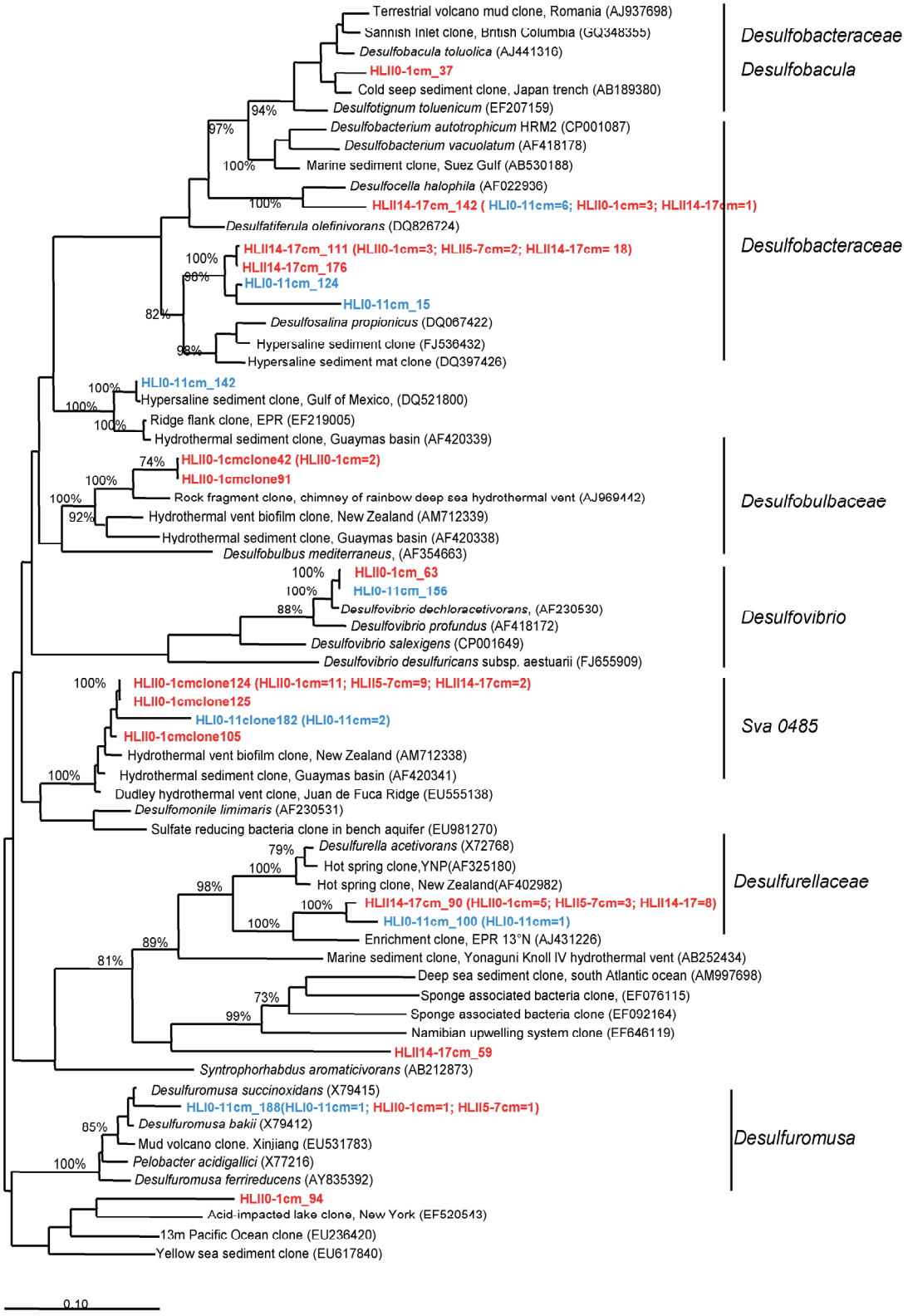

Terrestrial volcano mud clone, Romania (AJ937698)
Sannish Inlet clone, British Columbia (GQ348355)
Desulfobacula toluolica (AJ441316)
HLII0-1cm_37
Cold seep sediment clone, Japan trench (AB189380)
Desulfotignum toluenicum (EF207159)
Desulfobacterium autotrophicum HRM2 (CP001087)
Desulfobacterium vacuolatum (AF418178)
Marine sediment clone, Suez Gulf (AB530188)
Desulfocella halophila (AF022936)
HLII14-17cm_142 (HLI0-11cm=6; HLII0-1cm=3; HLII14-17cm=1)
Desulfatiferula olefinivorans (DQ826724)
HLII14-17cm_111 (HLII0-1cm=3; HLII5-7cm=2; HLII14-17cm= 18)
HLII14-17cm_176
HLI0-11cm_124
HLI0-11cm_15
Desulfosalina propionicus (DQ067422)
Hypersaline sediment clone (FJ536432)
Hypersaline sediment mat clone (DQ397426)
HLI0-11cm_142
Hypersaline sediment clone, Gulf of Mexico, (DQ521800)
Ridge flank clone, EPR (EF219005)
Hydrothermal sediment clone, Guaymas basin (AF420339)
HLII0-1cmclone42 (HLII0-1cm=2)
HLII0-1cmclone91
Rock fragment clone, chimney of rainbow deep sea hydrothermal vent (AJ969412)
Hydrothermal vent biofilm clone, New Zealand (AM712339)
Hydrothermal sediment clone, Guaymas basin (AF420338)
Desulfobulbus mediterraneus, (AF354663)
HLII0-1cm_63
HLI0-11cm_156
Desulfovibrio dechloracetivorans, (AF230530)
Desulfovibrio profundus (AF418172)
Desulfovibrio salexigens (CP001649)
Desulfovibrio desulfuricans subsp. aestuarii (FJ655909)
HLII0-1cmclone124 (HLII0-1cm=11; HLII5-7cm=9; HLII14-17cm=2)
HLII0-1cmclone125
HLI0-11clone182 (HLI0-11cm=2)
HLII0-1cmclone105
Hydrothermal vent biofilm clone, New Zealand (AM712338)
Hydrothermal sediment clone, Guaymas basin (AF420341)
Dudley hydrothermal vent clone, Juan de Fuca Ridge (EU555138)
Desulfomonile limimaris (AF230531)
Sulfate reducing bacteria clone in bench aquifer (EU981270)
Desulfurella acetivorans (X72768)
Hot spring clone,YNP(AF325180)
Hot spring clone, New Zealand(AF402982)
HLII14-17cm_90 (HLII0-1cm=5; HLII5-7cm=3; HLII14-17=8)
HLI0-11cm_100 (HLI0-11cm=1)
Enrichment clone, EPR 13°N (AJ431226)
Marine sediment clone, Yonaguni Knoll IV hydrothermal vent (AB252434)
Deep sea sediment clone, south Atlantic ocean (AM997698)
Sponge associated bacteria clone, (EF076115)
Sponge associated bacteria clone (EF092164)
Namibian upwelling system clone (EF646119)
HLII14-17cm_59
Syntrophorhabdus aromaticivorans (AB212873)
Desulfuromusa succinoxidans (X79415)
HLI0-11cm_188(HLI0-11cm=1; HLII0-1cm=1; HLII5-7cm=1)
Desulfuromusa bakii (X79412)
Mud volcano clone. Xinjiang (EU531783)
Pelobacter acidigallici (X77216)
Desulfuromusa ferrireducens (AY835392)
HLII0-1cm_94
Acid-impacted lake clone, New York (EF520543)
13m Pacific Ocean clone (EU236420)
Yellow sea sediment clone (EU617840)
94%
97%
100%
100%
100%
96%
82%
98%
100%
100%
100%
74%
100%
100%
92%
100%
100%
100%
88%
100%
100%
100%
79%
100%
98%
100%
100%
89%
81%
73%
99%
85%
100%
Desulfobacteraceae
Desulfobacula
Desulfobacteraceae
Desulfobulbaceae
Desulfovibrio
Sva 0485
Desulfurellaceae
Desulfuromusa
0.10

Figure 13. Phylogenetic tree showing the affiliation of 16S rRNA gene sequences from *Deltaproteobacteria*. The tree was calculated by maximum-likelihood analyses applying a 50% sequence conservation filter. Bootstrap values based on 100 replicates are given at the nodes (only that > 70%). The number of sequences with 97% identity is shown in brackets. The bar represents 10% estimated sequence changes. Sequences obtained in this study from Hot Lake I are indicated in blue and Hot Lake II in red.

Sequences related to *Bacteroidetes* affiliated with sequences from cave microbial mats, seamounts and deep sea hydrothermal vents (Figure 14). Most of sequences belonged to the group VC2.1 Bac22. This cluster had been detected before at other volcanic areas such as Vailulu'u Seamount (Sudek et al., 2009), East Pacific Rise (Alain et al., 2004) and Mid Atlantic Ridge (Reysenbach et al., 2000).

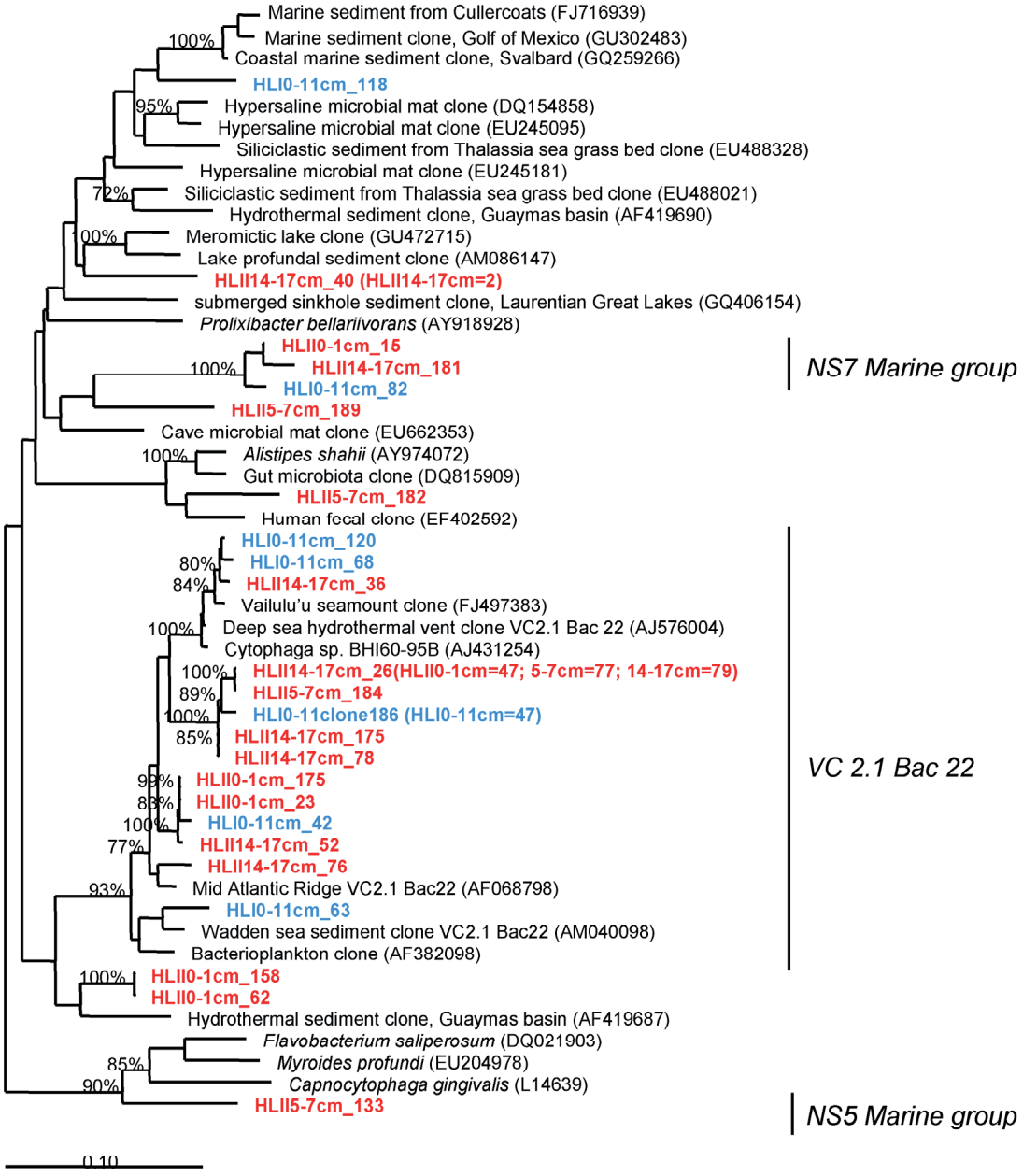


Figure 14. Phylogenetic tree showing the affiliation of 16S rRNA gene sequences from *Bacteroidetes*. The tree was calculated by maximum-likelihood analyses applying a 50% sequence conservation filter. Bootstrap values based on 100 replicates are given at the nodes (only that > 70%). The number of sequences with 97% identity is shown in brackets. The bar represents 10% estimated sequence changes. Sequences obtained in this study from Hot Lake I are indicated in blue and Hot Lake II in red.

Statistical approaches were used to evaluate species richness. An operational taxonomic unit (OTU) at 98% sequence similarity (Stackebrandt and Goebel, 1994) and rarefaction was applied to investigate the bacterial richness of the sediments (Figure 15). Rarefaction analysis at the 98% OTU level indicated that 16S rRNA gene sequences in the first 11 cm of Hot Lake I were not sampled to saturation. This was also the case for the three different depth layers at Hot Lake II indicating more species were still not detected yet.

