Bacterial niche adaptation at hydrothermal vents

Dissertation
zur Erlangung des Grades eines
Doktors der Naturwissenschaften
- Dr. rer. nat. –

dem Fachbereich Biologie/Chemie
der Universität Bremen
vorgelegt von

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Bremen
Mai 2016

Die vorliegende Arbeit wurde in der Zeit von Oktober 2012 bis Mai 2016 in der Abteilung für Molekulare Ökologie am Max-Planck-Institut für Marine Mikrobiologie in Bremen angefertigt.

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Tag des Promotionskolloquiums: 23. Juni 2016

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Summary

At deep sea hydrothermal fields, a mixing gradient between hot reduced fluids and cold oxygenated sea water creates a number of micro-environments with different physico-chemical conditions in direct proximity to each other. Although many of the key microorganisms in these environments have been identified and described, a systematic understanding of their distribution across the mixing gradient and their niche partitioning is still missing. In my doctoral thesis, I investigated the interplay of geochemical settings, microbial community structures, and diversification of microorganisms in three collaborative studies of hydrothermal vent fields in the Atlantic and Pacific Oceans.

The first study (Chapter II) addressed niche differentiation of two major clades of marine sulfur-oxidizing bacteria. We found strong evidence that the wide spread SUP05-clade *Gammaproteobacteria* are adapted to low sulfide concentrations, whereas *Sulfurovum* and *Sulfurimonas* related sulfur oxidizers occupy a dynamic niche with high sulfide levels closer to the venting orifices. Results of our analyses also suggest that the change of environmental parameters on a small spatial scale and temporal fluctuations of the fluid flow favor a diversification of *Sulfurovum* and *Sulfurimonas* into multiple subtypes. At the same time, the lack of stable conditions could prevent the selection of one specific subtype resulting in the observed diversity of these clades.

Investigating microbial communities on inactive hydrothermal chimneys (Chapter III) by metagenomics, we gained first insights into the genomes of ubiquitous groups of autotrophic *Gammaproteobacteria* likely specializing on active iron-sulfide minerals oxidation. We suggest that these microorganisms likely play an important role in sulfur, iron and carbon cycling at former hydrothermal fields and in marine sediments.

Finally, we were able to identify and locate heterotrophic microorganisms responsible for remineralization of organic carbon in hydrothermal environments (Chapter IV). Dominating the microbial communities in immediate vicinity of diffuse venting, they oxidize organic matter produced by the chemolithoautotrophs and vent fauna and likely also utilize organic molecules present in the venting fluids.

Taken together, this thesis provides a detailed overview of the microbial community structures in hydrothermal vents. It deepens our understanding of niche differentiation of major marine sulfur oxidizers, and offers valuable insights into microbial diversification.

Zusammenfassung

Hydrothermalfelder in der Tiefsee sind gekennzeichnet durch einen steilen Mischungsgradienten zwischen den heißen reduzierten Fluiden und kaltem sauerstoffreichen Seewasser, der eine Vielzahl von direkt benachbarten Mikroumgebungen generiert. Auch wenn viele Mikroorganismen aus diesen Habitaten bereits identifiziert und beschrieben wurden, fehlt immer noch ein systematisches Verständnis ihrer Verteilung innerhalb der Mischungsgradienten und ihrer Einnischung. In meiner Doktorarbeit habe ich die Wechselwirkungen zwischen den geochemischen Umweltbedingungen, Zusammensetzung von mikrobiellen Gemeinschaften und der Diversität von mikrobiellen Populationen im Rahmen von drei kollaborativen Studien der Hydrothermalfelder im Atlantischen und Pazifischen Ozean untersucht.

Die erste Studie beschäftigt sich mit der Einnischung von zwei verbreiteten Gruppen von marinen Schwefelbakterien. Wir haben solide Beweise dafür gefunden, dass die *Gammaproteobakterien* aus der SUP05-Gruppe an niedrige Sulfidkonzentrationen angepasst sein müssen, während die mit *Sulfurovum* und *Sulfurimonas* verwandten Schwefeloxiderer Nischen im Raum wechselnder hoher Sulfidkonzentrationen in der Nähe der Quellen einnehmen. Ferner, weisen unsere Ergebnisse darauf hin, dass die Änderung der Umweltparameter innerhalb einer kleinen räumlichen Distanz und die temporären Schwankungen des Flusses zu einer Diversifizierung von *Sulfurovum* und *Sulfurimonas* in mehrere Unterarten führen. Die instabilen Umweltbedingungen könnten eine Selektion von einer bestimmten Unterart verhindern, was in der beobachteten Diversität dieser Gruppen resultiert.

Bei der Untersuchung von mikrobiellen Gemeinschaften auf inaktiven hydrothermalen Schloten haben wir erste Einblicke in die Genome von ubiquitären autotrophen *Gammaproteobakterien*, die sich wahrscheinlich auf aktive Eisen- und Schwefeloxidation spezialisieren, gewonnen. Wir vermuten, dass diese Mikroorganismen eine wichtige Rolle in Schwefel-, Eisen- und Kohlenstoffkreisläufen an ehemaligen Hydrothermalfeldern und in marinen Sedimenten spielen.

Schließlich, konnten wir heterotrophe Mikroorganismen, die für die Veratmung von organischem Kohlenstoff in hydrothermalen Ökosystemen verantwortlich sind, identifizieren und lokalisieren. Diese dominieren die mikrobiellen Gemeinschaften in unmittelbarer Umgebung der Quellen und oxidieren das organische Material, das von

den Chemolithoautotrophen und der Hydrothermalfauna erzeugt wird, sowie vermutlich auch die in den Fluiden enthaltene organische Verbindungen.

Zusammengefasst bietet diese Arbeit einen detaillierten Überblick über die mikrobiellen Gemeinschaften in Hydrothermalhabitaten, erweitert unser Verständnis der Einnischung von wichtigen marinen Schwefeloxidierern und enthält wertvolle Einblicke in die mikrobielle Diversifikation.

List of abbreviations

APR	adenosine 5’-phosphosulfate reductase
AMP	adenosine monophosphate
ADP	adenosine diphosphate
ATP	adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
CARD	catalyzed reporter deposition
CBB cycle	Calvin-Benson-Bassham cycle
DMSO	dimethyl sulfoxide
DMSP	dimethyl sulfoniopropionate
FACS	fluorescence activated cell sorting
FISH	fluorescence in situ hybridization
HMM	hidden Markov models
IGT	isobaric gas-tight
ISMS	*in situ* mass-spectrometer
N4	tetranucleotides
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
rDsr	reverse dissimilatory sulfite reductase system
rRNA	ribosomal RNA
ROV	remotely operated vehicle
RSS	reduced sulfur species
SSU	small subunit
rTCA cycle	reductive tricarboxylic acid cycle
RuBisCo	ribulose 1,5-bisphosphate carboxylase/oxygenase
SOP	sulfur-oxidizing prokaryote
SOX	sulfur-oxidizing multi-enzyme system

I Introduction

1.1 Carbon fixation and chemolithoautotrophy in the ocean

The ocean and its seafloor are the largest inhabitable area on Earth. Both, macro- and microscopic life forms are ubiquitous here and participate actively in the geochemical cycling of elements such as carbon, nitrogen and sulfur. An estimated 48.5 Pg of inorganic carbon is fixed into organic matter by marine autotrophic organisms per year, which constitutes 46% of total carbon fixation on Earth (Field et al., 1998). One way of obtaining energy for carbon fixation is harvesting the energy of sunlight, as it is done by higher plants, algae and cyanobacteria. Even though sunlight penetrates into the water column only up to 300 m depth, the largest fraction of organic matter in the ocean is produced by photoautotrophs (Dunne et al., 2007; Middelburg, 2011). Many ecosystems of the dark ocean are therefore dependent on organic matter input from the photic zone in form of sinking particles. However, energy for carbon fixation does not have to come from the sunlight, as it can also be derived from chemical redox reactions. This light independent, “dark” carbon fixation or chemolithoautotrophy is only performed by prokaryotic microorganisms and has been reported to occur wherever suitable electron donors and acceptors are available and the thermodynamic conditions are favorable (Bach et al., 2006; Swan et al., 2011).

While inorganic carbon and potential electron acceptors such as e.g. oxygen, nitrate or sulfate are ubiquitous in sea water; electron donors such as sulfide, hydrogen, methane, ammonium or iron are commonly the limiting factor. In sediments, reduced sulfur compounds, hydrogen, and methane can be produced during anaerobic organic matter degradation (Claypool and Kaplan, 1974; Barnes and Goldberg, 1976; Jørgensen, 1977; Mah et al., 1977; Sørensen et al., 1981). Even higher concentrations of reduced compounds are emitted into the ocean at sites of geological activity such as cold seeps, mud volcanos and hydrothermal vents (Corliss et al., 1979; Paull et al., 1984; Milkov et al., 2003; Orcutt et al., 2011). These emissions fuel microbial chemolithoautotrophy which forms the basis for oases of life at the ocean floor populated by numerous microorganisms and various animals. Reduced iron and sulfur compounds can also be found at the ocean floor in precipitated form, as iron sulfides or poly-metal

sulfides (Jørgensen, 1977; Berner, 1984; Janecky and Seyfried, 1984; Binns and Scott, 1993). Such precipitates can occur as massive hydrothermal deposits (Francheteau et al., 1979) or be distributed in anoxic sediment layers (Morse and Cornwell, 1987). Contrasting geochemical conditions, large amounts and various forms of reduced compounds as well as temporal variations of the emissions make hydrothermally “fueled” ecosystems most dynamic interphases of geosphere and biosphere.

This work focusses on different aspects of the microbial ecology of hydrothermal vent ecosystems such as the structuring of microbial communities along geochemical gradients and the utilization of different energy sources, e.g. dissolved sulfur compounds, solid sulfur minerals or organic matter by various microbial guilds.

1.2 Hydrothermal vents

1.2.1 Geological and geochemical settings at hydrothermal vents

Hydrothermal emissions on the sea floor were first detected and observed at the Galapagos Rift in late 1970s (Weiss et al., 1977; Corliss et al., 1979). Today, several hundreds of marine hydrothermal fields are listed in the InterRidge vent database (Fig. 1). The emitted hydrothermal fluid is essentially seawater chemically altered during interaction with heated igneous rock and magma chambers (Fig. 2) (Butterfield et al., 1997; Tivey, 2007). Hydrothermal venting, therefore, occurs everywhere where seawater is entrained into the oceanic crust where it interacts with hot mantel rock. This mostly happens along the margins of tectonic plates. At spreading zones of mid-ocean ridges, new crust is formed by rising magma. At subduction zones, two tectonic plates collide and one tectonic plate is moving beneath another. In both cases tectonic movement creates cracks in the crust, through which sea water is entrained.

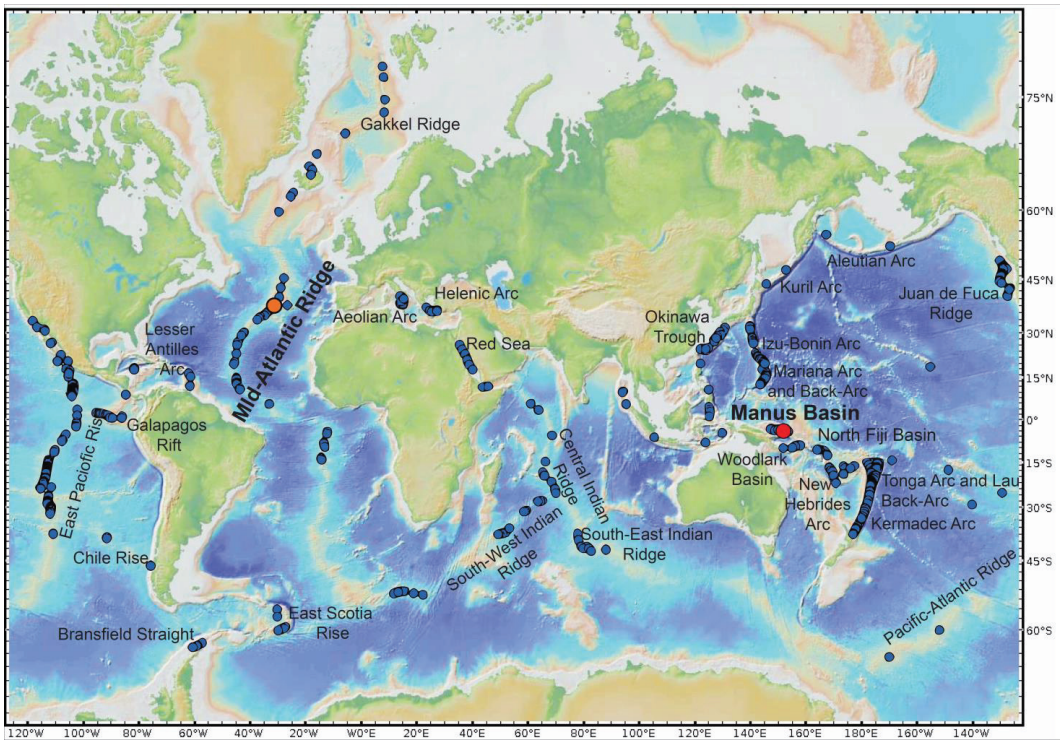

Figure 1: Global distribution of hydrothermal venting fields according to the InterRidge database (v.2.1). The Menez Gwen hydrothermal field on the Mid-Atlantic Ridge is marked in orange. The PACManus and SuSu Knolls hydrothermal fields in the Manus Basin are marked in red.