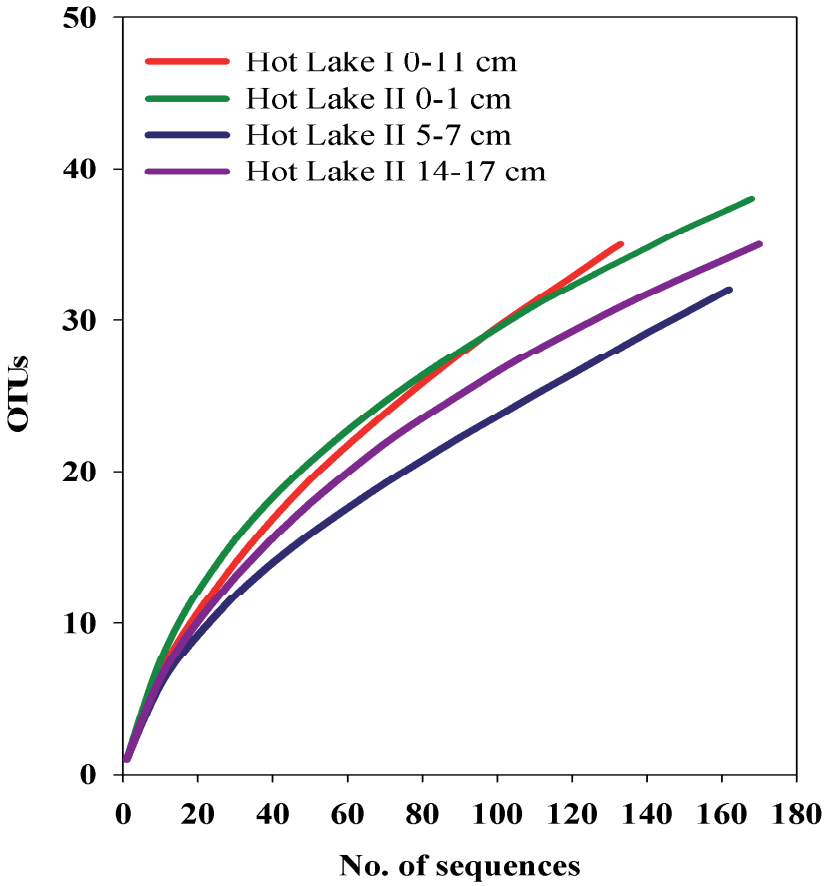


Figure 15. Relative bacterial richness from Hot Lake I and Hot Lake II shown through rarefaction analyses. The expected numbers of OTUs was calculated using cut-off values of sequence identity of 98%.

4. Diversity of archaeal 16S ribosomal RNA genes

For the pooled sample of Hot Lake I (0-11 cm) and the three layers of Hot Lake II: 0-1 cm, 5-7 cm and 14-17 cm, the archaeal diversity was as well assessed. The archaeal 16S rRNA gene composition for each sample is shown in Figure 16. At Hot Lake I and 0-1 cm, 5-7 cm from Hot Lake II, sequences affiliated to *Thermoplasmatales* and Marine Benthic Group D-Deep-Sea Hydrothermal Vent Group I (DHVE 1) were detected. Within the *Thermoplasmatales*, abundant sequences from Hot Lake I as well as from all three layers at Hot Lake II were retrieved. They were affiliated with uncultured archaea from other hydrothermal vent systems, e.g., those associated with the archaeal community of the polychaete *Alvinella pompejana* living on the walls of active hydrothermal chimneys along the East Pacific Rise (Moussard et al., 2006; Omoregie et al., 2008).

Terrestrial euryarchaeotal group (TMEG) sequences were found only at Hot Lake I sediment and 0-1 cm from Hot Lake II. Members of this group had been found in sediment overlying a natural CO_2 lake at the Yonaguni Knoll IV hydrothermal field, southern Okinawa Trough (Inagaki et al., 2006). Sequences affiliated to the Deep Sea Euryarchaeotic Group (DSEG) belonging to the Class *Halobacteria* appeared predominantly at Hot Lake I and in the upper layers at Hot Lake II (0-1 cm, 5-7 cm). This group was as well detected in deep sea hydrothermal vent areas (Takai and Horikoshi, 1999; Omoregie et al., 2008). Sequences related to the genus *Palaeococcus* were found at Hot Lake I and Hot Lake II.

Sequences belonging to *Crenarchaeota* and *Korachaeota* were detected at 14-17 cm from Hot Lake II exclusively. Sequences of these two phyla retrieved from this study are close to the hyperthermophilic relatives. All crenarchaeotal sequences found in this study affiliated with the family *Desulfurococcaceae*. Overall, the frequency of sequences shifted from *Halobacteria* towards *Thermococcaceae* and *Desulfurococcaceae* with depth at Hot Lake II. The community compositions of these four clone libraries showed different structures (Figure 16).

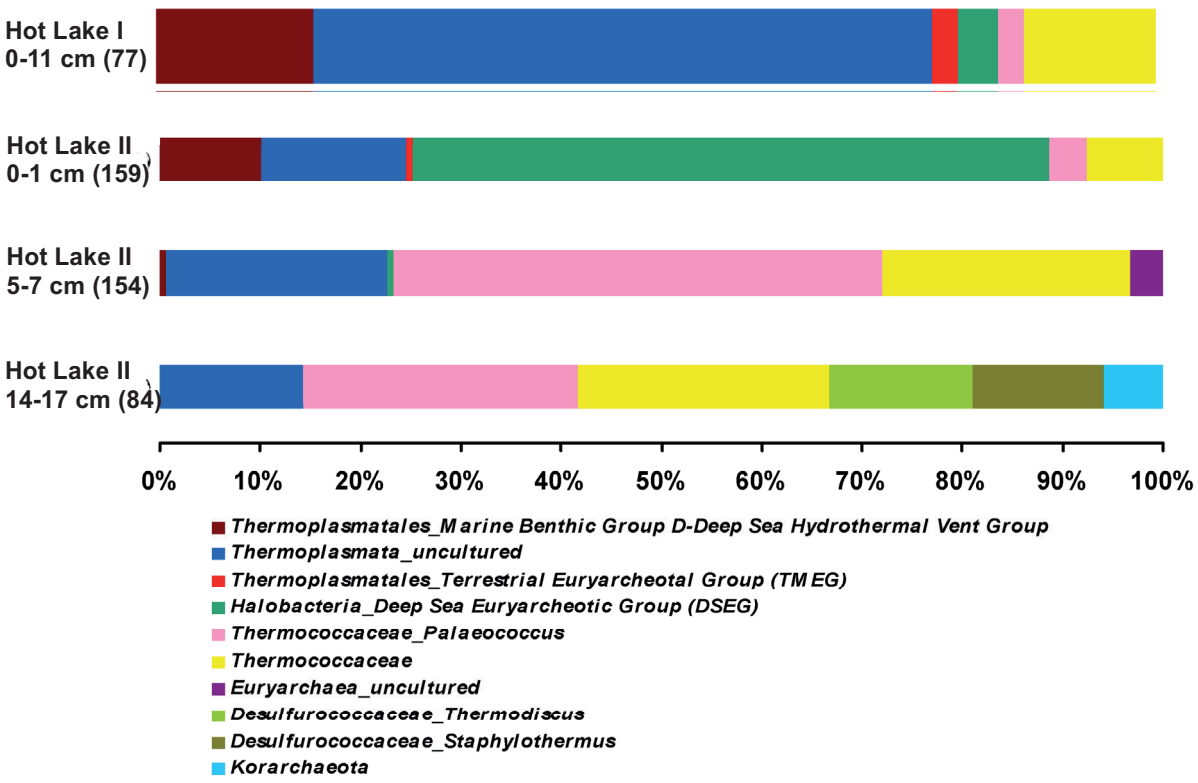


Figure 16. Clone affiliations and frequencies in archeal 16S rRNA libraries from sediment at Hot Lake I and Hot Lake II. The number of sequenced clones in each clone library is shown in parentheses.

5. Microbial community composition

The comparative analysis of 16S rRNA clone libraries of *Bacteria* and *Archaea* yielded an overview of the microbial diversity at Hot Lake. Subsequently, the abundance of certain groups of microorganisms was assessed and total cell counts were determined by SybrGreen I staining (Figure 17, Table 7). DAPI staining was applied initially and showed high background. SybrGreen I diluted in moviol solution revealed the best signals and reliable cell counts.

In the upper layers of Hot Lake I and Hot Lake II, total cell counts were in the range of 10^7-10^9 cells/ml. At 0-1 cm of Hot Lake I and Hot Lake II, 6 x 10^8 cells/ml were present. At Hot Lake I, total cells counts were still as high as in the upper layer of 10^8 cells/ml at 13 cm. It showed an increase of total cell counts within the first 3 cm at Hot Lake I. In contrast, at Hot Lake II, cell counts constantly decreased with depth to cell numbers of 10^7 cells/ml.

Catalyzed reporter deposition-fluorescence *in situ* hybridization (CARD-FISH) was applied to three different sediment layers of Hot Lake I and Hot Lake II. Distinct groups of *Bacteria* were enumerated with established and newly designed probes (Table 4). The results showed that *Bacteria* (probe: EUBI-III) were dominant in all sediment layers (Table 7). At Hot Lake I, the proportion of *Archaea* (probe: Arch915) increased from 6% to 24% with depth (Table 7). At Hot Lake II, *Archaea* accounted for ~20% relative to the total cell counts at all three layers. In the 5-7 cm layer of Hot Lake I and the 0-1 cm, and 5-7 cm layers from Hot Lake II, the detection rate of *Bacteria* and *Archaea* was together only ~60% of total cell counts indicating that many *Bacteria* and *Archaea* have not been covered or detected by the general probes.

Group specific probes (Table 4) were applied to quantify certain groups of bacteria based on the results of the comparative 16S rRNA gene analysis. At Hot Lake I, *Deltaproteobacteria* as detected by probes Delta495abc and competitors (Loy et al., 2002) increased with depth. In contrast, at Hot Lake II, the abundance of *Deltaproteobacteria* decreased with depth (Figure 18, Table 8). At Hot Lake I and Hot Lake II, *Epsilonproteobacteria* (probe: EPSY914) (Grote et al., 2007) increased in relative abundance from 0-1 cm to 5-7 cm whereas in the bottom layers, cell

frequencies decreased to ~5%. *Arcobacter* spp. have frequently been reported to be present the hydrothermal vents (Wirsen et al., 2002; Sievert et al., 2007). At Hot Lake II, *Arcobacter* counts were as high as the counts of *Epsilonproteobacteria*.

A general probe for *Bacteroidetes* (probe: CF319a) was initially used for *in situ* hybridization. However, an *in silico* analysis using ProbeCheck of the ARB package (Ludwig et al., 2004) revealed that this probe also targets many epsilonproteobacterial sequences retrieved from this study. As most of the 16S rRNA sequences grouping with the *Bacteroidetes* affiliated to the group VC2.1 Bac22, probes were designed for this group and utilized in this study. Two probes were required to target all sequences within the VC2.1 Bac22 group. At Hot Lake I, the frequency of cells detected with these probes increased with depth. At Hot Lake II, the trend was the same; nevertheless the detection rate was low.

Anoxygenic phototrophic bacteria were assumed to play an important role at shallow hydrothermal vents since light penetrates to this water depth. By comparative 16S rRNA gene analysis, two clusters of *Chlorobi* BSV 26 and OPB 56 were detected and probes were designed for further quantification (probe BSV 26 and OPB 56). At Hot Lake I, BSV 26 abundance reached up to ~13% of total bacterial counts at 15-17 cm. At Hot Lake II, 1-2% of BSV 26 were detected. The abundance of OPB 56 showed a similar trend as those of BSV26 (Figure 18, Table 8). Figure 19 shows the morphology of *Bacteria*, *Epsilonproteobacteria* and *Chlorobium* at Hot Lake.

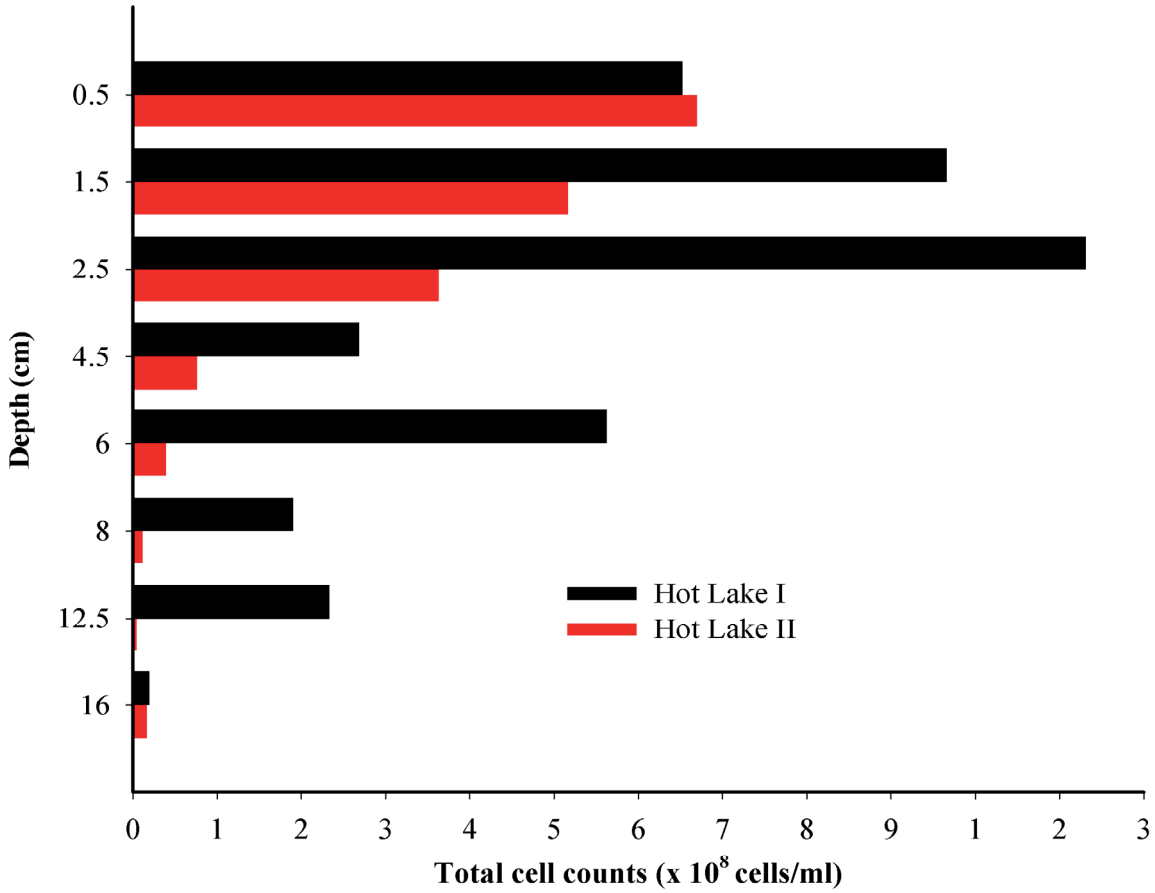


Figure 17. Total cell counts determined for the sediment cores from Hot Lake I and Hot Lake II.

Table 7. Total cell counts quantified by SybrGreen I staining at Hot Lake I and Hot Lake II. Absolute abundance of *Bacteria* (probe: EUB I-III). Percentage of *Bacteria* in total cell counts. Absolute abundance of *Archaea* (probe: Arch 915). Percentage of *Archaea* in total cell counts.

		CARD-FISH			
Hot Lake I	**Cell counts (cells/ml)**	**EUBI-III (absolute number)**	**EUBI-III (%)[a]**	**Arch 915 (absolute number)**	**Arch915%[b]**
0-1 cm	6.52E+08	5.38E+08	82.41%	4.28E+07	6.57%
1-2 cm	9.66E+08				
2-3 cm	1.13E+09				
4-5 cm	2.68E+08				
5-7 cm	5.63E+08	2.22E+08	39.38%	6.30E+07	11.20%
7-9 cm	1.91E+08				
11-13 cm	2.33E+08				
15-17 cm	1.95E+07	1.44E+07	74.09%	4.62E+06	23.70%
Hot Lake II					
0-1 cm	6.69E+08	2.44E+08	36.38%	1.15E+08	17.10%
1-2 cm	5.17E+08				
2-3 cm	3.63E+08				
4-5 cm	7.61E+07				
5-7 cm	3.92E+07	1.71E+07	43.62%	7.83E+06	19.97%
7-9 cm	1.14E+07				
11-14 cm	4.67E+06				
14-17 cm	1.66E+07	1.39E+07	83.47%	2.29E+06	13.77%

a: fraction of EUBI-III counts/ Cell counts
b: fraction of Arch915 counts/ Cell counts

Table 8. Absolute cell counts after CARD-FISH with specific probes for different bacterial and archaeal groups. Fraction of cell numbers detected with group specific probes.

	Hot Lake I						Hot Lake II					
Probes	**0-1 cm**		**5-7 cm**		**15-17 cm**		**0-1 cm**		**5-7 cm**		**14-17 cm**	
EUB338 I-III	5.38E+08		2.22E+08		1.44E+07		2.44E+08		1.71E+07		1.39E+07	
Arch915	4.28E+07		6.30E+07		4.62E+06		1.15E+08		7.83E+06		2.29E+06	
		% No.[a]		**% No.[a]**		**% No.[a]**		**% No.[a]**		**% No.[a]**		**% No.[a]**
Delta495abc	5.12E+07	9.52%	6.08E+07	27.43%	7.53E+06	52.11%	7.23E+07	29.70%	1.76E+06	10.28%	7.68E+05	5.54%
EPSY914	3.09E+07	5.74%	2.95E+07	13.29%	6.72E+05	4.65%	7.92E+06	3.25%	2.83E+06	20.43%	6.68E+05	4.82%
ARC94	4.74E+06	0.88%	7.26E+06	3.28%	1.70E+06	11.75%	9.11E+06	3.74%	3.11E+06	18.16%	9.84E+05	7.10%
GAM42a	6.24E+06	1.16%	2.14E+06	0.96%	1.11E+06	7.70%	5.66E+05	0.23%	1.04E+05	0.61%	3.96E+05	2.86%
CF319a	8.23E+07	15.31%	2.91E+07	13.13%	1.32E+07	91.53%	1.36E+07	5.59%	1.76E+06	10.29%	1.41E+06	10.14%
VC2.1_1	6.67E+06	1.24%	8.17E+06	3.69%	2.30E+06	15.96%	2.08E+06	0.85%	2.88E+05	1.69%	3.68E+05	2.65%
VC2.1_2	3.24E+06	0.60%	4.14E+06	1.87%	1.07E+06	7.42%	5.97E+05	0.25%	1.12E+05	0.66%	4.80E+04	0.35%
BSV26	2.83E+06	0.53%	4.87E+06	2.20%	1.82E+06	12.63%	1.75E+06	0.72%	4.32E+05	2.53%	1.92E+05	1.38%
OPB56	1.20E+06	0.22%	3.25E+06	1.47%	1.34E+06	9.31%	3.91E+05	0.16%	1.84E+05	1.08%	1.36E+05	0.98%
CREN512	1.68E+06	3.92%	3.95E+06	6.27%	1.92E+06	41.56%	2.16E+06	1.89%	2.40E+05	3.07%	4.08E+05	17.83%
EURY514	1.02E+07	23.82%	7.50E+06	11.90%	4.29E+06	92.73%	1.03E+07	8.99%	3.47E+06	44.27%	4.80E+05	20.98%

a: fraction of Probe counts/ EUBI-III counts(Delta495abc, EPSY914, ARC94, GAM42a, CF319a, VC2.1_1, VC2.1_2, BSV26, OPB56)

a: fraction of Probe counts/ Arch915 counts(CREN512, EURY514)

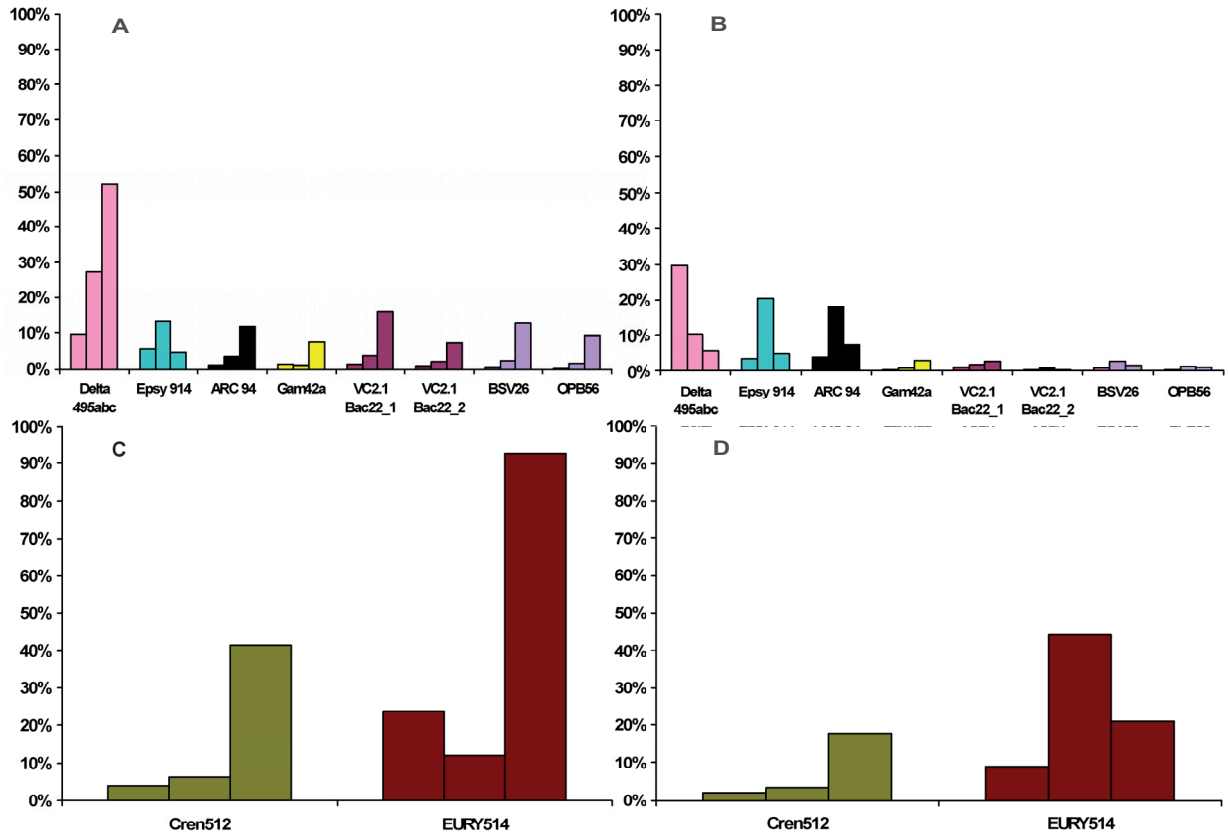


Figure 18. Abundance of bacterial and archael groups at Hot Lake. Relative abundance of bacterial groups in relation to total *Bacteria* counts at (A) Hot Lake I and (B) Hot Lake II. Relative abundance of archaeal groups in relation to total *Archaea* counts at (C) Hot Lake I and (D) Hot Lake II. Three bars correspond to 0-1 cm, 5-7 cm, 15-17 cm sediment depth at Hot Lake I and 0-1 cm, 5-7 cm, 14-17 cm at Hot Lake II (from left to right).

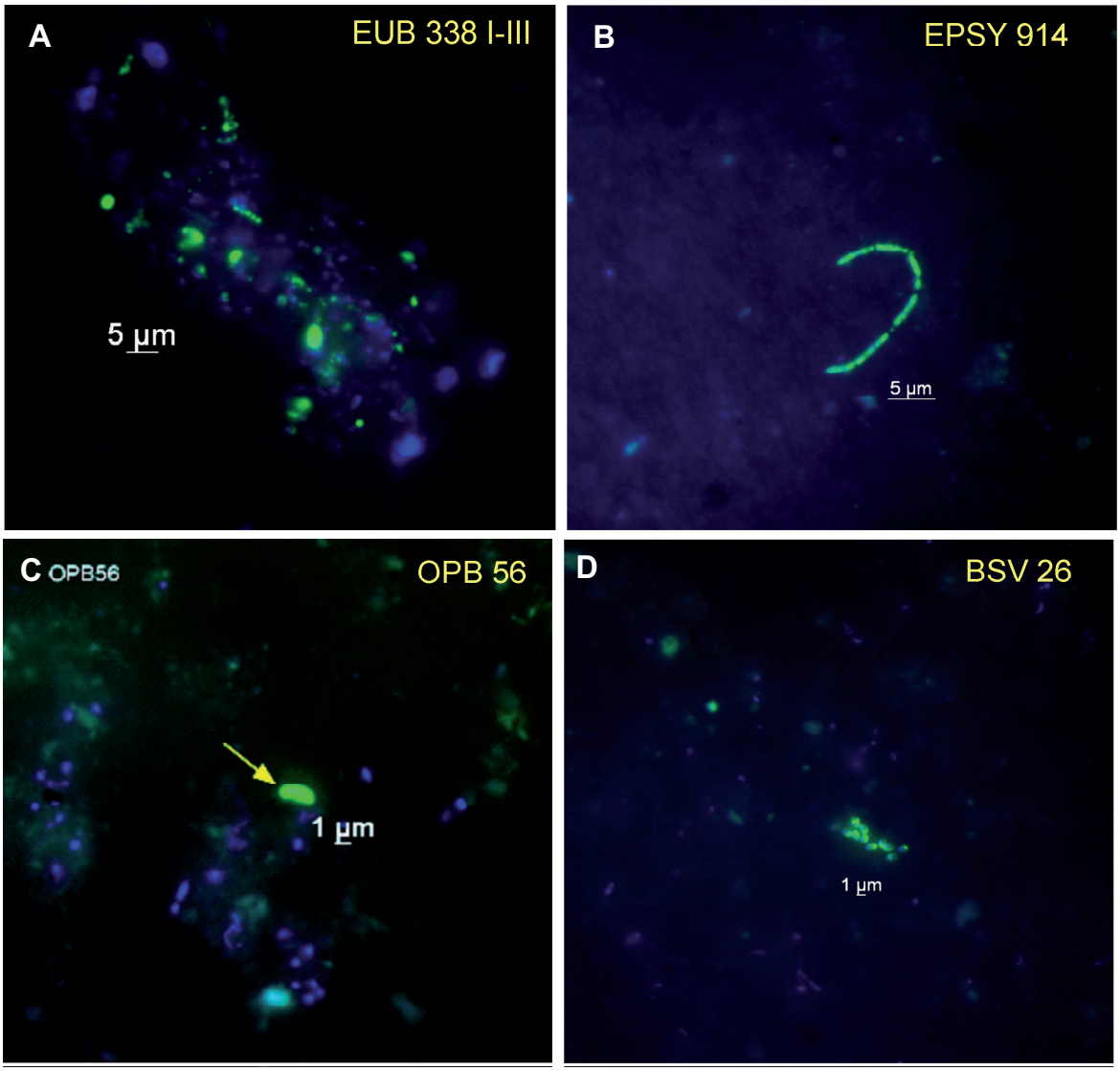


Figure 19. Epifluorescence micrographs of *Bacteria* from Hot Lake sampled in 2009. (A) Probe EUBI-III targeting most of the *Bacteria*. (B) Probe EPSY914 targeting >90% of epsilonproteobacterial sequences obtained from this study. (C) Probe OPB56 targeting cluster OPB56 sequences obtained from this study. (D) Probe BSV26 targeting cluster BSV26 sequences obtained from this study. Blue: DAPI signals of DNA containing cells. Green: positive signals after CARD-FISH using the probes.

6. Comparative analyses of microbial diversity and abundance retrieved by 454-pyrosequencing

Pyrosequencing of the metagenome of the surface sediment layers (0-2cm) from Hot Lake I and Hot Lake II produced about 186 and 203 Mbp, respectively, with an average read length of ~390 bp (Table 9). Of these, 5.87% and 9.62%, respectively, were identified as technical replicates (Gomez-Alvarez et al., 2009). These replicates occurred independently of sequence lengths, as indicated by similar sequence length distribution profiles before and after replicate removal (Figure 20).

Table 9. Characterization of raw data and after assembly pyrosequencing dataset from the Hot Lake I and Hot Lake II.