The interaction of sea water with hot igneous rock leads to precipitation of certain ions in the sea water, such as e.g. magnesium, sulfate and calcium. Other ions like manganese, iron, zinc, copper, and sulfide are dissolved from the rock and are carried back to the surface by the generated hydrothermal fluids (Tivey, 2007). Apart from enrichment with ions dissolved from the rock, seawater also receives gasses coming from the magma (Tivey, 2007). Depending on the hydrothermal system, the fluids can be enriched in hydrogen, methane and carbon dioxide (Butterfield et al., 1997). When the reduced hot fluid is emitted back into the cold oxygenated sea water, metal ions like zinc, iron, and copper precipitate with sulfides as mineral ores to form hydrothermal chimney structures (Fig. 2) (Haymon, 1983; Janecky and Seyfried, 1984).

The dissolved reduced contents of the fluids can be oxidized abiotically or used as electron donors by vent microbiota (Hannington et al., 1995; Luther et al., 2001; Gartman et al., 2011). Also precipitated sulfide minerals can be used by the microorganisms as energy source (Eberhard et al., 1995; Schippers and Sand, 1999; Bach and Edwards, 2003). Microbial chemolithotrophy in these environments is mostly linked to the fixation of inorganic carbon and this “dark” autotrophy provides the basis for the extensive ecosystems covering the hydrothermal vent fields (Jannasch and Wirsen, 1979; Karl et al., 1980; Jannasch and Mottl, 1985).

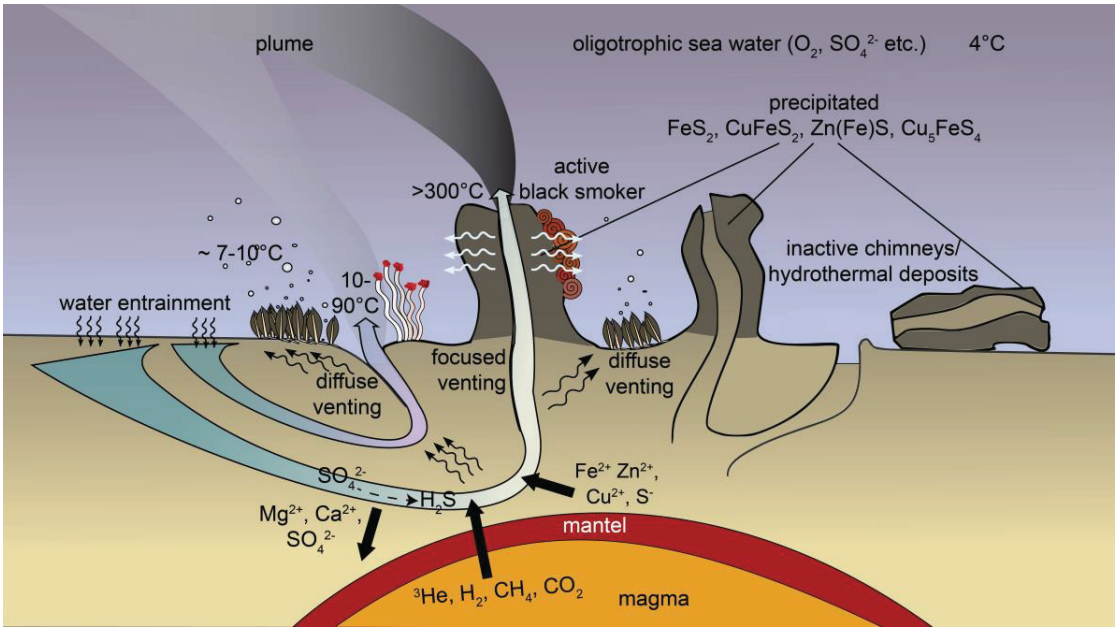

Figure 2: A schematic overview of the geological settings at hydrothermal venting sites. Sea water is entrained into the oceanic crust and heats up gradually while approaching the hot mantel rock. Magnesium and calcium are precipitated. Sulfate precipitates with calcium or is reduced to hydrogen sulfide. Various reduced compounds such as iron, zinc, copper and sulfide are dissolved from igneous rock and gasses like helium, hydrogen, methane and carbon-dioxide are released from the magma. The hot hydrothermal fluid is emitted back into the cold oxygenated sea water. At focused venting sites, metal sulfides precipitate creating massive chimney structures. After the venting stops, chimneys remain on the sea floor as massive sulfide deposits. Diffuse venting occurs, when hydrothermal fluids mixes with oxygenated sea water below the ocean floor, before it is emitted back into the bottom sea water.

1.2.2 Hydrothermal vents as microbial habitats

In hydrothermal ecosystems, living organisms are confronted with rapid changes and steep gradients of environmental conditions. Temperature, pH, and concentrations of electron donors as well as concentration of toxic heavy metals can vary within smallest spatial scales (Baross and Hoffman, 1985; Tivey, 2004; Flores et al., 2011; Sievert and Vetriani, 2012) Mixing models calculated based emitted fluid composition, composition of the bottom sea water, and diffusion and advection velocities, illustrate that contrasting redox conditions can be found within a single hydrothermal chimney wall (Tivey, 2004; Flores et al., 2011) (Fig. 3). However, fluid discharge does not have to happen as

focused emission of hot hydrothermal fluid. It can also appear in form of diffuse venting at the flanks of focused venting orifices (Bemis et al., 2012).

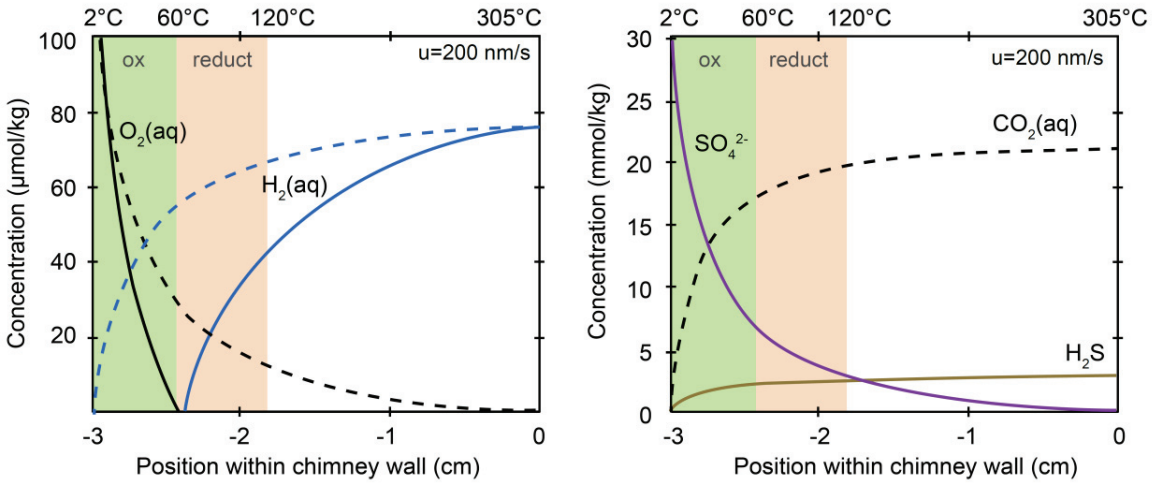

Figure 3: Example of modeled mixing gradients within a porous chimney adapted from Flores *et al.* (2011). Concentration gradients of H_2, O_2, H_2S, CO_2 and SO_4^{2-} within a 3 cm thick chimney wall of Marker 6 vent at Lucky Strike. Diffusion and advection through the porous chimney wall ($\varphi = 0.5$) occur from right to left at a rate of 200 nm/s. Orange shading indicates habitable space with reducing conditions. Green shading indicates habitable space with oxidizing conditions. Dashed lines in the left panel indicate H_2 and O_2 concentrations, when assuming inhibited of H_2 oxidation (Shock and Holland, 2004). "Calculations were performed as described by (Tivey, 2004). End-member concentrations were as follows: (…) H_2 = 77 µM, H_2S = 2.8 mM; CO_2 = 20.7 mM (from Charlou *et al.* 2000; 2002))" (Flores et al., 2011)

Diffuse venting is a term which describes more subtle discharges of diluted hydrothermal fluid, usually with temperatures below 100°C. Diffuse fluids originate from the subsurface of hydrothermal vent fields, when freshly entrained sea water mixes with hydrothermal fluids before those are emitted back into the ocean. The venting can be visible as shimmering water coming from a fissure or by bubbles rising from the sea floor. Due to milder geochemical and physical conditions, diffuse venting areas are usually densely populated by vent fauna. Sometimes hydrothermal fluids are diluted down to habitable temperatures already below the seafloor (Huber et al., 2003; Nakagawa et al., 2005b; Huber et al., 2010). Subsurface habitats filled with such fluids are often populated by mat forming sulfur oxidizing microorganisms (Huber et al., 2003;

Meyer et al., 2013). Accumulation of elemental sulfur in the process of sulfide oxidation gives the bacterial mats a white color and discharge of diffuse fluids from such subsurface habitats is usually visible by a milky plume containing washed out bacterial cells and filamentous elemental sulfur (Taylor and Wirsen, 1997; Taylor et al., 1999; Crowell et al., 2008; Meyer et al., 2013). Fluid and fauna surfaces samples presented in this work are mainly obtained from such diffuse venting sites. In addition, we also collected samples from both active and inactive hydrothermal chimneys.

1.3 Primary producers in deep sea hydrothermal ecosystems

Chemolithoautotrophic microorganisms are the only primary producers in deep sea hydrothermal vents (Jannasch and Wirsen, 1979; Karl et al., 1980; Jannasch and Mottl, 1985; Wirsen et al., 1993). Their main energy sources are the reduced compounds contained in the fluids of which hydrogen-sulfide, methane and hydrogen are the most common ones (Jannasch and Mottl, 1985). Thermodynamic calculations based on measured fluid data and mixing models show that aerobic oxidation of sulfide and methane in mesophilic conditions provide by far the highest amounts of energy per kg of fluid compared to other possible reactions (McCollom and Shock, 1997; Bach et al., 2006; Nakagawa and Takai, 2008; Amend et al., 2011).

Free-living sulfur, hydrogen and methane oxidizing organisms have been isolated from diffuse hydrothermal fluids, hydrothermally influenced sediment and chimney structures. They were also often detected by cultivation-independent methods such as 16S rRNA gene sequencing (for review see Orcutt et al., 2011; Sievert and Vetriani, 2012). Another important fraction of carbon fixing lithotrophic organisms are symbiotic bacteria living in vent fauna, which are until now only represented by candidate species and genome sequences, but no isolates (Kuwahara et al., 2007; Newton et al., 2007; Gardebrecht et al., 2012; Sayavedra et al., 2015). The diversity of chemolithotrophic prokaryotes found in hydrothermal ecosystems by far surpasses the variety of chemical energy sources present in their environment, which indicates that other environmental parameters such as substrate concentration, temperature and pH gradients play an important role in defining niches of these organisms (Campbell et al., 2006; Huber et al., 2010; Flores et al., 2011; Akerman et al., 2013).