	Raw data		**After assembly**	
	Hot Lake I	**Hot Lake II**	**Hot Lake I**	**Hot Lake II**
Total number of reads	515,111	476,604	9,674	4,235
Total base pairs	203,407,260	186,009,462	12,635,565	6,777,334
Mean read, bp	394±121	390±122	1,306 ± 1,763	1,600 ± 2,763
mean GC content	42±8%	46±7%	40 ± 5 %	42 ± 6 %
Percent of reads that are replicates:	5.87%	9.62%		

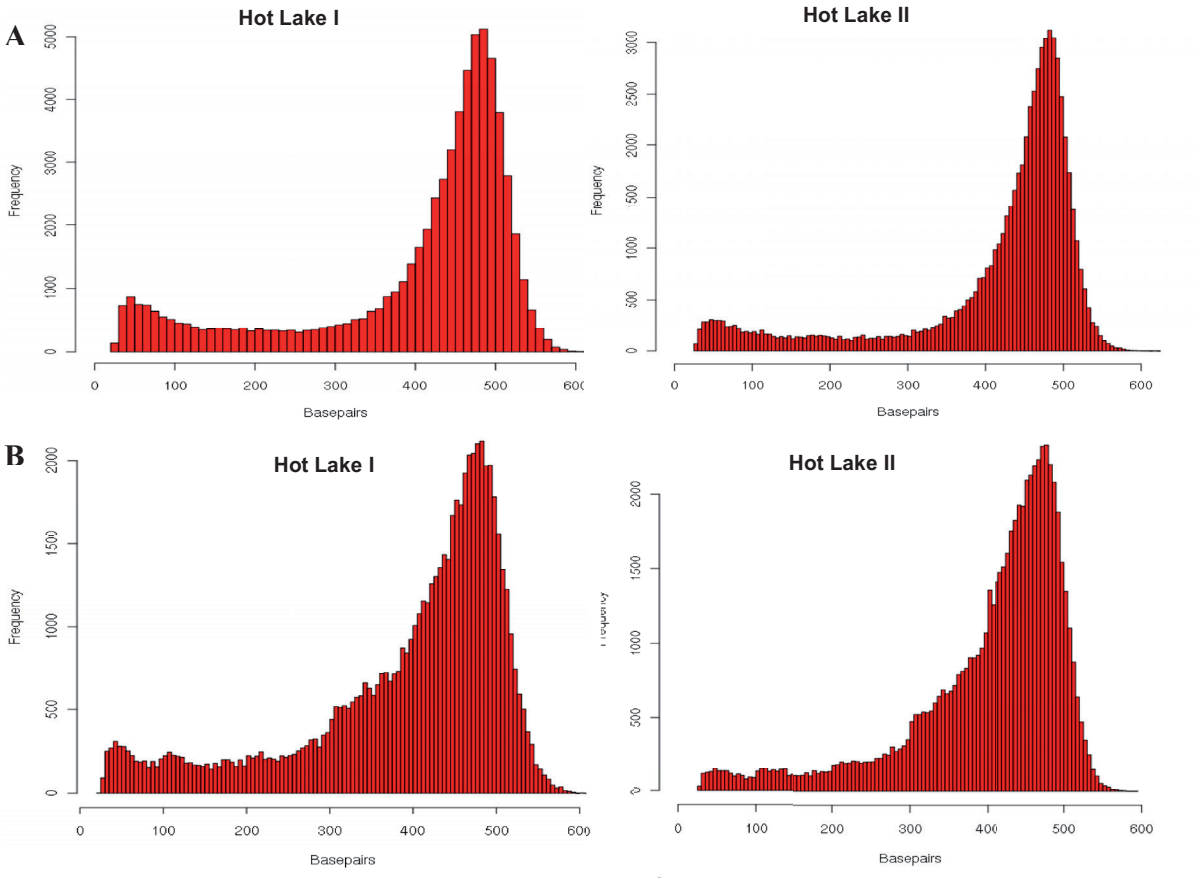


Figure 20. Overview of the read length distribution of raw and de-replicated metagenomic dataset from the surface sediment layer at Hot Lake I and Hot Lake II. Read length distribution of the raw reads (A) and the corresponding distribution of the de-replicated dataset (B).

For functional analysis, the datasets were assembled using the Newbler assembly software (Roche). The assembled dataset of Hot Lake I and Hot Lake II is shown in Table 9. The assembled contigs are from a minimum of 100 bp to a maximum of 31809 bp at Hot Lake I and a minimum of 100 bp to a maximum 41613 bp at Hot Lake II. All together 27276 open reading frames (ORFs) were predicted for Hot Lake I and 10308 ORFs for Hot Lake II. A BlastP search against the KEGG database was performed for the function analysis. The KEGG category distribution within the "energy metabolism category" is given in Figure 21. Generally, the KEGG category distribution was similar between Hot Lake I and Hot Lake II. In this study, it was focused on sulfur metabolism and reductive tricarboxylic acid cycle for further analyses.

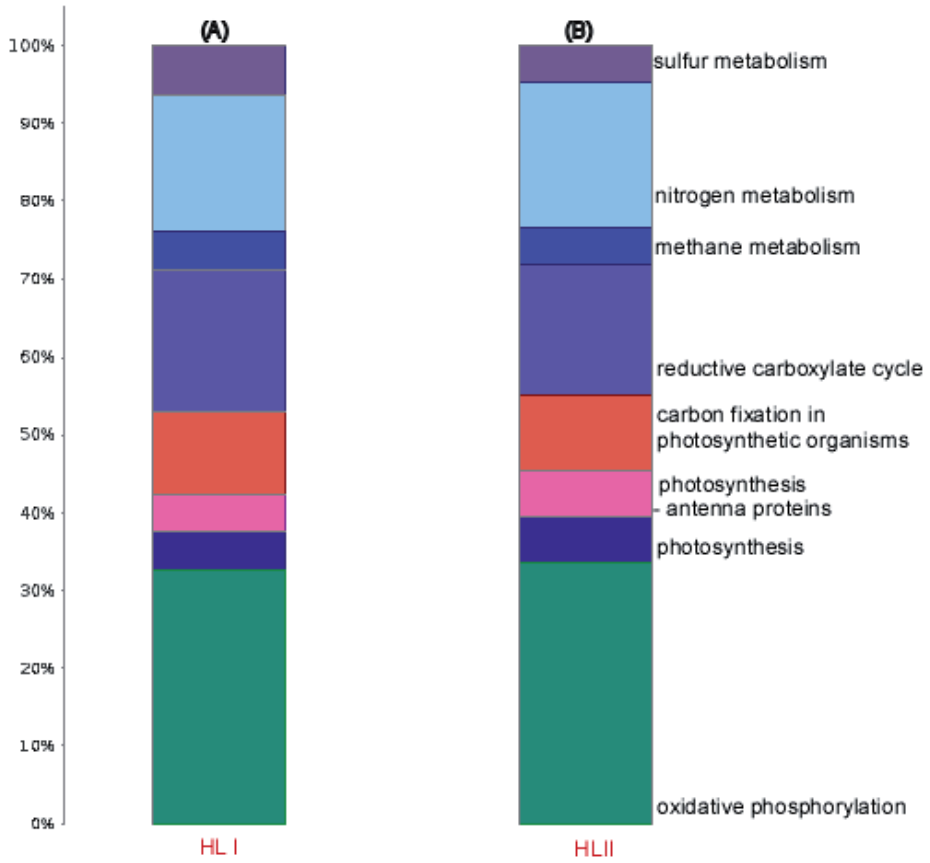


Figure 21. KEGG energy metabolism category distribution at Hot Lake I and II. Shown is the relative KEGG category distribution for the assembled metagenome of (A) Hot Lake I and (B) Hot Lake II.

6.1Taxonomic profiles based on MG-RAST

The taxonomic diversity of the metagenomic data was assessed through the MG-RAST metagenome annotation and analysis pipeline (Kent, 2002). MG-RAST uses BLAT (The Blast-like alignment tool) to find sequences in the metagenomic dataset which are homologous to sequences in large number of databases. For the taxonomic and function analyses, the dataset was assigned and annotated against the GenBank database (Benson et al., 2011) and the subsystems.

The MG-RAST pipeline revealed 99.6% bacterial and 0.2% archaeal reads hits for Hot Lake I after comparing the reads with GenBank using a maximum e-value of 1e-5, a minimum identity of 30%, and a minimum alignment length of 100. Same criteria were applied as well to the Hot Lake II dataset, resulting in 98.8% bacterial and 1.1% archaeal hits. At Hot Lake I, these bacterial gene sequences were found to be mainly affiliated to *Proteobacteria* (91.6%), *Bacteroidetes* (3.6%), *Firmicutes* (1.4%), *Chlorobi* (0.87%) and *Aquificae* (0.6%). Within the *Proteobacteria*, the sequences were assigned to *Epsilonproteobacteria* (88.6%), *Gammaproteobacteria* (8.30%) and *Deltaproteobacteria* (0.58%). At Hot Lake II, the affiliation of the bacterial gene sequences was quite similar to the one determined for Hot Lake I with 93.9% *Proteobacteria*, 1.6% *Firmicutes*, 1.6% *Bacteroidetes*, 0.4% *Chlorobi* and 0.3% *Aquificae*. Similar distribution was shown at Hot Lake II for the taxonomic classification of the proteobacerial reads with 92.1% *Epsilonproteobacteria*, 5.5% *Gammaproteobacteria* and 0.8% *Deltaproteobacteria.* However during CARD-FISH, little *Gammaproteobacteria* (Probe: Gam42a) and no *Aquificae* (Probe: Aqui338) were detected in the first one cm of sediment.

6.2 Taxonomic profiles based on 16S rRNA gene sequences

The taxonomic diversity of the metagenomic data was assessed in parallel by the analysis of 16S rRNA gene sequences within the pyrosequencing datasets (unpublished analysis pipeline designed by Jörg Peplies, Ribocon GmbH, Bremen, Germany). Multifasta files were imported into the ARB package for further taxonomic analyses. The results showed similar tendancy with MG-RAST analyses. *Bacteria* have the portion of 77-80% and *Archaea* have the portion of 0.5% of all the sequences at Hot Lake I and II, respectively.

At Hot Lake I and Hot Lake II, most of the bacterial sequences obtained belonged to *Epsilonproteobacteria* (Figure 22). At Hot Lake I, sequences affiliated to *Gammaproteobacteria*, *Deltaproteobacteria* and *Bacteroidetes* reached frequencies of 3-5%. At Hot Lake II, the same clusters account for less than 1%. Chlorobial sequences were detected at both sites. Sequences affiliated to *Thermodesulfobacteria* were found at Hot Lake II exclusively. However, the rarefaction curves of these two sites (Figure 23) showed that more sequences need to be gained in order to cover the microbial diversity.

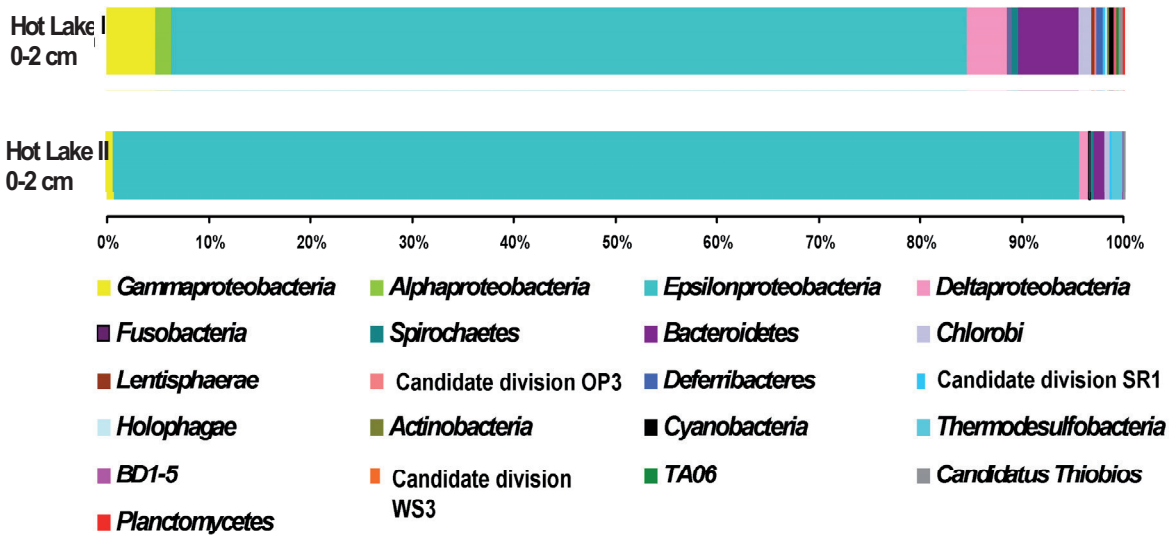


Figure 22. Taxonomic classification of 16S rRNA containing reads (> 200bp) detected in the metagenome of Hot Lake I and II.

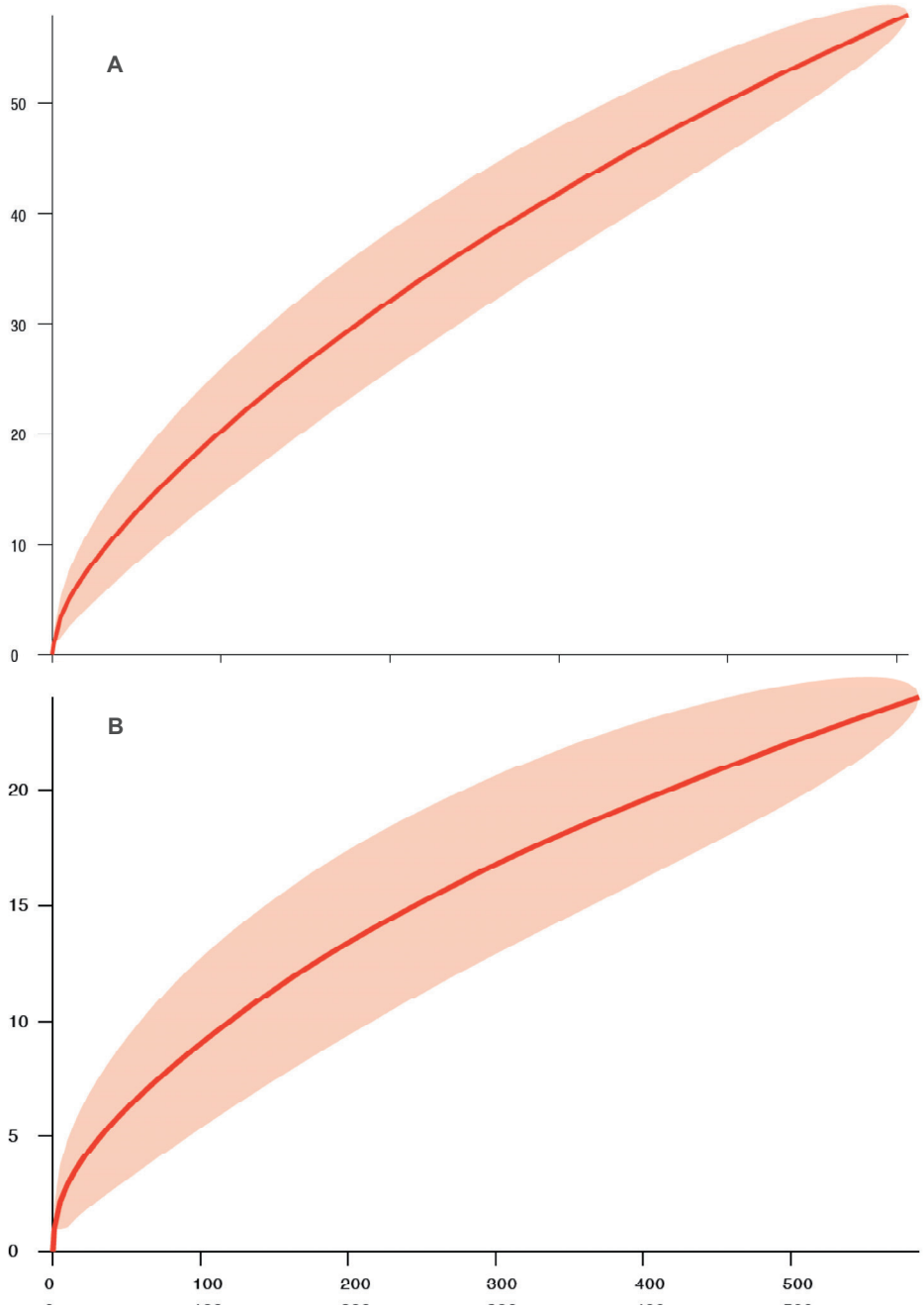


Figure 23. Relative bacterial richness based on the analysis of 16S rRNA reads contained in the metagenomic datasets. Rarefaction analysis for (A) Hot Lake I and (B) Hot Lake II. The expected number of OTUs was calculated based on 98% sequence identity.

6.3 Taxonomic profiles based on Taxometer

The taxonomic diversity of the metagenomic data was assessed in parallel with Taxometer pipeline. Both at Hot Lake I and Hot Lake II, more than 95% of the sequences were affiliated to *Bacteria* and less than 1% to *Archaea*. At Hot Lake I and Hot Lake II, most of the bacterial sequences obtained belonged to *Epsilonproteobacteria* (Figure 24). At genus level of phylogenetic classification, 83.5% sequences were assigned to this class at Hot Lake I and 50.7% at Hot Lake II. Nearly 40% of the sequences could not be categorized into a specific genus at Hot Lake II (Figure 25).

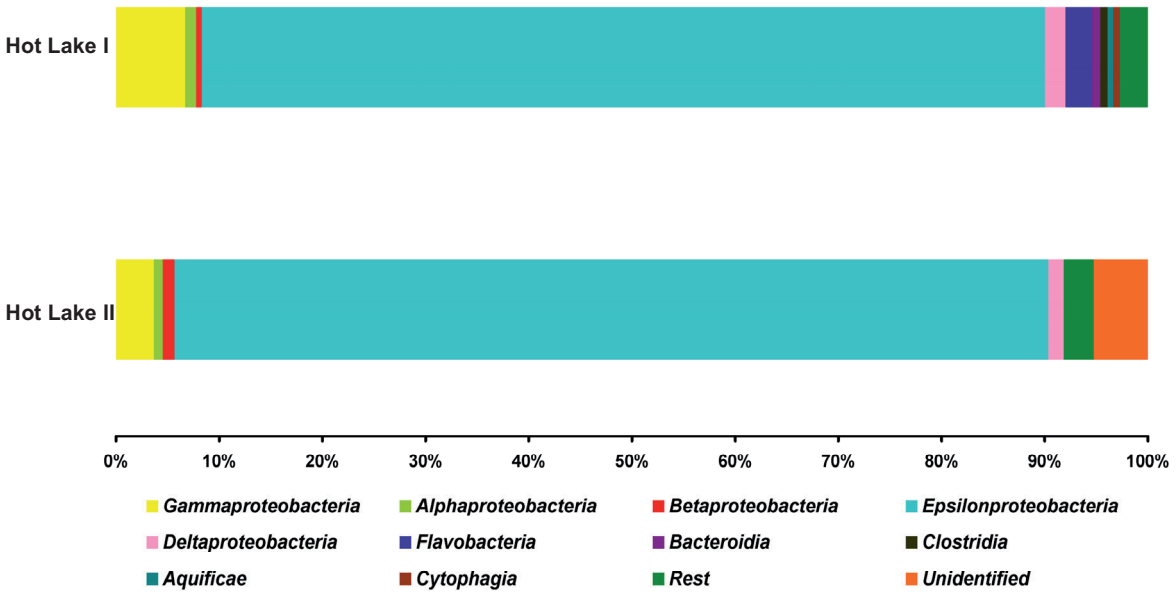


Figure 24. Phylogenetic comparison of *Bacteria* based on class level of the pyrosequencing-based dataset of 16S rRNA through the pipeline of Taxometer.

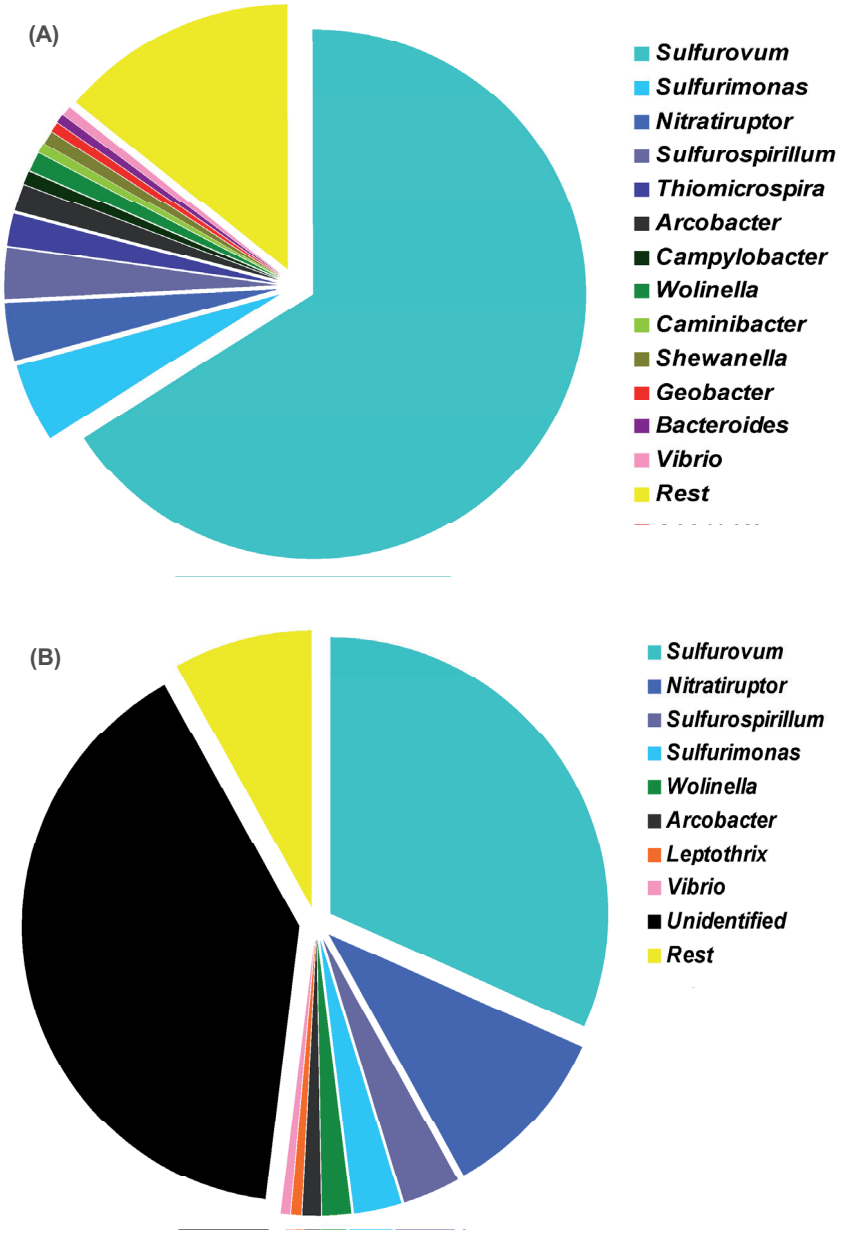


Figure 25. Phylogenetic comparison to genus level of the pyrosequencing-based dataset of 16S rRNA through the pipeline of Taxometer. (A) Hot Lake I. (B) Hot Lake II.

6.4 Functional profiles of Hot Lake I and Hot Lake II

6.4.1 Sulfur metabolism

Possible energy source at shallow-sea hydrothermal vents are either sunlight or reduced chemical compounds. The analyses of 16S rRNA clone libraries identified many sequences whose closest relatives could utilize sulfur to obtain their energy. Already in the previous study, it has been shown that a bacterium isolated from the hydrothermal fluids off Panarea is a mesophilic chemoautotrophic sulfur-oxidizing bacterium, resembling *Thiobacillus* sp. (Gugliandolo et al., 1999). A white mat composed of elemental sulfur and microbes has been reported for this area (Italiano and Nuccio, 1991; Gugliandolo et al., 2006). In order to identify energy generating pathways relevant for microbial life at Hot Lake, this study focused on the inorganic sulfur metabolism.

Genes related to sulfur metabolism were searched against the KEGG database, GenBank and subsystem from MG-RAST. Key genes for the Sox pathway were found both at Hot Lake I and Hot Lake II, including *sox*A, *sox*B and *sox*Y. The Sox multienzyme complex catalyzes the oxidation of hydrogen sulfide, elemental sulfur, sulfite and thiosulfate (Friedrich et al., 2000).

Sqr coding for sulfide:quinone oxidoreductase involved in sulfide oxidation appeared both at Hot Lake I and Hot Lake II. This enzyme has been detected in green sulfur bacteria (Frigaard et al., 2008) and deep sea *Epsilonproteobacteria* of the genus *Sulfurovum* (Yamamoto and Takai, 2011). It catalyzes the oxidation of sulfide with an isoprenoid quinone as the electron acceptor.

The key gene for sulfite oxidation *sorB* coding for sulfite oxidase was detected both at Hot Lake I and Hot Lake II. This enzyme catalyzes the direct oxidation of sulfite to sulfate (Kappler and Dahl, 2001). Sulfite could be alternatively oxidized to adenosine 5'-phosphosulfate (APS) and then to sulfate which is catalyzed by sulfate adenylyltransferase (Sat). Genes coding for the enzyme were detected both at Hot Lake I and Hot Lake II (Figure 26).

Genes coding for polysulfide reductase (Psr) involving sulfur respiration were detected at Hot Lake I and Hot Lake II. Elemental sulfur in the environment has little solubility. It converts to polysulfide in aqueous solutions containing sulfide. Polysulfide with electron acceptor such as hydrogen could then be catalyzed into hydrogen sulfide (Frigaard et al., 2008). Genes coding for thiosulfate sulfurtransferase involved in thiosulfate disproportionation were found both at Hot Lake I and Hot Lake II.

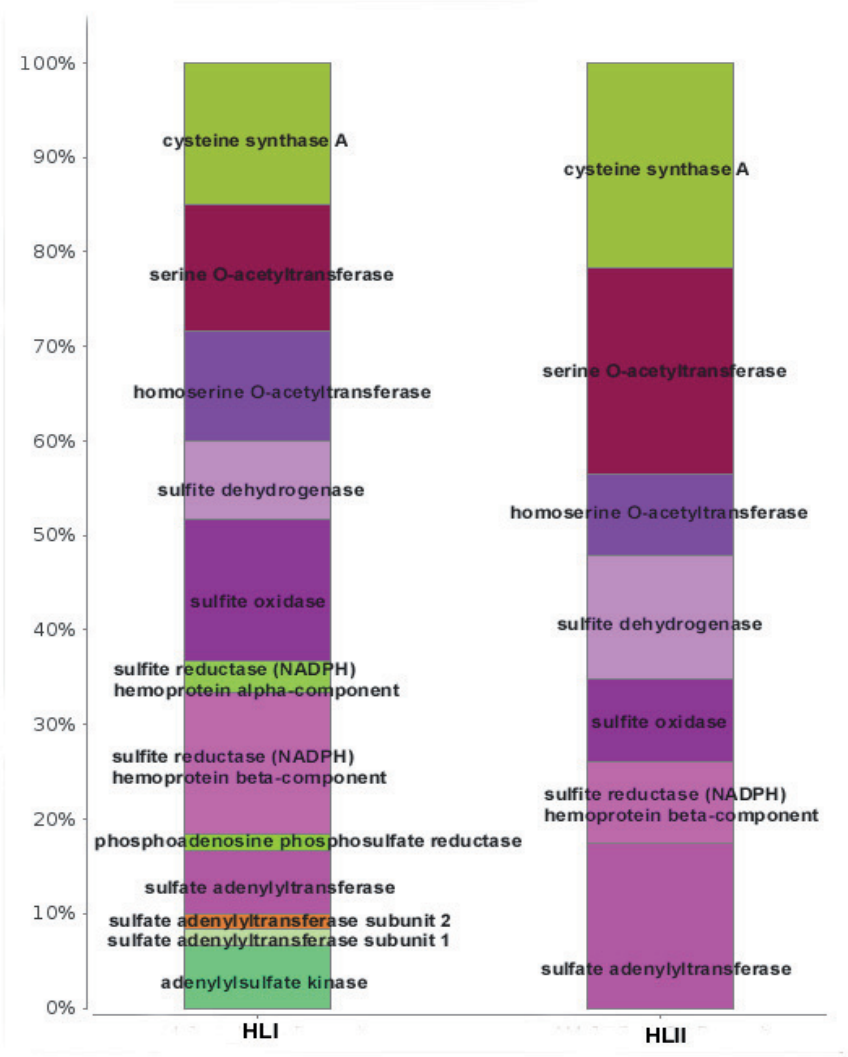


Figure 26. KEGG distribution for genes of the sulfur metabolism in the metagenome of Hot Lake I and Hot Lake II.