Table 1: Sulfur oxidizing microorganisms isolated from hydrothermal vents in pure culture (modified from Sievert and Vetriani (2012))

	Isolation site	Optimum T (°C)	Electron donor(s)	Electron acceptors	End product of nitrate respiration	Carbon source	Reference
Aquificales							
Persephonella marina	EPR	73	H_2, $S_2O_3^{2-}$, S^0	NO^{3-}, S^0, O_2	N_2	CO_2	(Götz et al., 2002)
Persephonella guaymasensis	Guaymas	70	H_2, $S_2O_3^{2-}$, S^0	NO^{3-}, O_2	N_2	CO_2	(Götz et al., 2002)
Hydrogenivirga okinawensis	SOT, Yonaguni Knoll IV	70–75	$S_2O_3^{2-}$, S^0	NO^{3-}, O_2	N_2	CO_2	(Nunoura et al., 2008)
Epsilonproteobacteria							
Sulfurovum lithotrophicum	MOT, Iheya, sediments	28–30	$S_2O_3^{2-}$, S^0	NO^{3-}, O_2	N_2	CO_2	(Inagaki et al., 2004)
Sulfurimonas paralvinellae	MOT, Iheya, *Paralvinella*	30	H_2, $S_2O_3^{2-}$, S^0	NO^{3-}, O_2	N_2	CO_2	(Takai et al., 2006)
Sulfurimonas autotrophica	MOT, Hatoma Knoll, sediments	25	$S_2O_3^{2-}$, S^0, H_2S	O_2		CO_2	(Inagaki et al., 2003)
Gammaproteobacteria							
Thiomicrospira crunogena	EPR, 21°N, Vestimentiferan tube	28–32	$S_2O_3^{2-}$, S^0, H_2S	O_2		CO_2	(Jannasch et al., 1985)
Thiomicrospira thermophila	Mariana Arc, diffuse flow	35–40	$S_2O_3^{2-}$, S^0, H_2S	O_2		CO_2, complex organic substrates	(Takai et al., 2004)
Salinisphaera hydrothermalis	EPR, 9°N, diffuse flow	30–35	$S_2O_3^{2-}$, complex organic substrates	O_2		CO_2, *n*-alkanes, acetate, complex organic substrates	(Crespo-Medina et al., 2009)
Halothiobacillus hydrothermalis	Fiji Basin	35–40	$S_2O_3^{2-}$, S^0, H_2S	O_2		CO_2, complex organic substrates	(Durand et al., 1993)
Thioprofundum hispidum	Izu-Bonin arc, Japan	39	$S_2O_3^{2-}$, S^0, $S_4O_6^{2-}$	NO^{3-}, O_2	?	CO_2	(Mori et al., 2011)
Thioprofundum lithotrophicum	MAR, TAG	50	$S_2O_3^{2-}$, S^0, $S_4O_6^{2-}$, SO_3^{2-}	NO^{3-}, O_2	?	CO_2	(Takai et al., 2009)

Abbreviations: “MOT: Mid-Okinawa Trough; EPR: East Pacific Rise; MAR: Mid-Atlantic Ridge; SOT: Southern Okinawa Trough; TAG: Trans-Atlantic Geotraverse” (Sievert and Vetriani, 2012)

1.3.1 Ecology of major sulfur oxidizers in deep sea hydrothermal habitats

As in many dark ocean habitats, reduced sulfur compounds are the major energy source for the hydrothermal systems studied in this thesis (McCollom and Shock, 1997; Charlou et al., 2000; Amend et al., 2011; Reeves et al., 2011b; Yeats et al., 2014). The most common groups of sulfur oxidizing bacteria found in hydrothermal environments belong to the phylum *Aquificeae* and proteobacterial classes of *Epsilonproteobacteria* and *Gammaproteobacteria* (present isolates summarized in Tab. 1).

Aquificeae are commonly found at thermophilic, anoxic or micro-oxic conditions (Huber et al., 1992; L'Haridon et al., 1998; Reysenbach, 2001; Nakagawa et al., 2004; Hugler et al., 2007). They oxidize reduced sulfur compounds and hydrogen, using nitrate or elemental sulfur as electron acceptors and fix carbon via the reverse tricarboxylic acid (rTCA) cycle (Huber et al., 1992; Beh et al., 1993; Nakagawa et al., 2004; Hugler et al., 2007).

Gamma- and *Epsilonproteobacteria* oxidizing reduced sulfur compounds occur in a broader range of sulfidic environments. Many of the sulfur oxidizing *Gammaproteobacteria* are known to form filaments and biofilms in marine sediment and limnic environments such as sulfidic springs and caves (Nelson and Castenholz, 1982; Jørgensen and Revsbech, 1983; Macalady et al., 2008; Grunke et al., 2011; Salman et al., 2011). Also at hydrothermal vents, filamentous *Gammaproteobacteria* have been observed (Jannasch et al., 1989; Crepeau et al., 2011; Schauer et al., 2011; Winkel et al., 2014a). Yet the most abundant sulfur oxidizing *Gammaproteobacteria* detected in diffuse fluids and hydrothermal plumes seems to be the SUP05-clade bacteria (Sunamura et al., 2004; Anderson et al., 2013; Glaubitz et al., 2013; Marshall and Morris, 2013; Shah and Morris, 2015; Sheik et al., 2015), also known from oxygen minimum zones and stratified, sulfidic marine water columns (Lavik et al., 2009; Schmidtova et al., 2009; Walsh et al., 2009; Zaikova et al., 2010; Glaubitz et al., 2013; Marshall and Morris, 2013). Members of the SUP05-clade are also found as endosymbionts of vent bivalves (Duperron et al., 2005; Kuwahara et al., 2007; Newton et al., 2007; Duperron et al., 2011; Petersen et al., 2011). While data from oxygen minimum zones suggests their adaptation to low sulfur concentration (Lavik et al., 2009; Walsh et al., 2009; Canfield et al., 2010; Glaubitz et al., 2013), their niches at hydrothermal vents are yet not fully resolved.

Sulfur oxidizing *Epsilonproteobacteria* have been found to dominate many sulfidic environments from shelf sediments to sulfidic caves and oxyclines of the Baltic Sea

(Engel et al., 2003; Campbell et al., 2006; Lin et al., 2006; Grote et al., 2008; Macalady et al., 2008; Sievert et al., 2008c). Also at many hydrothermal vent systems *Epsilonproteobacteria* are reported to be the dominating chemolithotrophs (Polz and Cavanaugh, 1995; Huber et al., 2003; Inagaki et al., 2003; Lopez-Garcia et al., 2003; Nakagawa et al., 2005a; Opatkiewicz et al., 2009; Huber et al., 2010; Akerman et al., 2013; Meyer et al., 2013; Perner et al., 2013). Here two major groups are consistently found. .One of them is the *Nautiliaceae* family which is currently represented by moderately thermophilic autotrophic or mixotrophic hydrogen oxidizers (Miroshnichenko et al., 2004; Takai et al., 2005a; Smith et al., 2008). The other group consists of two genera of mesophilic sulfur and hydrogen oxidizing *Epsilonproteobacteria*, named *Sulfurovum* and *Sulfurimonas* (Nakagawa et al., 2005a; Campbell et al., 2006). Several isolates and closed genomes of each genus are currently represented in the databases. They exhibit metabolic versatility in terms of electron donors and acceptors as well as high tolerance to harsh environmental conditions such as heavy metal toxicity and nitrosative and oxidative stress (Inagaki et al., 2003; Inagaki et al., 2004; Takai et al., 2006; Nakagawa et al., 2007; Sievert et al., 2008c; Yamamoto et al., 2010; Park et al., 2012). Colonization experiments on surfaces exposed to hydrothermal venting showed *Sulfurimonas* and *Sulfurovum* to be the primary colonizers in diffuse venting habitats (Lopez-Garcia et al., 2003). They could also be enriched on native sulfur particles due to their ability to use cyclooctasulfur (S_8) (Pjevac et al., 2014).

In culture independent studies of diffuse hydrothermal fluids *Sulfurovum* and *Sulfurimonas* have often been observed together with SUP05-clade bacteria (Sunamura et al., 2004; Bourbonnais et al., 2012; Akerman et al., 2013; Anderson et al., 2013; Sheik et al., 2015). Niche separation between these two groups based on different sulfur concentrations optima has been proposed (Anderson et al., 2013), but not yet shown *in situ*. The niche differentiation between *Sulfurovum* and *Sulfurimonas*, which are mostly found to be co-occurring, is also yet to be clarified, as currently available isolates seem to have similar optimal growing conditions (Inagaki et al., 2003; Inagaki et al., 2004; Takai et al., 2006; Yamamoto et al., 2010; Park et al., 2012). Furthermore, first hints at high internal diversity within the *Sulfurovum* and *Sulfurimonas* genera (Huber et al., 2007; Huber et al., 2010; Akerman et al., 2013; Sheik et al., 2015) raise the question how meaningful differentiation between the different species is realized.

As mentioned above, hydrothermal habitats comprise a wide range of environmental condition ordered in steep gradients between the emitted fluids and background

seawater. An example of a mixing gradient within a porous chimney wall modeled by Flores and colleagues (2011) is shown in Figure 3. The spectrum of these conditions exceeds growth limits currently known from isolates representing the main bacterial clades of hydrothermal microorganisms. This suggests that the different groups of microorganisms have to be positioned in a structured way within such mixing gradient. Until now, only few studies attempted to sample different parts of the gradient (Sievert et al., 1999; Sunamura et al., 2004; Akerman et al., 2013; Anderson et al., 2013; Perner et al., 2013; Sheik et al., 2015). Ecological studies at hydrothermal fields were often limited by the number of samples and the potential of molecular analysis available (Sunamura et al., 2004; Anderson et al., 2013; Perner et al., 2013). Still, these studies revealed differences such as those between diffuse fluid and plume (Sunamura et al., 2004; Anderson et al., 2013) or between diffuse fluids and solid surfaces (Huber et al., 2003; Lopez-Garcia et al., 2003). An elaborate study by Sheik and colleagues followed the hydrothermal plume from the orifice into the water column (Sheik et al., 2015). However, at the hydrothermal field itself, Sheik and colleagues (2015) merely state the difference in microbial community between sulfide deposits and diffuse fluids, as well as between the diffuse fluids at the orifice of venting and the plume. Other studies investigating microbial community composition at hydrothermal vents mostly focused on comparing different venting sites within a field or across different hydrothermal fields to each other (Huber et al., 2007; Perner et al., 2007; Huber et al., 2010; Flores et al., 2011; Xie et al., 2011; Flores et al., 2012). Differences in microbial communities observed by these studies were either attributed to differences in fluid chemistry (Huber et al., 2007; Perner et al., 2007; Flores et al., 2011) or to geographical isolation (Huber et al., 2010; Akerman et al., 2013). The ecology of co-occurring bacterial species with seemingly overlapping functions, such as sulfur oxidation, therefore remains largely unresolved.

1.3.2 Sulfur oxidation and carbon fixation in *Gamma*- and *Epsilonproteobacteria*

Gamma- and epsilonproteobacterial sulfur oxidizers differ in their sulfur oxidation and carbon fixation pathways (Friedrich et al., 2005; Hügler and Sievert, 2011). Both utilize the SOX multi-enzyme complex to oxidize reduced sulfur compounds (Fig. 4) (Friedrich et al., 2005; Sievert et al., 2008a). Originally, the complex was discovered and studied in detail in terrestrial *Alphaproteobacteria* of the genus *Paracoccus* as thiosulfate oxidizing enzyme complex (Friedrich et al., 2000; Rother et al., 2001). In the first step, covalent binding of thiosulfate to the SoxYZ complex is catalyzed by SoxXA heterodimeric c-type cytochrome (Quentmeier and Friedrich, 2001). Next SoxB, a di-manganese enzyme, catalyzes the release of sulfate from the SoxYZ-cysteine-thiosulfonate by hydrolysis (Quentmeier et al., 2003). SoxCD, a complex of the molybdoprotein SoxC and a di-heme c-type cytochrome SoxD, further oxidizes the second sulfur atom, still bound to SoxYZ to yield another sulfate (Zander et al., 2011). The second sulfate is finally released from SoxYZ via hydrolysis again involving the SoxB protein. Although the canonic pathway describes the oxidation of thiosulfate and is found as “thiosulfate oxidation” pathway in databases, such as MetaCyc, the proteins of the SOX complex have also been shown to oxidize sulfite, elemental sulfur and hydrogen sulfide (Fig. 4) (Rother et al., 2001).