6.4.2 Autotrophic carbon fixation

Autotrophic bacteria catalyze inorganic redox reactions to obtain energy and reducing equivalents for the formation of organic molecules from CO_2. There are six pathways known for carbon fixation – Calvin-Benson-Bassham (CBB) cycle (Raven, 2009), the reductive tricarboxylic acid (rTCA) cycle (Ljungdahl, 1986), the 3-hydroxypropionate (3-HP) cycle (Berg et al., 2007), the reductive acetyl coenzyme A pathway (Buchanan and Arnon, 1990), the 3-hydroxypropionate/4-hydroxybutyrate (3-HP/4-HB) cycle (Huber et al., 2008), and the dicarboxylate/4-hydroxybutyrate (DC/4-HB) cycle (Zarzycki et al., 2009).

In this study, ~20% of the ORFs in the KEGG category "energy metabolism" were assigned to rTCA cycle specific enzymes both at Hot Lake I and Hot Lake II (Figure 21). The reductive TCA cycle is mainly the reverse cycle of the catabolic TCA cycle. Rather than breaking down acetyl-CoA with the release of 2 CO_2 and the generation of energy, acetyl-CoA is synthesized by the incorporation of 2 CO_2 and the input of 8 H (in the form of NADH and/or FADH) and 2 ATP. Most of the enzymes of the TCA cycle function reversibly and could catalyze both directions. Genes coding for these enzymes were detected both at Hot Lake I and Hot Lake II (Figure 27).

There are three critical steps which differentiate these two pathways that are non reversible. These three steps include the conversion of citrate to oxaloacetate and acetyl-CoA, the conversion of fumarate to succinate, and the conversion of succinyl-CoA to 2-oxoglutarate. Genes coding for these enzymes were found both at Hot Lake I and Hot Lake II. Based on the best BlastP hits, the ORFs originated mostly from *Epsilonproteobacteria*. Only a minor fraction gave hits to *Chlorobium.* Due to the oxygen sensitivity of the enzyme 2-oxoglutarate and pyruvate synthase, the rTCA cycle appears to be restricted to anaerobic or microaerophilic bacteria which corresponds to the physico-chemical profiles at Hot Lake.

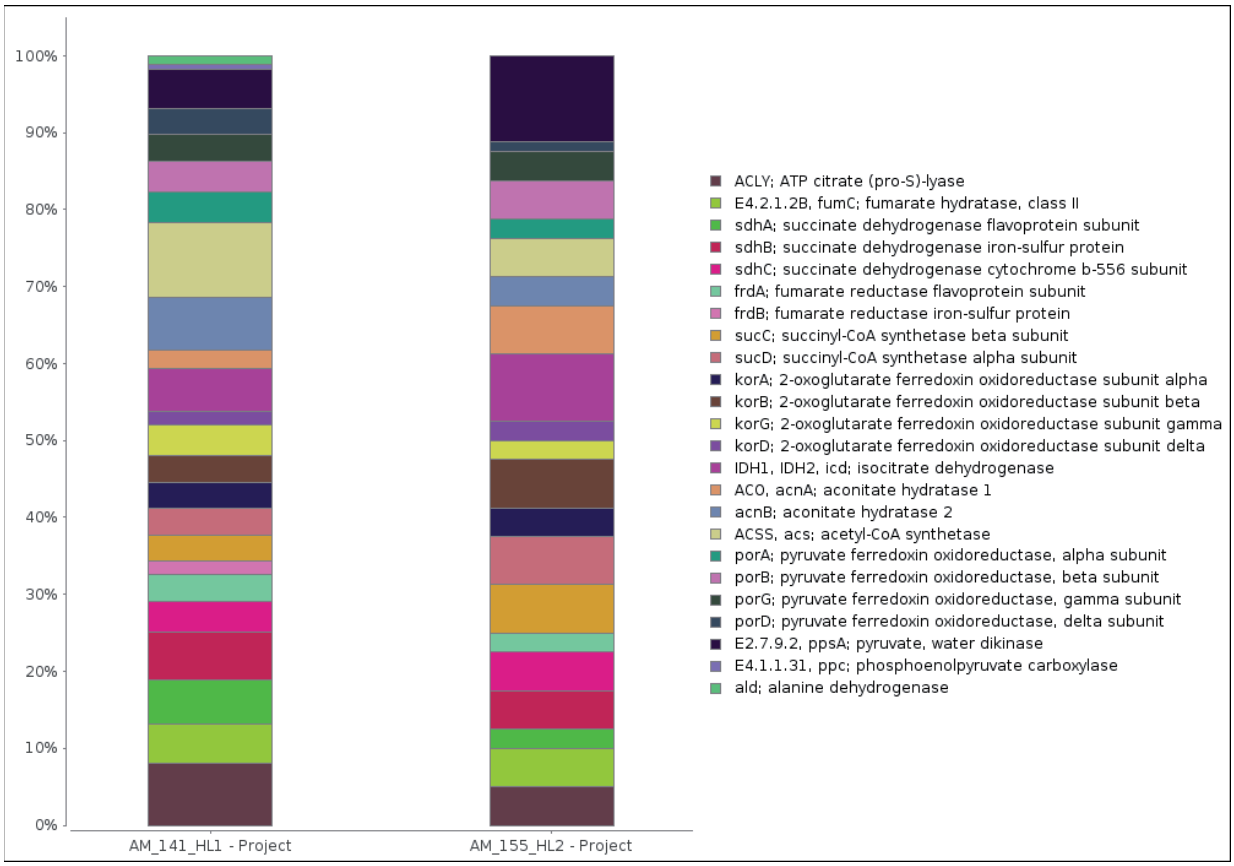


Figure 27. KEGG distribution for genes of the reductive tricarboxylic acid cycle in the Hot Lake I and Hot Lake II metagenome.

V Discussion

1. General physico-chemical parameters and potential metabolic pathways

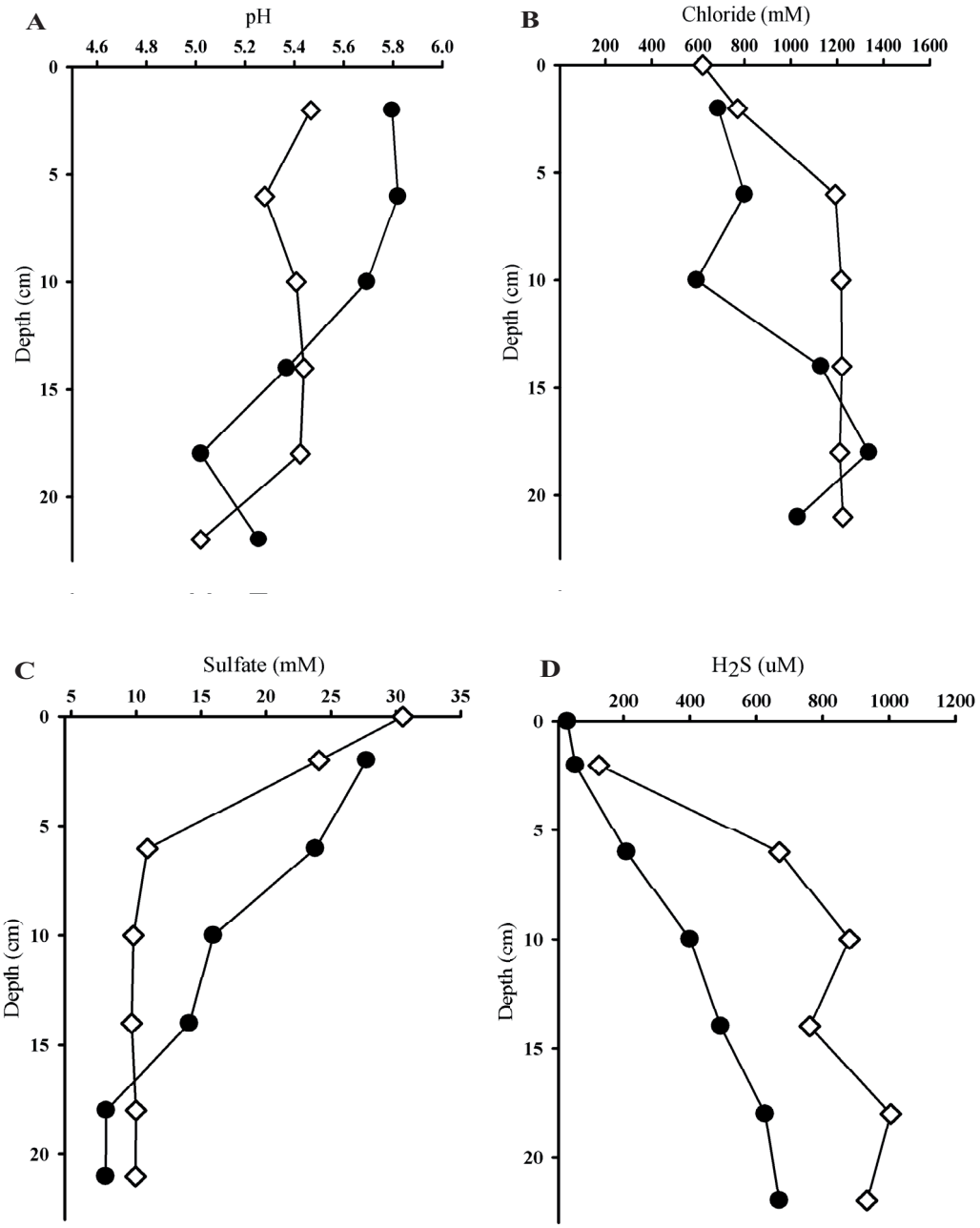
The Panarea hydrothermal system is characterized by a high rate of water circulating at several geothermal bodies (Italiano and Nuccio, 1991). Mineral analysis revealed large amounts of mineral precipitates, such as pyrite, sphalerite and barites at Hot Lake indicating water rock interaction. The abundant elemental sulfur (Figure 7) is formed either by abiotic oxidation of H_2S present in the vent fluids or by biological reactions (Gugliandolo et al., 1999). While heated water ascends towards the seafloor due to lower buoyancy, it may reach boiling point due to reduced hydrostatic pressure. On boiling, gases including water vapor and other volatile species partition into the vapor phase and the remaining water phase increases in concentration of dissolved constituents (Nicholson, 1993). This process the so called phase separation results in a residual high-saline liquid phase which could be observed and confirmed by the pore water analysis at Hot Lake (Figure 28 (B)) (Roy Price, USC, USA, unpublished data). At Hot Lake the chloride concentration was two times higher than that of normal sea water. The temperature at Hot Lake I and Hot Lake II showed distinct differences and increased slightly with sediment depth (Figure 29). However, at the sediment surface the temperature should be similar with the surrounded sea water. From the chemical profiles of pore water analysis and temperature measurements at Hot Lake I and Hot Lake II, two distinct mixing patterns of thermal fluids and sea water can be deduced. At Hot Lake I the flux is less intense than that at Hot Lake II.

The flux of the reduced hydrothermal fluids into oxidized zones creates redox pairs which supply energy to the microbial community. An increase of sulfide concentration and a decrease of sulfate concentration with depth had been observed by Steinbrückner in the thermal fluids of Hot Lake (Steinbrückner, 2009). The measured $\delta^{34}S$ in H_2S indicated that most of the H_2S is derived from leaching of rocks (Ono et al., 2007). Trace elements such as Fe and Mn were enriched in the pore water and thermal water samples (Sieland, 2009). Based on the thermodynamic modeling conducted in the present study, all fourteen investigated chemolithotrophic reactions were suggested to be thermodynamically favorable for the microorganisms.

Hydrogen oxidation with oxygen (knallgas reaction) was observed to be the most favorable reaction. This suggests that oxygen will be immediately utilized by the microorganisms at Hot Lake. Sulfide oxidation and sulfur reduction are also exergonic. In a previous study, sulfide oxidizing bacteria have been detected at or were cultivated from the hydrothermal vent systems off Panarea (Maugeri et al., 2009).

In addition to sulfur metabolism, metal compounds can also be used as electron donors or electron sinks. Several microorganisms have been identified to be capable of exploiting energy through Fe(III) reduction, such as the genera *Desulfuromusa* (Fredrickson and Gorby, 1996) and *Desulfovibrio* (Lovley et al., 2006). Sequences of these genera have also been detected during comparative 16S rRNA analysis in the present study. Mn (IV) is also known as favorable electron acceptor under anaerobic conditions when nitrate and oxygen are limited (Nealson and Saffarini, 1994). At Hot Lake, the discovery of pyrite suggested that the reaction of Fe (III) oxides with sulfur or H_2S could provide metabolic energy for the microorganisms (Edwards, 2004; Schoonen, 2004).

Sulfate reduction and methanogenesis seem to be less favorable under the condition at Hot Lake. Nitrate is known to be an efficient electron acceptor, yet nitrate concentration in the pore water was below the detection limit. A similar phenomenon has been observed in a Black Sea study (Schubotz et al., 2009). This suggested that nitrate is used up by the microorganisms.