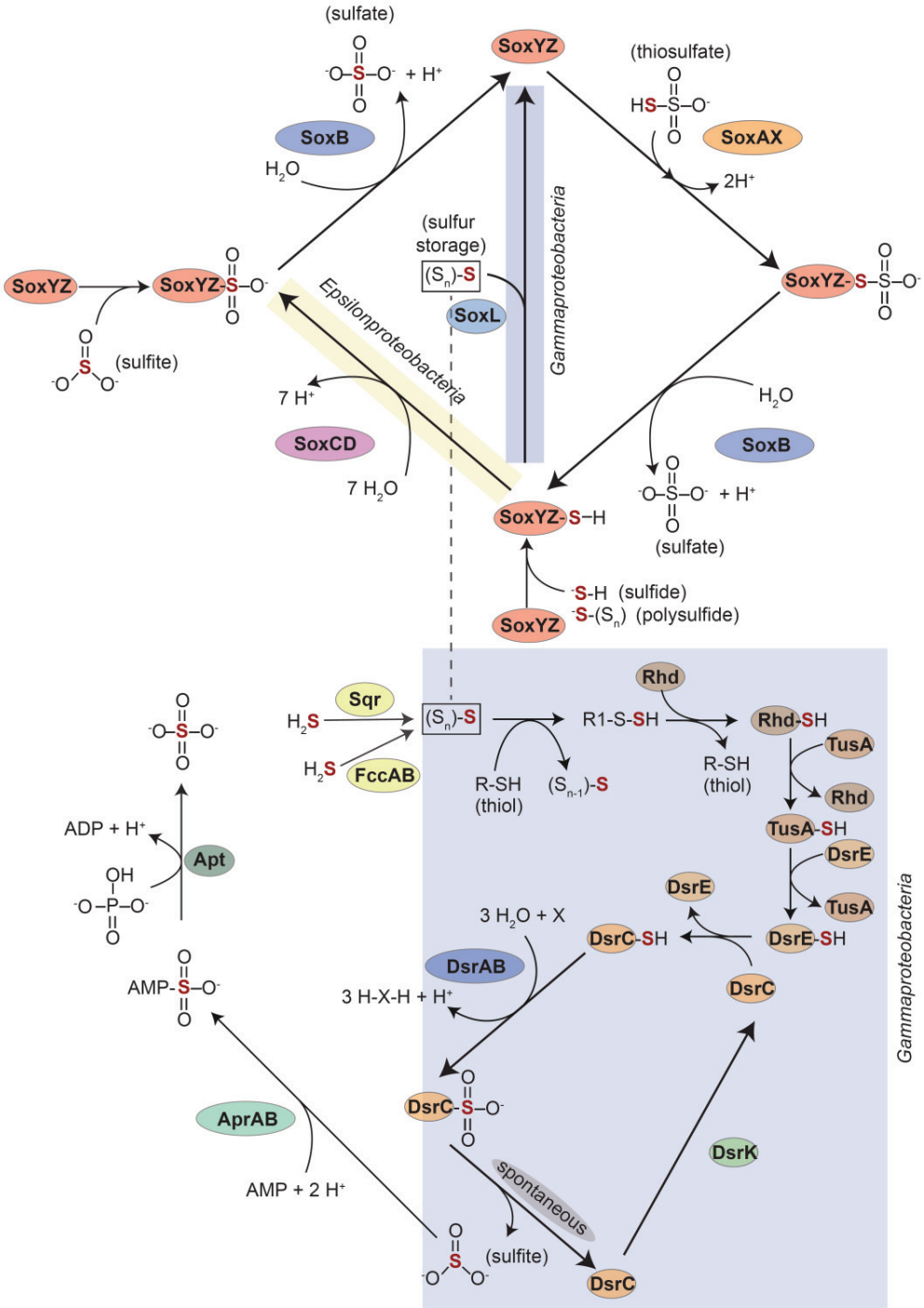

Figure 4: Schematic overview of reduced sulfur compounds oxidation in Epsilon- and Gammaproteobacteria. Colored ellipses mark catalytic enzymes and carrier proteins. The sulfur atom covalently bound to the carrier proteins is marked in red. Blue and yellow squares frame the pathways specific only for Gammaproteobacteria or for Epsilonproteobacteria, respectively. Metabolic map was generated based on MetaCyc database v. 20.0 (Caspi et al., 2014)

The pathway can be found in the described form in prominent vent *Epsilonproteobacteria* of the genera *Sulfurovum* and *Sulfurimonas* (Inagaki et al., 2003; Nakagawa et al., 2005a; Takai et al., 2006; Nakagawa et al., 2007; Yamamoto et al., 2010; Park et al., 2012), although the organization of the *sox* genes in the genomes differs from the model system (Sievert et al., 2008a; Sievert et al., 2008c). In *Gammaproteobacteria*, however, the SoxCD complex is missing from the SOX system (Friedrich et al., 2005; Sievert et al., 2008a). The lack of SoxCD would not matter for the oxidation of sulfite to sulfate. However, the oxidation of thiosulfate and hydrogen sulfide can only be performed until the stage of elemental sulfur, since further steps require an oxidation of the sulfur atom to sulfate by SoxCD (Fig. 4) (Hensen et al., 2006; Welte et al., 2009). The sulfur can then be accumulated in intracellular sulfur globules or oxidized via the reverse dissimilatory sulfate reduction pathway (rDsr) (Schedel et al., 1979; Pott and Dahl, 1998; Dahl et al., 2008; Grein et al., 2010). Sulfur atoms at the end of a polysulfide chain can be spontaneously reduced to thiols. The thiol can then dissociate and be passed through a number of sulfur carrier proteins covalently binding it to a cysteine residue (Rhodanase, TusA, and DsrE) until it is transferred to the DsrC sulfur carrier protein (Fig. 4) (Dahl et al., 2008; Stockdreher et al., 2012). The thiol bound to the DsrC protein is oxidized to sulfite by the dissimilatory sulfate reductase (DsrAB) (Pott and Dahl, 1998; Dahl et al., 2008). The sulfite is further transferred to an AMP molecule by the dissimilatory adenylyl-sulfate reductase resulting in an adenosine 5'-phosphosulfate molecule (Peck, 1968; Hipp et al., 1997; Sanchez et al., 2001). Finally, an ATP molecule is generated from the AMP moiety of adenosine 5'-phosphosulfate and a pyrophosphate while a sulfate molecule is released by the dissimilatory sulfate adenylyltransferase (Kappler and Dahl, 2001).

Also the carbon assimilation pathways of chemolithoautotrophic *Gamma*- and *Epsilonproteobacteria* are different. *Epsilonproteobacteria* employ the energetically more efficient reverse tricarboxylic acid cycle, whereas *Gammaproteobacteria* fix carbon via the Calvin-Benson-Bassham (CBB) cycle known from phototrophic *Cyanobacteria* and plants (Hugler et al., 2005; Takai et al., 2005b; Tabita et al., 2007; Hügler and Sievert, 2011). The advantage of the CBB over the rTCA cycle is its higher oxygen tolerance. Ferredoxin dependent reactions of the rTCA cycle performed by the 2-oxoglutarate:ferredoxin oxidoreductase and pyruvate:ferredoxin oxidoreductase are oxygen sensitive and are thought to function best in micro-aerophillic or anaerobic environments (Shiba et al., 1985; Beh et al., 1993; Yoon et al., 1996; Campbell et al.,

2006; Hügler and Sievert, 2011). Yet, less efficient, aerotolerant variants of 2-oxoglutarate:ferredoxin oxidoreductase are known (Yun et al., 2002; Yamamoto et al., 2003, 2006).

1.4 Microbial utilization of iron-sulfur minerals

Reduced iron and sulfur can be used as energy sources by microbes not only when they are dissolved in water. Also precipitated minerals can serve as an energy source. Pyrite, for example, can be oxidized abiotically by dissolved oxygen or iron (III), releasing sulfide from the mineral (Lowson, 1982; Luther et al., 1982; Moses et al., 1987). Microorganisms can either utilize this abiotically released sulfide or actively oxidize the iron atom of pyrite to accelerate the sulfide release (Boon et al., 1998; Nemati et al., 1998; Schippers and Sand, 1999; Sand et al., 2001). Microbial oxidation of iron-sulfides such as pyrite is long known from acid drainage associated with coal mines (Colmer and Hinkle, 1947; Hoffert, 1947; Baker and Banfield, 2003). The organisms involved in iron and sulfur oxidation at acid mine drainage (AMD) sites are well known and represented by several isolates (Edwards et al., 2000; Hippe, 2000; Kelly and Wood, 2000). Chemical and microbial oxidation of pyrite has also been studied in coastal and limnic sediments (Luther et al., 1982; Thamdrup et al., 1994; Schippers and Jørgensen, 2002). However, presence and identity of microorganisms involved in pyrite oxidation in sediments remains mainly unclear.

At hydrothermal vents large amounts of potential chemical energy in form of reduced sulfur are deposited on the sea floor as massive sulfide chimneys (Tivey, 2007). Consisting of pyrite, chalcopyrite ($CuFeS_2$), iron-sulfide and other metal sulfides they represent a potential source of energy available for chemolithotrophic microorganisms even after hydrothermal venting has ceased. Inorganic carbon fixation in biofilms of inactive sulfide deposits was first detected by Wirsen and colleagues (1993) and recently has been shown to largely occur via the CBB cycle present in chemolithoautotrophic *Gammaproteobacteria* (Reeves et al., 2014b). *Gammaproteobacteria* belonging to the sulfur oxidizing genus *Thiomicrospira* isolated from hydrothermal chimney structures were shown to grow autotrophically with poly-metal sulfides as sole energy source (Eberhard et al., 1995). Also, iron oxidizing *Gamma-* and *Alphaproteobacteria* related to heterotrophic species were isolated from sulfide deposits (Edwards et al., 2003). However, their exact identity was never fully resolved. Later 16S rRNA amplicon based

studies confirmed dominance of *Gammaproteobacteria* related to sulfur oxidizing clades on inactive chimney structures (Kato et al., 2010; Sylvan et al., 2012). Taken together, these findings show that hydrothermal sulfide deposits should be considered as another important energy source for microbial primary production in the dark ocean. Related organisms might also be involved in metal sulfide oxidation and carbon fixation in marine sediments and are therefore of major importance for the marine carbon cycle (Lenk et al., 2011; Dyksma et al., 2016).

1.5 Organic carbon and heterotrophy at hydrothermal vents

Another poorly studied aspect of hydrothermal vent ecology is microbial heterotrophy at vent sites. As in any other ecosystem, the organic matter generated by autotrophic organisms is utilized and largely remineralized back to CO_2 by heterotrophic organisms. For hydrothermal ecosystems, until recently, it was assumed that carbon fixed by chemolithoautotrophic microorganisms would be transferred to heterotrophic vent fauna (Jannasch and Mottl, 1985). In the case of symbiotic CO_2-fixing bacteria the organic carbon is indeed taken up and largely respired by the host (Polz et al., 1998; Bright et al., 2000). Also free-living, mat-forming bacteria are subject to grazing by, e.g., gastropods or worms (Van Dover and Fry, 1989; Bergquist et al., 2007; Stokke et al., 2015). This can be considered the main food chain which in other habitats is completed by heterotrophic microorganisms playing a crucial role in remineralization of dead organic matter.

At hydrothermal vent systems, carbon freshly fixed by the autotrophs is also not the only source of organic matter. There is mounting evidence that hydrothermal fluids themselves contain various types of organic substrates such as methane-thiols (Reeves et al., 2014a), short chain hydrocarbons (Brault et al., 1988; Konn et al., 2009) and fatty acids (Lang et al., 2006; Lang et al., 2010). Abiotic genesis of organic molecules during hydrothermal processes and its extent has become subject to speculation shortly after the discovery of hydrothermal vents (Baross and Hoffman, 1985; Miller and Bada, 1988; Marshall, 1994; McCollom and Seewald, 2001; Proskurowski et al., 2008; McDermott et al., 2015). Small organic molecules like methane or formate are thought to be produced abiotically (Proskurowski et al., 2008; McDermott et al., 2015), while larger molecules are rather considered to originate from organic matter input from the ocean floor and sediments (Brault et al., 1988; Simoneit and Fetzer, 1996; Lein et al., 2003; Reeves et

al., 2014a). This partly explains why hydrothermal systems located close to sedimented continental slopes emit hydrocarbon rich fluids (Brault et al., 1988; Bazylinski et al., 1989; Simoneit and Fetzer, 1996). Recently, it has been proposed that hydrothermal fluids in general contain high amounts of organic matter, which is derived from refractory dissolved organic compounds in the sea water and is made bioavailable during the hydrothermal transformation (Hawkes et al., 2015; Rossel et al., 2015). For example, for one of the sites studied in Chapter IV, a high content of volatile organic matter was shown by Rossel and colleagues (2015).

Consistent with the accumulating knowledge about types and origins of organic carbon at hydrothermal vents, 16S rRNA gene sequences related to those of heterotrophic bacteria have been detected in several studies investigating microbial diversity of vents. (Lanzen et al., 2011; Akerman et al., 2013; Meyer et al., 2013; Perner et al., 2013; Sheik et al., 2015). These sequences were not investigated in detail as the focus of hydrothermal microbial research was traditionally on chemolithotrophic organisms. Until now, only few studies focused on heterotrophic organisms. Several heterotrophic thermophilic *Archaea* species were isolated from deep sea vents (Gonzalez et al., 1998; Marteinsson et al., 1999; Jolivet et al., 2003; Gorlas et al., 2014; Price et al., 2015). Heterotrophic bacterial isolates from hydrothermal fluids, sediments or vent fauna have been studied with respect to production of unusual exopolysaccharides (Vincent et al., 1994; Raguenes et al., 1997a; Raguenes et al., 1997b), use of unusual electron acceptors such as e.g. arsenite (Handley et al., 2009) or their capability of growth on hydrocarbons as sole carbon and energy source (Bertrand et al., 2013). Their ecological roles, as well as distribution and abundance in hydrothermal ecosystems were barely discussed.

In a recent case study of a hydrothermal biofilm Stokke and colleagues (2015) demonstrated a trophic relation between a filamentous exo-polysaccharide producing *Sulfurovum* and heterotrophic *Bacteroidetes* species. In another culture independent study by Winkel and colleagues (2014b) found rapid growth of heterotrophic organisms upon addition of acetate to the fluids investigated in Chapter IV.

1.6 Cultivation-independent approaches in microbial ecology

Our knowledge about the microbial world to a large part comes from isolated strains and cultivation or enrichment experiments. Although, it is long accepted that microbial diversity present in the environment is only poorly represented by culturable strains (Whitman et al., 1998), isolates remain a valuable source of information about physiology and metabolism or high quality genome information (Leadbetter, 2003; Alain and Querellou, 2009). Derived from well-defined experiments and controlled tests, results of cultivation studies provide high confidence information about the studied organism. However, for various reasons, there can be no guarantee that the organism in culture is a typical, characteristic representative which is autochthonous to the studied environment (Amann et al., 1995). Cultivation media and conditions might lack unknown, yet important environmental factors and thus select for allochthonous microorganisms which are present in low numbers under environmental conditions and originate from other sites (Alain and Querellou, 2009). Thus, to truly understand the functioning of whole microbial communities and their key players in the environment, obtaining additional *in situ* information on true clade abundances and activities is absolutely necessary.

Cultivation-independent molecular ecology methods are currently based on direct extraction and analysis of microbial material from the environment.

Environmental DNA or RNA can be used as template to amplify genes of interest such as that encoding small subunit ribosomal RNA (SSU rRNA) (Amann et al., 1995) or shotgun-sequenced in approaches known as metagenomics and metatranscriptomics, respectively (Riesenfeld et al., 2004; Cardenas and Tiedje, 2008). In metaproteomics, proteins extracted from environmental samples are related to the (meta)genomic information (Wilmes and Bond, 2006). Environmental lipidomics employ structure, composition and carbon isotope ratio of microbial lipids as sources of information about taxonomic profiles of microbial communities and carbon flow pathways (White et al., 1979; Fang and Barcelona, 1998; Fang et al., 2000; Biddle et al., 2006; Lipp et al., 2008). Metabolomics adds profiles of metabolites present in an environmental samples to the community picture (Miller, 2007).

Molecular profiling can be combined with incubation experiments conducted immediately upon retrieval of environmental sample. The sample can be amended with specific substances and incubated at defined conditions in order to test for enrichment of

different community members. The amended substances can also be labeled with stable or radioactive isotopes, allowing tracing of the uptake and incorporation of the label into the biomass (Boschker et al., 1998; Radajewski et al., 2000). For example, stable or radioactive carbon isotopes are commonly used to identify organic and inorganic carbon assimilating microorganisms (Wirsen et al., 1993; Polz et al., 1998; Bright et al., 2000; Webster et al., 2006; Herrmann et al., 2010; Fortunato and Huber, 2016). However, these methods rely on survival and "normal" functioning of the microorganisms after sample retrieval.

For hydrothermal vent studies, cultivation-independent methods represent a promising way to overcome the limitations of cultivation-dependent methods and maximize the information retrieved from samples which rare and technologically challenging to obtain.

1.6.1 SSU rRNA based methods

The establishment of the SSU rRNA gene as a universal phylogenetic marker (Woese, 1987) opened many possibilities for culture independent microbial community surveys (Amann et al., 1995). On the one hand, amplification of this gene or a fragment of it from environmental DNA allowed for an assessment of microbial diversity and community composition (e.g. Schmidt et al., 1991; Acinas et al., 2004; Turnbaugh et al., 2007; Ley et al., 2008). On the other hand, SSU rRNA molecules could now be targeted by taxon specific oligonucleotide probes carrying fluorescent dyes or reporter enzymes in order to visualize certain microbial community members (Amann et al., 1990; Pernthaler et al., 2002).

Advances in DNA sequencing techniques, especially the introduction of massive parallel "next-generation" sequencing by 454 and Illumina (Mardis, 2008), made the profiling of microbial communities by comparative analysis of environmental SSU rRNA genes a standard technique in molecular ecology (Huse et al., 2008; Degnan and Ochman, 2012). Although, profiling of community composition based on 454 or Illumina sequencing of short fragments of the gene can be effective in detecting community shifts and provide a solid resolution of the community members (e.g. Bartram et al., 2011; Caporaso et al., 2011; Koenig et al., 2011; Sylvan et al., 2012), precise phylogenetic placement of detected organisms requires full length sequences of the SSU rRNA gene (Yarza et al., 2008; Yarza et al., 2014). Until recently, preparation of clone libraries and

Sanger sequencing remained necessary to obtain full-length sequences of the SSU rRNA gene. Since a few years, PacBio circular consensus sequencing provides an alternative way of obtaining full length SSU rRNA gene sequences from environmental samples bypassing the construction of clone libraries, and increasing the quantities of sequences obtained in the same time and for the same cost dramatically (Fichot and Norman, 2013; Mosher et al., 2014).

Combined, SSU rRNA based techniques are powerful tools to rapidly identify, enumerate and track microorganisms in many samples: while full length 16S rRNA sequences provide a robust phylogenetic placement of microorganisms, massive parallel amplicon sequencing provides high resolution of the community composition and its variation across samples. In addition, oligonucleotide probes designed to target key community members can be used to identify, visualize and enumerate microorganisms directly in environmental samples, also yielding information on lifestyles.

The drawbacks of the rRNA approach lie mainly in the fact that the majority of the sequences obtained from the environment come from yet undescribed organisms and can only be attributed to broader taxonomic clades like genera or families, but not to cultured and described species (Yarza et al., 2014). In order to still be able to describe the diversity in a microbial ecosystem, environmental sequences are usually grouped into abstract “operational taxonomic units” (Schloss and Westcott, 2011). Such units are traditionally generated based on comparison of the sequences to each other via pairwise alignment with a certain percentage similarity threshold considered to correspond to sequence variability within a species (Seguritan and Rohwer, 2001; Schloss and Handelsman, 2005). The used thresholds are largely empiric and might not apply in the same manner to all taxonomic groups or to all regions of the SSU rRNA gene (Youssef et al., 2009; Yarza et al., 2014). Further drawbacks to the OTU generation are errors introduced during the gene amplification or sequencing (Huse et al., 2007; Kunin et al., 2010). This “noise” is considered to cause an inflation of observed microbial diversity over the real one. Several approaches exist to minimize this noise including stringent quality filtering, removal of singleton sequences, alternative clustering methods (Huse et al., 2010; Reeder and Knight, 2010), and platform specific noise removal algorithms like “Pyronoise” (Quince et al., 2009).

Regardless of other shortcomings which can potentially be fixed with improved methods, the ultimate limit of the rRNA based techniques is their inability to accurately predict the function of an identified microorganism. Methods trying to predict

functionalities of microbial communities from SSU rRNA data exist (Langille et al., 2013), yet they rely on functions of close cultured representatives which are often missing (Tringe et al., 2005; Fuhrman, 2009). For some environmental SSU rRNA gene sequences, the closest isolate might be from a different family, order, class or even phylum. At the same time, even organisms with almost identical SSU rRNA gene sequence might have significant differences in their metabolisms (Fox et al., 1992; Jaspers and Overmann, 2004; Thompson et al., 2005; Woebken et al., 2008; Hoefman et al., 2014). A solid linkage of microbial identity and function can therefore only be accomplished via cultivation and/or by obtaining almost complete genomic information, if possible, combined with gene expression data.

1.6.2 Environmental genome analysis techniques

There are two major cultivation-independent ways of obtaining genomic information from environmental samples: i) shotgun sequencing of bulk DNA extracted from an environmental sample, known as metagenome sequencing (Riesenfeld et al., 2004), and ii) sequencing of randomly amplified single cell genomes (Lasken, 2007; Ishoey et al., 2008). In both cases, genome(s) need to be assembled from short sequence reads, which poses significant algorithmic challenges (Tyson and Hugenholtz, 2004; Lasken, 2007). In case of metagenomes, this task is further complicated by presence of potentially hundreds different genomes of different complexity and abundance (Tyson and Hugenholtz, 2004; Luo et al., 2012; Peng et al., 2012; Howe et al., 2014). Yet, the advantage of metagenome sequencing is a relatively unbiased retrieval of genomic information of the whole community, with the main sources of potential bias being the DNA extraction (Martin-Laurent et al., 2001; de Lipthay et al., 2004) and sequencing platform specific issues such as Illumina's bias against very low and very high GC content (Minoche et al., 2011; Nakamura et al., 2011).

In contrast to metagenome sequencing and assembly, genomic information assembled from single cell sequencing almost certainly originates from one organism. However, the confidence does not reach the absolute level due to possibility of two attached cells being sorted together, free DNA, and other contamination sources (reviewed in Blainey, 2013). Besides challenges in cell sorting, which is mostly done in flow cytometers based on optical properties of cells or fluorescence signals via fluorescence activated cell sorting (FACS), the amplification of a rather complete single

cell genome currently poses severe problems. Multiple displacement amplification (MDA) is currently employed to generate a sufficient amount of DNA for sequencing. This method tends to generate chimeric sequence fragments and provides highly uneven coverage of the genome (Lasken, 2007, 2012; Nurk et al., 2013). Single cell genomes therefore often end up being incomplete, missing important genes like the SSU rRNA gene required for the phylogenetic placement of the organism or genes needed for an accurate prediction of metabolic capabilities (e.g. Martinez-Garcia et al., 2012; Rinke et al., 2013).