A
pH
4.6 4.8 5.0 5.2 5.4 5.6 5.8 6.0
0 5 10 15 20
Depth (cm)
B
Chloride (mM)
200 400 600 800 1000 1200 1400 1600
0 5 10 15 20
Depth (cm)
C
Sulfate (mM)
5 10 15 20 25 30 35
0 5 10 15 20
Depth (cm)
D
H2S (uM)
200 400 600 800 1000 1200
0 5 10 15 20
Depth (cm)

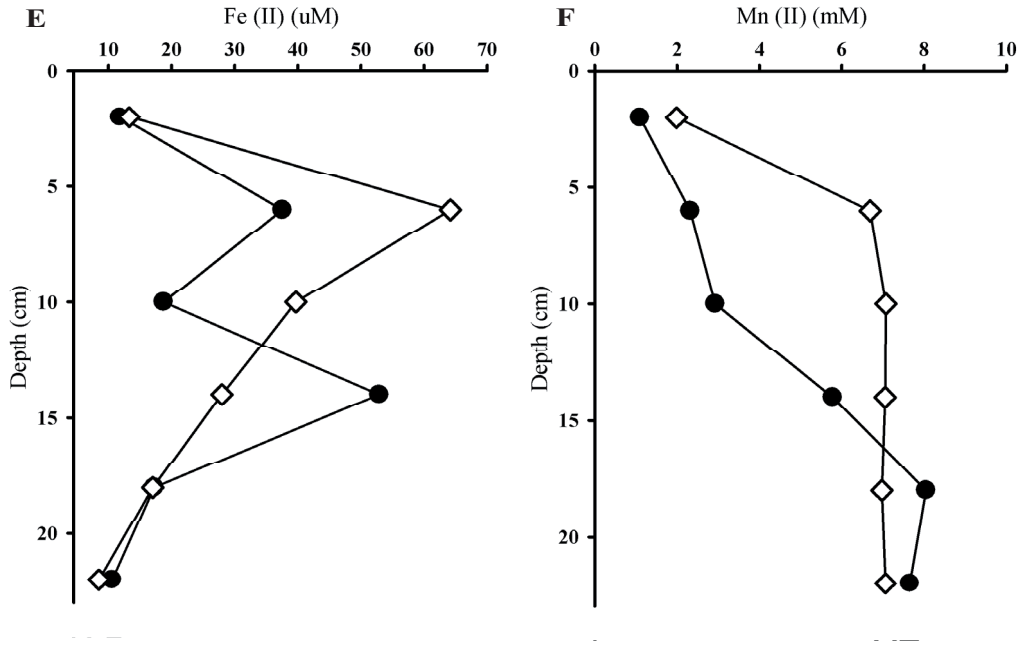


Figure 28. Pore water analysis of different sediment layers. (A) pH values. (B) Chloride concentration. (C) Sulfate concentration. (D) H_2S concentration. (E) Iron concentration. (F) Manganese concentration. Hot Lake I (circles), Hot Lake II (diamonds).

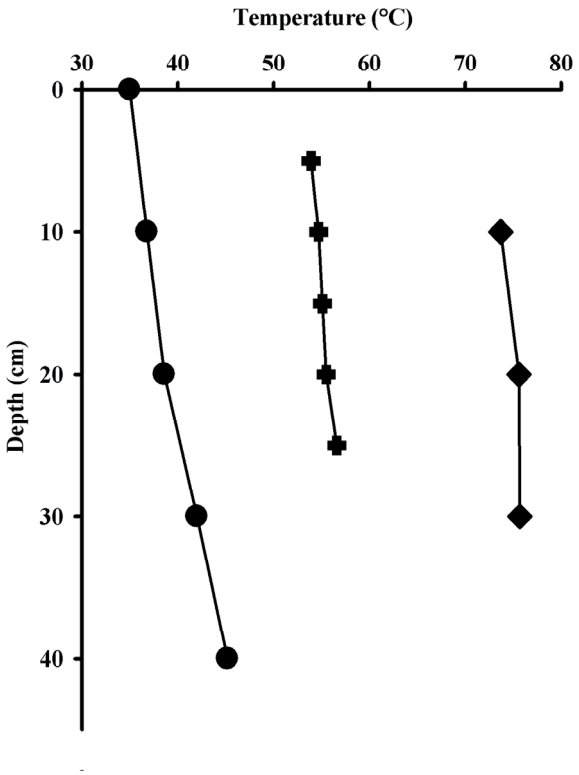


Figure 29. Depth profiles of temperature (*in situ*) of different sediment layers. Hot Lake I (circles), Hot Lake II (diamonds) and Hot Lake 2010 (cross).

2. Microbial diversity and community structures

Through thermodynamic modeling, fourteen chemolithotrophic reactions were characterized. This yielded hypotheses of exploitable metabolic energy sources for the microorganisms. Hydrothermal fluids originate from thermal body in the deep subsurface and mix with entrained sea water. When the fluids arise, mixing occurs and is reflected in the different depth profiles of physico-chemical parameters.

The difference intensity of fluid fluxes at Hot Lake I and II resulted in different microbial community structures which are shown on the ordination plot of ARISA results. Especially, the bottom layers of Hot Lake I and II appeared distant from each other. Samples taken at Hot Lake in 2010 at a medium temperature site (50ºC at 10 cm) revealed a distribution in between Hot Lake I and Hot Lake II on the ordination plot. It confirms that different physico-chemical characteristics likely result in a variation of the microbial diversity.

2.1 Deeper layers (11-17 cm bsf)

Whereas the cell counts at the surface and 14-17 cm below seafloor (bsf) were almost the same at Hot Lake I and II, the profiles indicated a constant decrease of the total cell counts with more extreme conditions for Hot Lake II. At Hot Lake I, characterized by a less steep temperature gradient, drop of cell counts was more discontinuous (Figure 17).

In the lower sediment layer at 14-17 cm bsf *in situ* temperature differed by 37°C between Hot Lake I and Hot Lake II, with significantly higher sulfide concentrations at Hot Lake II. The total cell counts determined for both layers were similar with 10^7 cells/ml and the bacterial abundance was equally high. CARD-FISH revealed a clear dominance of *Bacteria* (74-83%) over *Archaea* (14-24%) at both sites with detection rates around 96%. The analysis of intact polar lipids (IPLs) provides complementary information on the *in situ* community as only living prokaryotes still have the polar head groups of the membrane lipids. This IPL analysis did not detect known archaeal lipids at Hot Lake I, but revealed predominantly archaeal lipids at Hot Lake II. Although IPL analysis cannot be considered to be a quantitative method, this difference is striking and may reflect the importance and viability of certain *Archaea* at Hot

Lake II (unpublished data, Florence Schubotz, MIT, USA) (Sturt et al., 2004) (Figure 30) with characteristically higher *in situ* temperatures at depth.

Comparative archaeal 16S rRNA gene analysis of sequences obtained from 14-17 cm at Hot Lake II identified crenarchaeotal and koracheaeotal sequences exclusively in this sediment layer. These were related to those of known hyperthermophiles, which had already been described from hydrothermal fluids off Panarea (Maugeri et al., 2009). Within the phylum *Crenarchaeota*, all the sequences retrieved in this study belonged to *Desulfurococcaceae.* Members of this family have been shown to be thermophilic autotrophic or heterotrophic sulfate reducers and fermenters (Huber and Stetter, 1998; Kormas et al., 2006; Zhou et al., 2009). The candidate division *Korarchaeota* comprises a group of uncultivated microorganisms. They may have diverged early from *Crenarchaeota* and *Euryarchaeota* according to their small subunit rRNA phylogeny. Genomic study of a close relative has shown gene functions of peptide fermentation for carbon and energy (Elkins et al., 2008).

Euryarchaeal sequences detected in this layer were related to known hyperthermophilic archaea, such as *Thermococacceae*. This group is common at several hydrothermal vent systems (Pagé et al., 2008; Zhou et al., 2009). Cultured relatives are anaerobic and the presence of sulfur enhances greatly their growth (Godfroy et al., 1997). The corresponding layer at Hot Lake I was not investigated by comparative 16S rRNA analysis. Nevertheless, from the IPLs analysis it may be deduced that the archaeal groups present at Hot Lake II either were absent at Hot Lake I or they exhibited IPLs which have not yet been described or they are not viable. Generally, CARD-FISH counts with group specific probes were difficult to determine for the deeper sediment layers due to low cell counts and high background fluorescence.

Bacterial 16S rRNA gene diversity at the deepest investigated layer at Hot Lake II showed the presence of hyperthermophilic *Thermodesulfobacteria.* This group is known to comprise obligate anaerobic sulfate reducing bacteria. Moreover, sequences related to *Nitratiruptor* belonging to *Epsilonproteobacteria* were found exclusively in the deeper layer at Hot Lake II. This genus belongs to the deeply branching order *Nautiliales*. Cultured representatives of *Nitratiruptor* are thermophiles isolated from Mid-Okinawa Trough and are

able to grow by respiratory nitrate reduction with H_2 (Nakagawa et al., 2005). Altogether, *Epsilonproteobacteria* accounted for nearly 15% of the 16S rRNA sequences in the layer 14-17 cm at Hot Lake II. Members of related orders and families affiliate predominantly with genera of known sulfur metabolizing microorganisms. They made up ~5% of the microbial community at Hot Lake I and II.

Deltaproteobacteria constituted another significant fraction of the bacterial community at 14-17 cm of Hot Lake II reflected by approx. 6% of the bacterial fraction in CARD-FISH and 20% of sequences in the 16S rRNA gene clone library. Most of the deltaproteobacterial sequences detected affiliated with *Desulfosalina* spp. and *Desulfocella* spp. within the *Desulfobacteraceae*. Cultivated relatives are halophilic sulfate reducers (Kjeldsen et al., 2010) which corresponded well with the higher salinity at Hot Lake II at this depth. Moreover, sequences related to *Desulfurella* spp. were found. They are known to grow by sulfur reduction (Rainey et al., 2005). *Deltaproteobacteria* were also detected by means of CARD-FISH in the deeper layer of Hot Lake I (50%). This suggests the presence of microorganisms capable of sulfate reduction or sulfur reduction at lower temperatures.

Concurrent with 16S rRNA analysis, the IPL analysis revealed the dominance of betaine and ornithine lipids in the deeper layer of Hot Lake I (Figure 30). These IPLs had been detected in other sulfur rich environments (Schubotz et al., 2009). Betaine lipids are common constituents in marine algae and have been found as well in purple non sulfur bacteria (Benning et al., 1995; Dembitsky, 1996; Brett and Mueller-Navarra, 1997). Ornithine lipids have been detected in sulfate reducing bacteria (*Desulfovibrio*) and sulfur oxidizing bacteria (Shively and Knoche, 1969; Makula and Finnerty, 1975). At Hot Lake, these lipids might derive from sulfate reducers or from algae detritus. In the layer of 11-14 cm of Hot Lake II, only PE lipids were found. A great amount of PE has been detected in sulfate reducing bacteria thriving at suboxic and anoxic zones (Schubotz et al., 2009). Taken together, the physiologies of cultivated relatives of species detected in the lower sediment layers are indicating a relevant role of sulfur cycling in energy generation.

2.2 Middle layers (5-11 cm bsf)

At the middle layers (5-11 cm), the ordination of Hot Lake I and Hot Lake II suggested the presence of different microbial communities. The chloride and sulfide concentrations were enriched at 5-7 cm compared to sea water. Nevertheless, at Hot Lake II, mixing of sea water and hydrothermal fluids is stronger and faster compared to that at Hot Lake I (Figure 28). At Hot Lake I, cell numbers increased to 10^8 cells/ml. At Hot Lake II, total cell numbers were still low and at the same range of that at deeper layers. The abundance might be strongly influenced by the temperature. At Hot Lake I, the fraction of *Archaea* decreased to 10% and no archaeal lipids were detected.

The archaeal 16S rRNA clone library at 5-7 cm at Hot Lake II showed a dominance of *Thermococcaceae* related sequences. Members of this euryarchaeotal order have been detected in the vent fluids at southern Okinawa Trough (Nunoura et al., 2010). Cultivated relatives are known as anaerobic organotrophs (Antoine et al., 1995). Some species are known to reduce elemental sulfur to hydrogen sulfide while oxidizing organic carbon at high temperature. Only few sequences of this order were retrieved at Hot Lake I. CARD-FISH counts showed as well the increase of *Euryarchaeota* over *Crenarchaeota*.

Sequences affiliated to the thermophilic *Thermotogae* were found exclusively in bacterial 16S rRNA clone library at Hot Lake II. Cultivated relatives carry out sulfate reduction or iron reduction or heterotrophic reaction to gain energy (Huber et al., 1991; Reysenbach and Shock, 2002). The alphaproteobacterial 16S rRNA sequences found at this layer at Hot Lake II were closely related to those of pelagic SAR11 clade. These quite abundant sequences (10%) therefore indicate the mixing of marine surface water into this depth of sediments.

Sequences affiliated to *Sulfurovum* were the most abundant within *Epsilonproteobacteria*. Cultured relatives are known to reduce elemental sulfur or oxidize reduced sulfur compounds (Campbell et al., 2006). CARD-FISH counts revealed 13% and 20% of *Epsilonproteobacteria* at Hot Lake I and Hot Lake II respectively. At this mixing zone, *Epsilonproteobacteria* might adapt to the changing environment based on their versatile metabolisms (Campbell et al., 2006).

The high abundance of ornithine lipids in the surface layers of Hot Lake II might be contributed by *Epsilonproteobacteria*. 2Gly-DAG and SQ-DAG lipids are main constituents of photosynthetic membranes suggesting the presence of viable green sulfur bacteria (Siegenthaler and Norio, 2004).

2.3 Upper layers (0-5 cm bsf)

The upper layers of Hot Lake are stongly influenced by sea water, input of elemental sulfur and sea grass detritus (organic matter). The ordination plot of ARISA results showed that upper layers of Hot Lake I and II group closely with each other. *Bacteria* are dominant in the upper layer which was also consistent with the metagenomic and IPLs analyses. The composition of detected IPLs was similar between Hot Lake I and II. The presence of DPG indicated the presence of viable green sulfur bacteria (Imhoff et al., 2004).

At Hot Lake II, the detection rate of *Bacteria* and *Archaea* were only 36% and 17% by means of CARD-FISH. The low detection rates of general bacterial and archaeal probes have been observed in a previous study at hydrothermal sediments at Vulcano (Rusch et al., 2005). The sequences of the probes are based on known sequences in the database and thus new populations that may not contain the target signature sites are not detected. Environments such as hydrothermal vents are suspected to bear many unknown or new populations (Barns et al., 1996; Hugenholtz et al., 1998; Takai and Sako, 1999; Dando and Kiel, 2010; Kubo et al., 2011). Total cell counts were enumerated with SYBR green staining. RNA might be already degraded in the dead cells which could not be detected by CARD-FISH.

Sequences affiliated to Marine Benthic group D-Deep sea hydrothermal vent group (DHVEG I) were detected both at Hot Lake I and Hot Lake II. This group was suggested to be endemic to hydrothermal chimneys, however, the physiological characterization was still barely studied (Teske et al., 2002; Kormas et al., 2006). Sequences affiliated to the Deep Sea Euryarcheotic Group (DSEG) belonging to the class *Halobacteria* accounted for 70% of the clones in the 0-1 cm at Hot Lake II. Closest relatives have been found in deep sea hydrothermal

vents close to group DHVE3 (Takai and Horikoshi, 1999). Members of this order could thrive at high saline environments and obtain energy from sunlight (Oren et al., 2006).