The pendulum is therefore going back to metagenomics. Recent developments in metagenome analysis allow us retrieving of much more complete microbial genomes from metagenomic datasets (Iverson et al., 2012; Albertsen et al., 2013; Di Rienzi et al., 2013; Kantor et al., 2013; Sharon and Banfield, 2013). These developments include improved assembly algorithms combined with sophisticated methods of grouping assembled metagenome sequences referred to as metagenome binning (Sharon and Banfield, 2013). In general, current metagenome binning techniques rely on a combination of sequence composition information (e.g. GC content, k-mer frequencies), sequence abundance information (read coverage in one sample, coverage variation across samples) and reference database derived data (conserved single copy genes, taxonomic classification) to group sequences likely belonging to the same organism (Fig. 5) (Strous et al., 2012; Albertsen et al., 2013; Alneberg et al., 2014; Imelfort et al., 2014; Wu et al., 2014; Kang et al., 2015). However, due to the likely presence of multiple closely related strains in an environmental sample, current consensus of the scientific community is to consider genomic bins derived from metagenomes rather “population genomes” containing information from several strains of a species (Luo et al., 2012; Sharon et al., 2013). In studies of low complexity microbial communities or community members with distinctly different genomes, metagenome sequencing and binning showed impressive success in retrieving almost complete genomes of yet uncultured, poorly studied microbial clades (e.g. Woyke et al., 2006; Iverson et al., 2012; Albertsen et al., 2013; Di Rienzi et al., 2013)

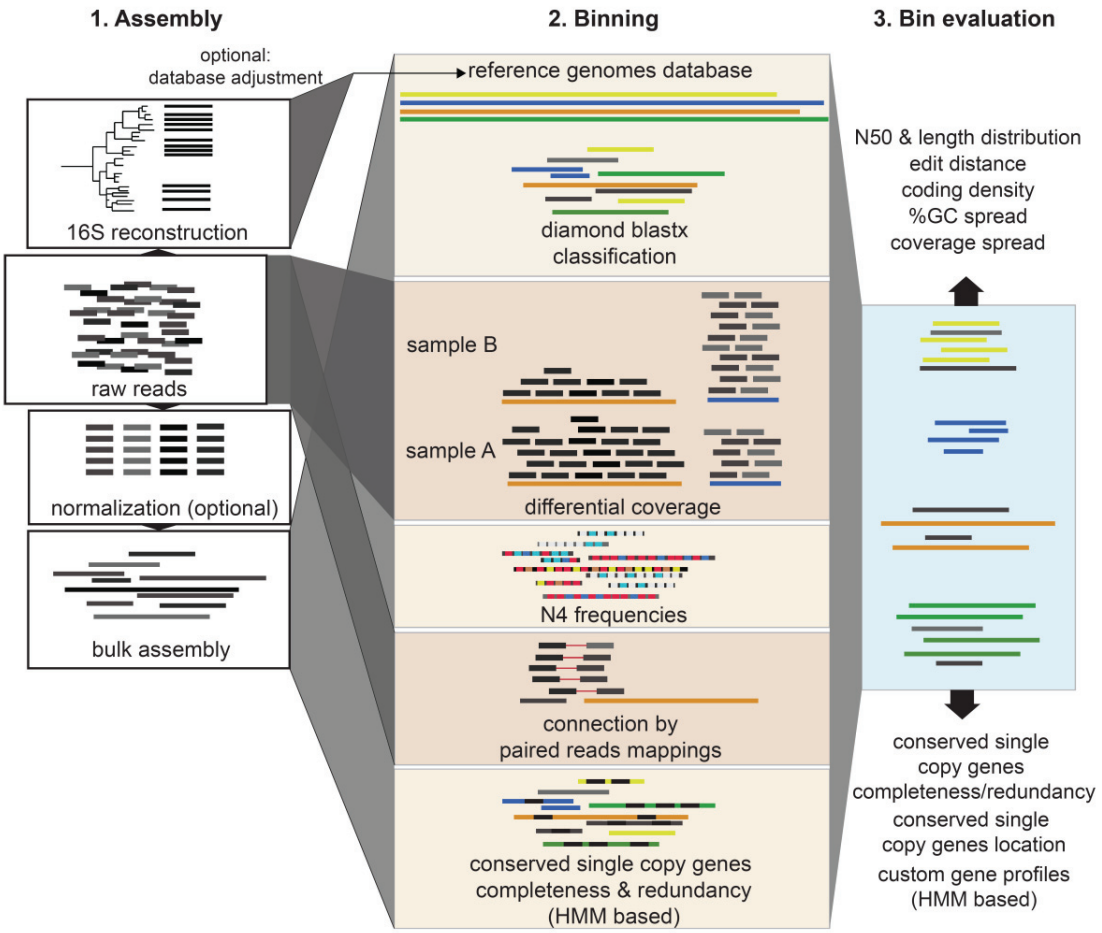

Figure 5: A schematic illustration of the metagenome assembly and binning process. Binning and bin evaluation as performed by the current version of MetaWatt (3.5.2) (Strous et al., 2012). A consensus of multiple criteria is used to group contigs into bins. After binning is finalized, the user can evaluate the bins by several displayed metrics.

1.7 Geochemical habitat characterization

For microbial ecology studies in such dynamic environments as hydrothermal vents, a precise assessment of physico-chemical conditions at the point of sampling is crucial. Temperature, pH, and concentrations of electron donors and acceptors determine the composition and function of microbial communities, and therefore must be determined as precise as possible.

Determination of fluid composition can be done on board, after retrieval of a fluid/water sample. The drawback of the shipboard analysis is the loss of pressure in the sample leading to partial loss of gaseous components from the fluid. The development of isobaric gas-tight (IGT) samplers, which keep the sample at *in situ* pressure and prevent gases from escaping, was a major improvement towards more precise measurements of fluid compositions (Seewald et al., 2002; Reeves et al., 2011b). It has also been shown that the *in situ* pH of hydrothermal fluids might differ significantly from the shipboard measured values (Ding and Seyfried, 1996). Thus, another important tool for hydrothermal fluid sampling is an *in situ* pH-sensor (Le Bris et al., 2001; Ding et al., 2005). A further promising tool is a membrane-ionization mass-spectrometer adapted for deep sea *in situ* measurements of gas concentrations (Wankel et al., 2010; Petersen et al., 2011; Perner et al., 2013). Mounted on a submersible, it can be run in parallel to *in situ* pumping and filtering system providing real-time data on gas concentrations at the very moment of biological sampling.

An alternative is modeling. Since many geo-chemical parameters of hydrothermal environments are a result of mixing of hydrothermal fluid and sea water, they can be modeled if the precise compositions of the two are known and a proxy for the mixing grade is available. For example, a combination of mixing models based on IGT data and continuous real-time temperature data as mixing proxy can be applied to estimate reduced compounds concentrations at points of sampling lacking *in situ* chemical data.

1.8 Hydrothermal fields investigated in this study

The samples were collected at two different hydrothermal fields. The Menez Gwen hydrothermal field is a basalt-hosted low sedimented system located around a young axial volcano at the Mid-Atlantic Ridge (MAR) spreading zone, South of the Azores (Ondreas et al., 1997; Charlou et al., 2000; Marcon et al., 2013). The main hydrothermal

activity occurs on the southern and eastern flanks and mainly consists of less hot and less iron rich grey smokers or clear fluids of up to 300°C. The fluids are rich in sulfide and methane and poor in hydrogen (Charlou et al., 2000; Amend et al., 2011; Reeves et al., 2011a). Areas of diffuse venting are densely covered by *Bathymodiolus* mussels. Also various shrimps (*Remicaris, Maricaris* and others), crabs and gastropod are present (Ondreas et al., 1997; Desbruyères et al., 2001; Galkin and Goroslavskaya, 2010; Marcon et al., 2013).

The Manus Basin is a young fast spreading back-arc basin located next to the New Britain trench and the Manus trench subduction zones in the Bismarck Sea off Papua New Guinea. Its basalt hosted hydrothermal systems are highly active and exhibit a wide range of fluid compositions (Binns and Scott, 1993; Scott and Binns, 1995; Reeves et al., 2011b). Common to all fluids of the Manus Basin are high concentrations of hydrogen sulfide, less methane and almost no hydrogen (Reeves et al., 2011b; Yeats et al., 2014). The fauna of the Manus Basin is more diverse than fauna at Menez Gwen and includes abundant *Ifremeria* snails and vestimentiferan tubeworm colonies along with *Bathymodiolus* related *Gigantidas* mussels, shrimps and others (Galkin, 1997; Desbruyères et al., 2006; Pante et al., 2012).

Aims of the study

Hydrothermal fields and associated microbiota have been studied for decades. Substantial knowledge about the geochemical conditions as well as the identities and functions of microorganisms populating the venting sites has been accumulated. On the other hand, a systematic understanding of how the distribution of different microorganisms and the composition of microbial communities is affected by the dynamic conditions was still missing when this doctoral thesis was started. Rare sampling opportunities and technically challenging sampling conditions had prevented a coherent and extensive biological and geochemical sampling necessary for elucidation of robust correlations.

The overarching goal of this thesis was to generate a systematic overview of microbial community structuring at hydrothermal vent fields with respect to physico-chemical gradients and different energy sources. A secondary general goal of the project was as well to maximize the information obtainable without cultivation from the preserved

samples by a combination of up-to-date DNA sequencing and data analysis techniques. These goals can be divided into following specific aims:

- First aim of this study, was to create a spatially resolved (cm scale) and extensive overview over microbial community composition shifts across geochemical gradients accompanied by comprehensive assessment of relevant geochemical data. This aim was determining the sampling strategy at all studied hydrothermal fields.
- Second aim, was to test if niche differentiation between dominant hydrothermal sulfur oxidizing bacteria in this dynamic environment occurs according to the same principles as it does on land and in fresh water streams. For this purpose we collected a large set of samples from different venting sites within the Manus Basin covering different dilution rates of hydrothermal fluids as well as biofilms on solid surfaces.
- The third aim was to investigate the identity and function of microbial organisms populating the inactive hydrothermal chimneys. These structures represent a mineralized storage of reduced iron and sulfur which could be potentially used my microorganisms for energy generation. At the Manus Basin hydrothermal field we collected poly-metal sulfide deposits not exposed to visible venting in order to identify the key players in the hosted microbial assemblages.
- Finally the fourth aim of the thesis was to study the distribution, identity and potential function of heterotrophic microorganisms in hydrothermal habitats. Until now only few studies addressed heterotrophy in hydrothermal vent systems, although remineralization of organic matter is an important part of carbon cycling in every ecosystem. At the Menez Gwen hydrothermal field, we conducted a spatially resolved sampling of diffuse venting orifices and their immediate surroundings in search for areas of high abundance of heterotrophic microorganisms.

Chapter II

Niche partitioning of diverse sulfur-oxidizing bacteria at hydrothermal vents

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Manuscript in preparation

Contributions:

D.M, P.P. and A.M. developed concepts and ideas. P.P., A.M. and W.B. collected samples at the PACManus and SuSu Knolls hydrothermal fields. D.M. performed experiments and data analysis, conceived and wrote the manuscript. P.P. performed experiments on samples of solid surfaces. S.H. performed geochemical measurements during sampling. C.V., S.H. and P.R.G. assisted with ISMS raw-data analysis. C.V. ran gas concentrations calculation based on ISMS data. W.B. assisted in modeling and thermodynamic analysis. P.P., W.B., S.H., P.R.G., C.V., R.A., and A.M. conceived and edited the manuscript.

Niche partitioning of diverse sulfur-oxidizing bacteria at hydrothermal vents

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Running title: Niches and microdiversity of sulfur-oxidizing bacteria

Keywords: diffuse fluids, geochemical gradients, metagenomes, targeted assembly, 16S rRNA

Summary

In the dark ocean primary production is carried out by chemolithoautotrophic microorganisms, with the oxidation of reduced sulfur compounds being a major driver for microbial carbon fixation. At highly sulfidic hydrothermal fields a variety of microorganisms oxidizing sulfur compounds can be observed in high abundance. Yet, the principles of niche differentiation and distribution of the different sulfur oxidizing prokaryotes (SOP) across geochemical gradients remain poorly understood.

Here we addressed niche differentiation of SOP by extensive sampling of active sulfidic vents at the hydrothermal fields of the Manus Basin, off Papua New Guinea. For this study, we collected 33 diffuse fluid and rising plume samples as well as 23 samples from surfaces of chimneys, rocks and biota from six different venting sites. Based on our detailed analyses of 16S RNA gene sequences, metagenomes and real-time *in situ* measured geochemical parameters, we are now able to describe the distribution and potential niches of the highly diverse *Epsilonproteobacteria* genera *Sulfurimonas* and *Sulfurovum* and the rather uniform SUP05-clade *Gammaproteobacteria* within the geochemical mixing gradient. While *Sulfurovum* was found mainly attached to surfaces exposed to diffuse venting, SUP05 was found as planktonic in areas of high fluid dilution. We further propose that the high diversity within *Sulfurimonas* and *Sulfurovum* related bacteria observed in this study derives from the high variation of environmental parameters like sulfide concentrations across small spatial and temporal scales within their niche.

Introduction

Reduced sulfur compounds are widely distributed in the environment and sulfur oxidation is one of the most ancient microbial metabolisms (reviewed in Canfield and Raiswell, 1999). The long evolutionary history of sulfur oxidation is reflected in high diversity of sulfur oxidizing prokaryotes (SOP) which inhabit many different environments (reviewed in Canfield and Raiswell, 1999; Friedrich et al., 2005). In aphotic ecosystems, such as the deep sea or terrestrial cave systems, chemolithotrophic SOP are often the main primary producers (e.g. Jannasch and Wirsen, 1979; Engel et al., 2003; Nakagawa et al., 2005; Grote et al., 2008). To successfully co-exist, SOP have adapted to different ecological niches, commonly defined by environmental factors such as pH, temperature, salinity, light availability and substrate concentrations. In some environments (e.g. anoxic water column, sulfidic cave systems, and sulfur-oxidizing microbial mats) the mechanisms of niche partitioning between SOP are rather well understood (Jørgensen and Revsbech, 1983; Jørgensen and Des Marais, 1986; Macalady et al., 2008; Grunke et al., 2011; Headd and Engel, 2013). At hydrothermal vent sites, a systematic study investigating niche-partitioning of SOP is still missing.

Chemolithotrophic SOP are ubiquitous in hydrothermal environments. They can be found as free-living microorganisms, but also as ecto- and endosymbionts of vent fauna (reviewed in Nakagawa and Takai, 2008). Key sulfur oxidizers at hydrothermal vent sites are the *Epsilonproteobacteria* and the *Gammaproteobacteria* (reviewed in Sievert et al., 2008a) while sulfur oxidizing *Aquificae* only occupy a narrow thermophilic niche (Reysenbach, 2001; Alain et al., 2003; Hugler et al., 2007), and sulfur oxidizing *Archaea* (order *Sulfolobales*) are generally rare in the marine environment (reviewed in Friedrich et al., 2005). Both, cultivation-dependent and -independent studies show that *Sulfurovum*- and *Sulfurimonas*-related (SVr and SMr) species are the most dominant and widespread SOP in hydrothermal environments (Inagaki et al., 2003; Lopez-Garcia et al., 2003; Inagaki et al., 2004; Nakagawa et al., 2005; Meyer et al., 2013). The most prominent gammaproteobacterial sulfur oxidizers are giant mat forming sulfur oxidizing bacteria, such as *Beggiatoa* or filamentous *Thiomicrospira* species (Jannasch et al., 1985; Jannasch et al., 1989; Takai et al., 2004; Brazelton and Baross, 2010) and SUP05-clade bacteria, known from hydrothermal plumes, oxygen minimum zones, and symbioses with vent fauna (Sunamura et al., 2004; Duperron et al., 2005; Lesniewski et al., 2012; Anderson et al., 2013; Glaubitz et al., 2013; Marshall and Morris, 2013).

Culture independent studies at hydrothermal vents and other marine sulfidic environments found SUP05 often as co-occurring with *Epsilonproteobacteria* (Sunamura et al., 2004; Labrenz et al., 2007; Bourbonnais et al., 2012; Sheik et al., 2015). First hypotheses suggest a niche separation between these two groups based on sulfur/oxygen ratio (Schmidtova et al., 2009; Grote et al., 2012; Anderson et al., 2013) analogous to niche differentiation of gamma- and epsilonproteobacterial sulfur oxidizers in cave systems and sulfidic springs (Macalady et al., 2008; Headd and Engel, 2013).

At hydrothermal vents niche separation of SOP would have to occur within steep physico-chemical gradients (Baross and Hoffman, 1985). At sites of focused discharge, hot hydrothermal fluids (up to 400°C) enriched in reduced compounds such as sulfide, hydrogen, methane, ferrous iron, also referred to as geofuels (Bach et al., 2006) gush into cold oxygenated seawater. Thereby, a turbulent mixing zone with extreme gradients is formed (Tivey, 2004). Microbial life is mainly found on hydrothermal chimneys formed by precipitation of metal-sulfides (Harmsen et al., 1997; McCollom and Shock, 1997; Flores et al., 2011; Reeves et al., 2014) or in areas of diffuse venting (McCollom and Shock, 1997; Amend et al., 2011; Bemis et al., 2012; Meyer et al., 2013), where hydrothermal fluids and seawater mix already within the ocean crust or sediment deposits (reviewed in Bemis et al., 2012). Temperatures of diffusely venting fluids are more moderate, allowing microorganisms to thrive in subsurface chambers, at orifices of fluid emission and in the fluids themselves (reviewed in Orcutt et al., 2011).

Here we tested to what extent does niche partitioning of SOP occur in these turbulent and complex environments and investigated the factors driving it. Hydrothermal fields of the Manus Basin with their highly sulfidic fluids, at the same time poor or depleted of other energy sources like methane or hydrogen offer this variety (Scott and Binns, 1995; Reeves et al., 2011b; Yeats et al., 2014; McDermott et al., 2015). We collected a for hydrothermal studies unprecedented dataset of 56 samples from six different venting sites, among them 33 samples from diffuse venting fluids and hydrothermal plumes and 23 samples from solid surfaces, such as chimney structures and shells of vent fauna. Extensive molecular diversity and function analysis of the sampled microbial communities was correlated with real-time geochemical data. As a result, we are able to assign tentative niches to key SOP populations and phrase a hypothesis on diversification patterns among closely related epsilonproteobacterial SOP species.

Materials and methods

Site description and sample collection

Samples consisting of fluids, rocks, hydrothermal chimneys, and vent fauna were collected during R/V Sonne expedition SO-216 in June/July 2011 to the Manus Basin (Bismarck Sea, Papua New Guinea), a back-arc fast spreading center located between New Britain and New Ireland in the Bismarck Sea (Tab S1). Its basaltic to intermediate and felsic lavas generate vigorous venting of sulfidic fluids with varying properties (Binns and Scott, 1993; Reeves et al., 2011a). Venting sites sampled in this study are located at PACManus and SuSu Knolls hydrothermal fields, at a depth of 1150 – 1775 m (Fig. S1, Tab. S1). Fluid samples were collected with the remotely controlled flow-through system KIPS (Kiel Pumping System; Schmidt et al., 2007) mounted on the remotely operated vehicle (ROV) *Quest* (Marum, Bremen). Samples for metagenome sequencing were collected by pumping fluids directly onto 142 mm diameter cellulose-acetate (CA) or polyethersulfone (PES) membrane filters (0.22 µm pore size, Millipore, Darmstadt, Germany). Collection time ranged between 13 and 33 minutes. Additionally, fluid samples were collected into 675 ml flasks (Savillex, Eden Prairie, MN, USA). These were prefilled with deionized water, which was exchanged for ambient seawater during ROV descent. At the fluid sampling sites, flask volume was exchanged with sample volume at least 4 times (3 min pumping at a rate of 1 L min-1) prior to sample collection. Temperature and pH of all sampled fluids was recorded with in-line sensors attached to the KIPS sampling nozzle (Tab S1). An inlet of an *in situ* mass spectrometer (ISMS) was attached parallel to the KIPS nozzle in order to record gas concentrations in real-time with (Tab. S1).

Rock, hydrothermal chimney and macrofauna samples were collected with the ROV's hydraulic arm and kept in closed bio-boxes during ROV ascent. Samples of hydrothermal plume were taken collected in Niskin bottles attached to a CTD-rosette. Directly after shipboard retrieval, *in situ* collected CA and PES membrane filter were transferred to -80°C. Fluids collected in flasks were passed through PES membrane filters (0.22 µm pore size) and the filters were stored at -20°C. Retrieved rocks and hydrothermal chimney structures were subsampled and directly frozen at -20°C for DNA extraction.

Thermodynamic calculations

Gibb's free energies available from one mol of substrate were calculated as described in Meier *et al.* (2016) using concentrations measured with the ISMS instead of activities. To determine the energy available per kg of fluid-water mix, calculated Gibbs' free energies were multiplied by concentration of the limiting compound of the reaction.
Modeling of the mixing gradient was performed with the REACT module of the Geochemist's Workbench software (Aqueous Solutions LLC, Champaign, IL), using the thermodynamic database of Amend *et al.* (2011) and endmember values for the Fenway vent as published in Reeves *et al.* (2011b).

16S rRNA gene sequencing and analysis

DNA was extracted from ca. 1.5 x 1.5 cm membrane filter pieces or 0.5 – 1 g of crumbled solid material (chimney pieces and fauna shells) as described previously (Meier et al., 2016), with an additional 1 h Proteinase K digestion (80 µg/ml final concentration) step at 37°C and a 2 h incubation at 65°C after addition of SDS containing buffer S1 (MO BIO Laboratories, Carlsbad, CA, USA) prior to applying the kit protocol. The V3-V4 region of the 16S rRNA gene was amplified as described previously (Meier et al., 2016). The amplicons were sequenced on an Illumina MiSeq sequencer at the Max Planck Genome Centre (Cologne, Germany). After trimming of 3'-ends with quality below q10, paired-end reads were merged using BBmerge (BBmap package v.33.57, http://sourceforge.net/projects/bbmap/) with a minimum overlap of 50 bp.
Full-length 16S rRNA genes were amplified using the GM3F and GM4R primer set (Muyzer et al., 1995) and sequenced on a Pacific Biosciences RSII sequencer in circular consensus mode at the Planck Genome Centre (Cologne, Germany).

Reads were de-multiplexed and randomly subsampled to 5000 reads per sample using Mothur v.1.34 (Schloss et al., 2009). Reads of the whole dataset were decomposed into "nodes" by MED v2.0 (Eren et al., 2015) with 4 discriminant locations and minimum substantive abundance (count of the most abundant sequence in a node) of 3. Finally, percentage similarity independent operational taxonomic units (OTUs) were generated based on representative sequences of MED nodes (sequence with the highest number of copies, ca. 70-80% of all reads, in a node) using SWARM (Mahe et al., 2015). Basically, each sequence in an OTU is either identical or differs by only one position to at least one another sequence within an OTU. Any sequence in an OTU

differs by two or more positions to any sequence in other OTUs (Mahe et al., 2014, 2015). SWARM was run with the “fastidious” option and 20 as the number of sequences in a node for it to be considered “big” (otherwise default parameters).

Full length 16S rRNA sequences obtained by PacBio sequencing and PhyloFlash reconstruction were quality trimmed with Mothur v 1.34 as follows: in a sliding window of 10 bp the average quality should remain above q21 and never fall below q10. Otherwise, the sequence was trimmed at this point. After trimming only sequences over 1000 bp were kept. Subsequently sequences were clustered with vsearch v.1.9.10 (github.com/torognes/vsearch) at 94.5% minimum sequence identity level corresponding to a genus-level cut-off according to Yarza *et al.* (2014).

Metagenome sequencing and assembly

High molecular weight genomic DNA for metagenomic analysis was extracted from a quarter of a 142 mm diameter CA or PES membrane filter as well as from rock and hydrothermal chimney samples following the same protocol used to extract DNA for 16S rRNA amplicon sequencing. The genomic DNA was shotgun sequenced on an Illumina HiSeq2500 sequencer at the Max Planck Genome Centre (Cologne, Germany) after library construction using the Ovation Ultralow Library system kit (NuGen, San Carlos CA, USA) (15 cycles of amplification).

Bulk assembly of the metagenomes as well as reconstruction of full length 16S rRNA reads was performed as described previously (Meier et al., 2016). K-mer depth for read normalization was adjusted to 40 according to the number of reads obtained.

Statistical analysis

All statistical analyses were performed in R using the “vegan” package (Oksanen et al., 2013). Permutational multi-variate analysis of variance (perMANOVA) (Anderson, 2001) was performed with the “adonis” function. Distance based redundancy analysis was performed with the “capscale” function. “Simper” function was used for the similarity percentages breakdown analysis.

Targeted re-assembly of metagenomic bins

Binning of the metagenomes based on differential coverage, tetranucleotide frequencies, taxonomic classification, paired end read mapping and conserved single-copy genes profiles was performed using the Metawatt binning software (version 3.5.2) (Strous et al.,

2012). Targeted de-novo assemblies of bins of interest were performed with the SPAdes assembler V3.1.1 (Bankevich et al., 2012) as described in (Meier et al., 2016) with 3 re-assembly rounds per bin. The generated assemblies were automatically annotated with the standard RAST annotation pipeline (Aziz et al., 2008) and loaded into the GenDB (Meyer et al., 2003) annotation system for comparative analyses using the JCoast frontend (Richter et al., 2008). Completeness and quality of final assemblies was assessed by CheckM (Parks et al., 2015) using the translated protein sequences exported from RAST and a *Proteobacteria* specific set of single copy marker genes. Average nucleotide identities between the assemblies and to the next sequenced relative were calculated with JSpeciesWS web service (Richter et al., 2015).

The annotation of selected genes, referred to in this study, was manually inspected and, if necessary, curated: results of RAST annotations were compared to hidden Markov model based HMMER3 (Eddy, 2011) searches against the Pfam-A database (Finn et al., 2014) and BLASTP searches against the NCBI-Nr database.