Via bacterial 16S rRNA clone libraries and metagenomic analyses, sequences affiliated to *Chlorobi* were detected. Cultivated *Chlorobi* are known as green sulfur bacteria which combine phototrophy with oxidation of reduced sulfur species or ferrous iron (Heising et al., 1999). Probes designed in this study for the *Chlorobi* of Hot Lake were applied to the environmental samples. The counts of *Chlorobi* were less than 1% which is consistent with the results of metagenomic analyses. However, rarefaction curves showed insufficient sequencing, and more probes for this cluster should be designed in order to elucidate the abundance.

Several *Deltaproteobacteria* have been identified to be capable of conserving energy through Fe (III) reduction, such as members of the genus *Desulfuromusa* (Fredrickson and Gorby, 1996) and *Desulfovibrio* (Park et al., 2008). Sequences related to these genera were detected at the upper layers of Hot Lake I and Hot Lake II. Members of the genus *Desulfuromusa* are known to comprise mesophilic, obligate anaerobic bacteria which are able to grow by sulfur reduction with H_2 (Liesack and Finster, 1994). Cultivated relatives of *Desulfovibrio* have been shown to grow heterotrophically with sulfate reduction (Hirayama et al., 2007). CARD-FISH counts of *Deltaproteobacteria* revealed 30% of total bacteria at Hot Lake II indicating the importance of sulfate reduction as well as sulfur reduction.

At the upper layers of Hot Lake I and Hot Lake II, metagenomic analyses revealed 80% of pyrosequencing reads belonging to *Epsilonproteobacteria*. Sequences affiliated to *Sulfurovum* dominated at both sites. Close relatives had been found at deep sea hydrothermal vents. They are able to gain energy from oxidation of reduced sulfur compounds or reduction of elemental sulfur (Yamamoto et al., 2010). Sequences related to *Sulfurimonas* were found at both Hot Lake I and Hot Lake II. Cultivated *Sulfurimonas* sp. could also oxidize hydrogen or reduced sulfur compounds lithotrophically (Takai et al., 2006). The abundance of *Epsilonproteobacteria* detected by CARD-FISH was between 5-20% at 0-1 cm of Hot Lake I and Hot Lake II. The inconsistency between metagenomic analyses and CARD-FISH was probably due to methodical limitation such as permeabilization issues or incomplete cell lysis.

Sequences related to *Bacteroidetes* were found both at Hot Lake I and Hot Lake II within all layers. More than 90% of the *Bacteroidetes* sequences detected belonged to uncultured clade VC2.1 Bac22. Probes were designed for this group and detected 1-3% of the cells. This agreed with metagenomic analyses which suggested fractions of 3.6% and 1.6% *Bacteroidetes* at Hot Lake I and Hot Lake II, respectively. *Bacteroidetes* are known to be heterotrophic and many members can degrade polymers (Reichenbach and Dworkin, 1992). Sequences of the clade VC2.1 Bac22 had been detected as well in Milos. Members of this group likely have a heterotophic life style utilizing allochthonous organic matter (Sievert et al., 2000a). Sequences related to *Thermococcus* were detected also in the upper layers. It is not likely that they are active there.

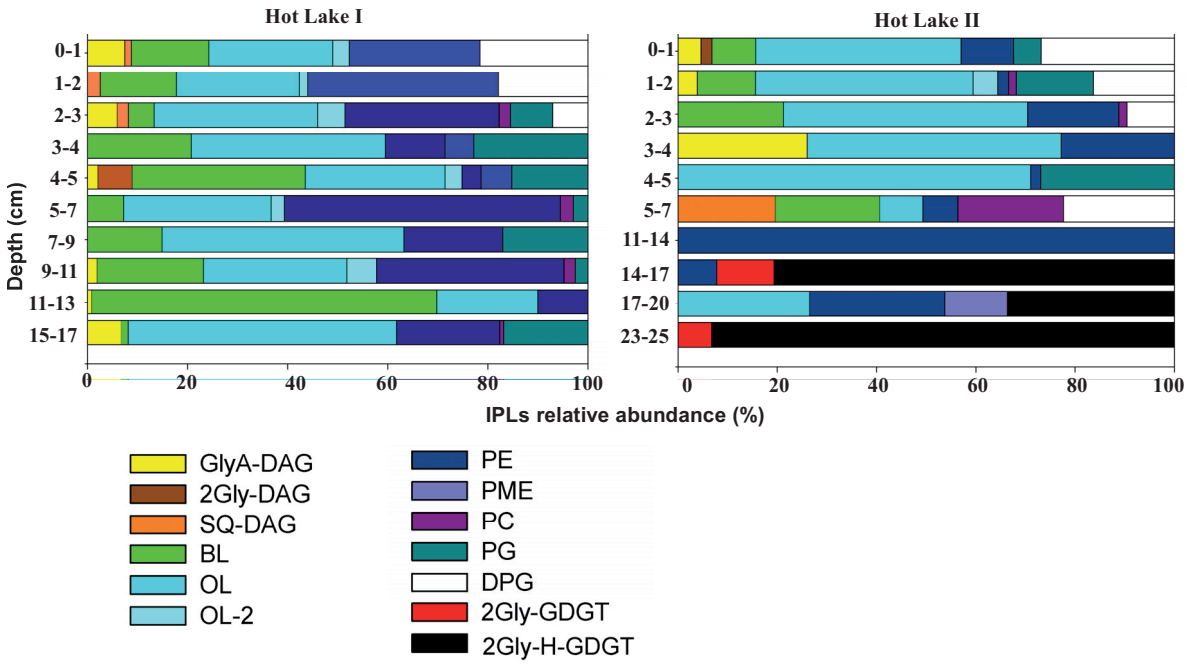


Figure 30. Intact polar lipids analysis of Hot Lake I and Hot Lake II.

GlyA-DAG: Monoglycosydiacylglecerols. **2Gly-DAG**: Diglycosyldiacylglycerols. **SQ-DAG**: sulfoquinovosyldiacylglycerols. **BL**: Betanine. **OL**: Ornithine. **PE**: Phosphatidylethanolamines. **PME**: methylated derivatives of Phosphatidylethanolamines. **PC**: Phosphatidylcholine. **PG**: Phosphatidylglycerols. **DPG**: diphosphatidylglycerols. **2Gly-GDGT**: Archaeal glycosidic Glyceroldibiphytanyltetraethers.

3. Metagenomic analyses provide insights into carbon fixation and sulfur metabolism in the upper layers of Hot Lake

Our thermodynamic modeling had revealed that the oxidation of hydrogen sulfide or sulfide could be exploited by the microorganisms as energy source at Hot Lake. In addition, partially reduced inorganic sulfur compounds such as polysulfide and thiosulfate could serve as both electron donor and acceptor in a variety of energy metabolism (McCollom and Shock, 1997; Nakagawa and Takai, 2008).

Our diversity analyses provided further evidence of sulfur related metabolism. *Epsilonproteobacteria* have been identified before to be important primary producers in sulfur enriched habitats. They were also found to be the most abundant group in the upper layers of Hot Lake I and II through metagenomic analysis.

Past studies have shown that *Epsilonproteobacteria* could sustain by utilization of reduced inorganic sulfur compounds (Takai et al., 2003; Inagaki et al., 2003; Inagaki et al., 2004; Takai et al., 2005) or by bacterial sulfur-oxidation pathways and sulfur reduction pathways. The detection of *sor* within this study indicated the presence of direct sulfite oxidation which was observed previously in the genome of *Sulfurovum* sp. NBC37-1 and *Sulfurimonas autotrophica* (Inagaki et al., 2003; Yamamoto et al., 2010). Cultivated *Sulfurovum* sp. showed the constitutive expression of Sox enzyme systems whereas the expression of sulfur reduction enzymes varied under different cultivation conditions. Deep-sea *Epsilonproteobacteria* are versatile. They change their energy metabolism in response to variable physical and chemical conditions in mixing zones between hydrothermal fluids and sea water (Yamamoto et al., 2010). The dominant CO_2 fixation mechanism of *Epsilonproteobacteria* is the rTCA cycle that was also dominant in the metagenome from Hot Lake.

The rTCA cycle was originally discovered in green sulfur phototrophs (i.e. *Chlorobium*) (Evans et al., 1966). It has been also discovered in many chemoautotrophs including a sulfate-reducing deltaproteobacterium (i.e. *Desulfobacter hydrogenophilus*), thermophilic *Aquificales* (e.g. *Hydrogenobacter* and *Aquifex*) and *Thermoproteales* (e.g. *Thermoproteus*) (Hügler et al.,

2007). Most important, it has been shown that deep sea *Epsilonproteobacteria* can utilize rTCA cycle as carbon fixation pathway (Takai et al., 2005; Hügler et al., 2007; Nakagawa et al., 2007).

Genes coding for the rTCA cycle-specific enzymes were detected both at Hot Lake I and Hot Lake II. The ORFs obtained at both sites showed almost full length proteins. After comparing against NCBI-nr database, the most close relatives were found to be *Nitratifractor*, *Sulfurovum* and *Sulfurimonas* with >90% similarity at Hot Lake II. Cultivated *Nitratifractor* spp. grows at opitimum temperature of 55°C (Nakagawa et al., 2005). At Hot Lake I, most close relatives were found to be mesophilic *Epsilonproteobacteria*, such as *Sulfurimonas*, *Sulfuricurvum* and *Sulfurovum*. It suggested at Hot Lake surface, autotrophs (mostly *Epsilonproteobacteria*) are able to assimilate inorganic carbon using the energy from oxidation of inorganic sulfur compounds.

ORFs related to other carbon fixation pathway were as well detected, such as genes coding for succinate dehydrogenase. This enzyme catalyzes the oxidation of succinate into fumarate in the 3-hydroxypropionate bicycle. So far it has been only found in *Chloroflexaceae* (Zarzycki et al., 2009). Other enzyme such as pyruvate kinase involves in carbon fixation in photosynthetic organisms were observed at both Hot Lake I and II.

VI Conclusion and Perspectives

Hot Lake is an oval-shaped (~10 by 6 meters) shallow (~2.5 m deep) depression in the seafloor at ~18 m water depth. It showed spatial differences in the mixing patterns of hydrothermal fluids and sea water within the depression. ARISA showed diversified microbial community structure depending on physico-chemical parameters. *Bacteria* were dominant at all layers of sediments both at 35°C and 74°C sites. *Archaea* abundance increased with depth and temperature. The pore water analyses, IPLs analysis and molecular analyses concluded that sulfur cycling prevailed in the microbial metabolisms. The correspondent metagenomic analyses of the surface sediments showed more than 80% sequences originated from *Epsilonproteobacteria*. Several key genes of inorganic sulfur metabolism and rTCA cycle were abundant. This indicated that at Hot Lake, primary production was contributed mainly by chemolithotrophs that generate their energy by oxidation of reduced sulfur and to a less degree by phototrophic *Chlorobi*.

Still, in order to decipher the link between geosphere and biosphere, more analyses need to be conducted. Metagenomic analyses of nitrogen metabolisms, photosynthesis or genes coding enzymes catalyzing for e.g. Fe (III) reduction, Fe (II) oxidation and Mn (IV) reduction need to be carried out. Nitrate is a favorable electron acceptor in the absence of oxygen. However, nitrate and also ammonium concentration were below detection limit at Hot Lake. Nitrogen related metabolism at this ecosystem remains ambiguous.

Another question addressed in this study is how the geofuels nourish the microbial community. For that, also colonization experiments were conducted at Hot Lake. In previous mineral analyses, sulfur and iron compounds were found abundant at Hot Lake. Pyrite, orthoglas, olivine and elemental sulfur were cut into 1 cm x 1 cm chips, polished and mounted onto plastic stripes. Subsequently, the mineral chips were put on the sediment surface of Hot Lake at a site with 80°C at 10 cm bsf. Two sets of experiments with or without sunlight respectively were conducted with the time periods of 10 days and 2 months. After the retrieval, samples were treated and fixed for the following CARD-FISH experiment and directly frozen

for DNA extraction. Further analyses will identify the microorganisms that colonize on mineral surfaces and reveal the influence of light.

Incubation experiments with different substrates are essential to understand the ecophysiology of the microbial community. Together with F. Schubotz, sediment samples were taken from Hot Lake and divided into two parts. The surface sediments (0-4 cm) were incubated with $H^{13}CO_3$ (1 mM) and D_2O (1%) with light and without light. This experiment was targeting the enrichment of anoxygenic phototrophs. Samples were harvested T=0, after 24 hours, 72 hours and 1 week, followed by processing for lipid biomarker, CARD-FISH and nano-SIMS analysis. The same procedure was carried out for the deeper layers of sediments. The deeper layers of sediments were incubated in addition with H_2 and acetate, respectively. These experiments target the microorganisms which could use H_2 as electron donor or those that are heterotrophs. Such samples could be analyzed by nano-SIMS, a method that enables to quantify metabolic activities of single microbial cells in the environment. The uptake rates of different substrates can then be measured. At the same time, FISH will identify the targeting microorganisms phylogenetically. Also these experiments are currently ongoing.

In addition, samples were taken from Hot Lake in 2009 for further RNA extraction and analyses. Transcriptome analyses facilitate the investigation of biological processes underlying physiological adaptations to the environments. Characterization of transcriptomics at Hot Lake will give us more insights into the expression of certain genes and usage of certain metabolic pathways. There is no doubt that still much can be learnt from this unique shallow-sea hydrothermal vent off Panarea Island.

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ERKLÄRUNG

Hiermit erkläre ich, dass ich die Arbeit mit dem Titel:

Molecular Ecology of Free-Living Chemoautotrophic Microbial Communities at a Shallow-sea Hydrothermal Vent

selbstständig verfasst und geschrieben habe und außer den angegebenen Quellen keine weiteren Hilfsmittel verwendet habe.

Ebenfalls erkläre ich hiermit eidesstaatlich, dass es sich bei den von mir abgegebenen Arbeiten um drei identische Exemplare handelt.

……

(Unterschrift)