Orthologous proteins among the SOP genomes were identified by BLAST and OrthoMCL (Li et al., 2003) based FastOrtho tool (http://enews.patricbrc.org/fastortho/) with minimum percent identity set to 10%, minimum of matching amino acids to 20, and otherwise default settings.

Phylogenetic tree construction

Translated SoxY genes identified on contigs of the bins and in the bulk metagenomes were used to construct phylogenetic trees together with SoxY sequences from the UniprotKB database (Magrane and Consortium, 2011) including sequences from isolates of confirmed sulfur oxidizers. Protein sequences were aligned with MAFFT (Katoh and Standley, 2013), using the L-INS-I method and the Blosum62 scoring matrix.

A concatenated alignment of 138 conserved single copy genes was generated with HMMER3 (Eddy, 2011) implemented in CheckM (Parks et al., 2015).

16S rRNA sequences were aligned by SINA (v. 1.3.0, Pruesse et al., 2012) to a curated SILVA SSU123 NR99 database, where all sequences with a pintail value below 50 and alignment quality below 70 were removed. PacBio and PhyloFlash sequences longer than 1200 bp and together with high quality (>95) clade representative sequences from the SILVA database were used for tree calculations. Shorter metagenomic 16S rRNA

sequences and OTU-representative Illumina amplicon sequences were added to the calculated trees based on maximum parsimony in ARB (Ludwig et al., 2004).
Trees were calculated with various algorithms: neighbor-joining (Ludwig et al., 2004), PhyML (v. 3.1, Guindon et al., 2010), RaxML (v. 8.0.26, Stamatakis, 2014), and FastTree (v. 2.1.9, Price et al., 2009, 2010) to check the stability of basic topology. Position conservation filters of 20%, 25%, and 30% for proteins and 30%, 40%, 50%, and bacterial position variability filter of SILVA for 16S sequences were tested. Representative protein trees shown in this study were calculated with PhyML based on alignment positions conserved in at least 25% of the sequences. Phylogenetic tree of 16S rRNA genes shown was calculated with FastTree using positions conserved in 50% of the sequences. Multifurcations were assigned for branches with less than 50% support or branches shorter than 0.005 changes / base.

Nucleotide sequence accession numbers

All sequence data will be submitted to the European Nucleotide Archive and made public by the release of this study.

Results

Diversity and distribution of SOP in the Manus Basin

To assess total bacterial diversity, we performed high throughput 16S rRNA gene amplicon sequencing and data analysis by minimum entropy decomposition (MED, Eren et al., 2015) and SWARM (Mahe et al., 2015). MED generated a total of 9281 “nodes” for the whole dataset, which were further clustered into 1307 operational taxonomic units (OTUs) with SWARM.

Hierarchical clustering of samples according to their microbial community composition showed strong patterns based on whether the samples came from a solid surface or a fluid sample (Fig. 1). In contrast, significant clustering based on the venting site was not observed. Sequences affiliating with known SOP were present in all analyzed samples. However, different SOP occurred and dominated in different environments and sample types. A non-parametric permutational multivariate analysis of variance (perMANOVA) revealed that 30% of the community composition variance could be explained by the sample category alone (“fluid”, “plume”, “rock surface”, “fauna surface”, p=0.0001). All solid surface samples were dominated by sequences affiliated with the epsilonproteobacterial genus *Sulfurovum*, accounting on average for 30% (2 - 74%) of all reads (Fig. 1). Additionally, other sequences classified as sulfur-oxidizing epsilonproteobacterial genera and various uncultured and thiotrophic gammaproteobacterial clades were present (e.g. *Sulfurimonas*: 0 - 58%, 10% on average, *Nitratifractor*: 0 - 27%, 4% on average). *Sulfurovum*-related (SVr) and *Sulfurimonas*-related (SMr) sequences were also present in all collected fluid samples, but in lower relative abundance (on average 24%, compared to 41% on solid surfaces) and with lower proportion of SVr reads (0 - 47%, on average 11%) in comparison to solid surface samples (2 - 74%, 30% on average). Few fluid samples also showed elevated proportions of 16S rRNA sequences affiliated with the phylum *Aquificeae* harboring thermophilic SOP (over 1% in 13 of 33 fluid samples, 22% max.). Sequences related to SUP05-clade *Gammaproteobacteria* were found almost exclusively in fluid samples, with relative sequence abundances reaching up to 58% (15% on average, 1% min.) (Fig. 1). In 10 of 23 surface samples SUP05 sequences were completely absent, and in the remaining they stayed below 1%.

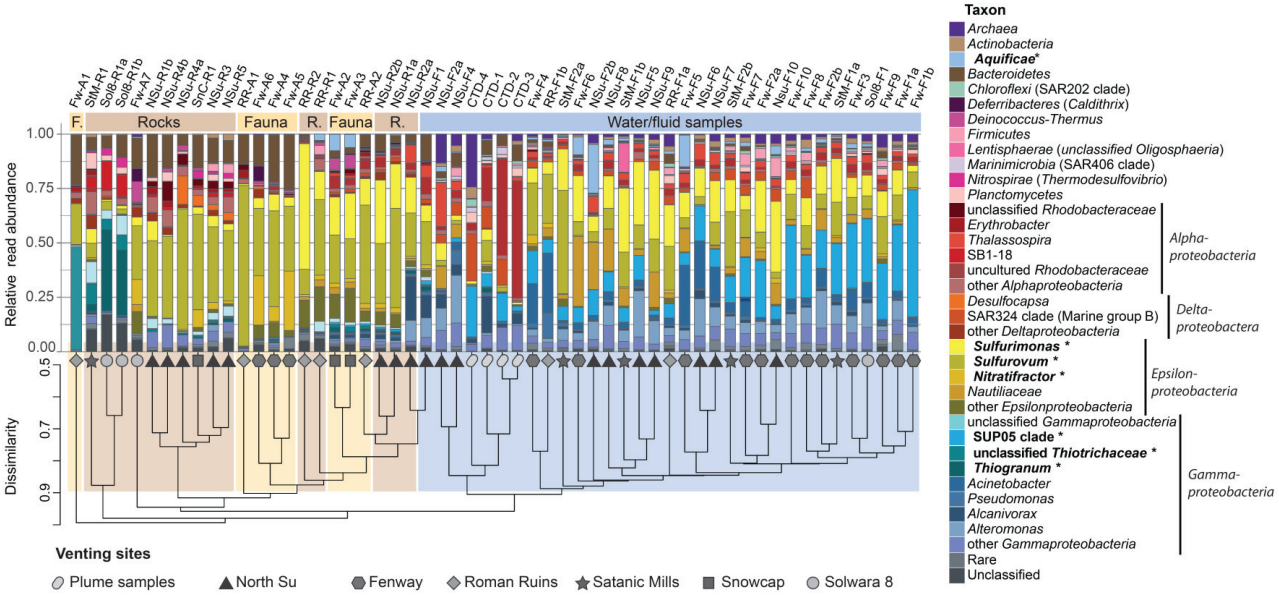

Figure 1: Relative abundances of 16S rRNA sequence reads according to their classification. Putative SOP are denoted in bold and marked with a "*". The cluster dendrogram depicts the average linkage hierarchical clustering based on a Bray-Curtis dissimilarity matrix of community compositions resolved down to MED node level.

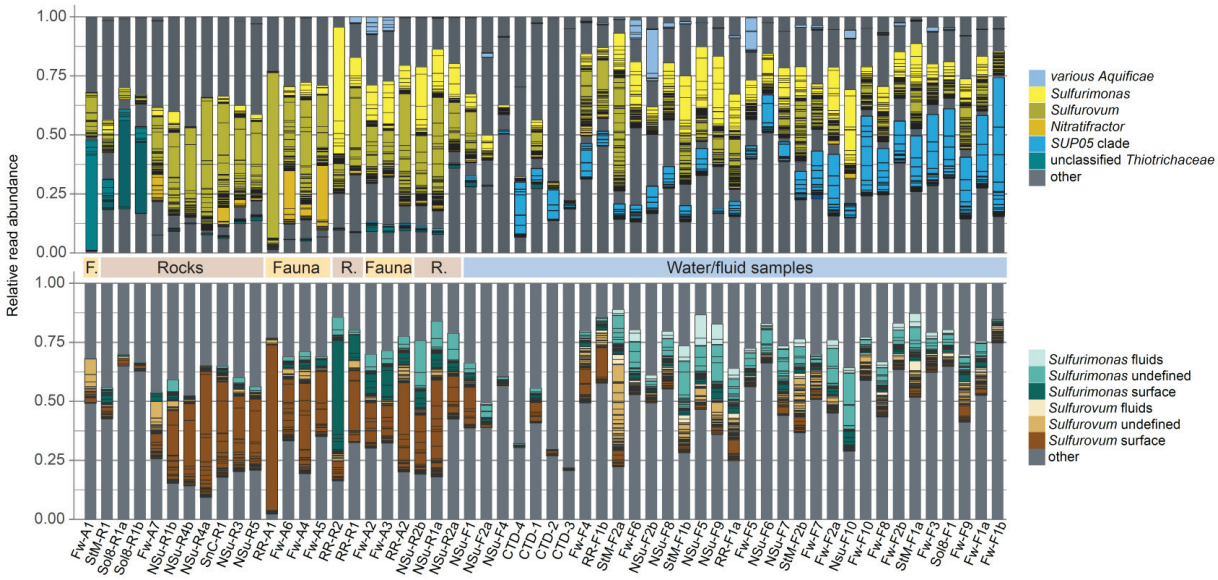

Figure 2: Upper panel: Distribution of 16S rRNA gene sequences of putatively sulfur oxidizing *Bacteria*. Horizontal lines dividing the bars indicate OTUs generated by SWARM. Lower panel: SVr and SMr OTUs in respect to their specificity for fluid or solid surface samples. The categories are assigned based on i) SIMPER p-values indicating if an OTU is contributing significantly to the difference between surface and fluid samples and ii) average abundances in respective sample category: “undefined”: $p > 0.05$, “fluids”: $p < 0.05$ & average abundance higher in fluids, “surface”: $p < 0.05$ & average abundance higher on surfaces.

A similarity percentages breakdown (SIMPER) calculated based on the relative abundances of OTUs in relation to sample category revealed that most SVr and SMr OTUs were significantly contributing to the overall community composition difference between fluids and surfaces samples ($p < 0.05$; Fig. 2). Most of these significantly differently abundant OTUs had higher average abundance in the surface than in the fluid samples (Fig. 2).

The diversity of 16S rRNA sequences the three most abundant SOP populations, SVr, SMr and the SUP05-clade differed significantly (Fig. S2). SVr sequence reads exhibited the highest level of diversity with 1602 nodes generated by MED and 149 OTUs generated by SWARM. SMr was the second most diverse group (1027 MED nodes, 99 OTUs). SUP05, showed comparatively low diversity (515 MED nodes, 24 OTUs), despite high relative abundances in fluid samples (15% on average, 58% max.). In addition, almost full length 16S rRNA gene sequences retrieved by PacBio amplicon sequencing and targeted 16S rRNA gene reconstruction from metagenomes confirmed the trends emerging from short read analyses. Clustering of the long 16S rRNA gene sequences by percentage identity at genus level (Yarza et al., 2014) resulted in a number of clusters slightly below the number of OTUs generated by SWARM from short amplicon reads. Again, SVr sequences were more diverse (100) than SMr (85), while SUP05-clade sequences (20) were the least diverse. A phylogenetic tree calculated based on the full-length sequences and relevant epsilonproteobacterial sequences from the SILVA SSU123 database showed that sequences classified as *Sulfurovum* and *Sulfurimonas* form two distinct monophyletic branches with several sub-clades each (Fig. S3). However, the branching of these sub-clades within *Sulfurovum* and *Sulfurimonas clades* clade was instable between different tree calculations.

Niche partitioning along an environmental gradient

During fluid sampling, in-line temperature and pH probes as well as *in situ* mass spectrometry (ISMS) were used to record the geochemical conditions the microbiota was exposed to at the time of collection. Therefore, a comprehensive set of physico-chemical *in situ* parameters is available for the majority of diffuse fluid samples (21/29) (Fig. 3). Pearson and Spearman's correlation indices show that most of the recorded parameters exhibit a strong pairwise covariance, while pH and oxygen concentration show a lesser degree of relation to other parameters (Fig. 3, Table S2).

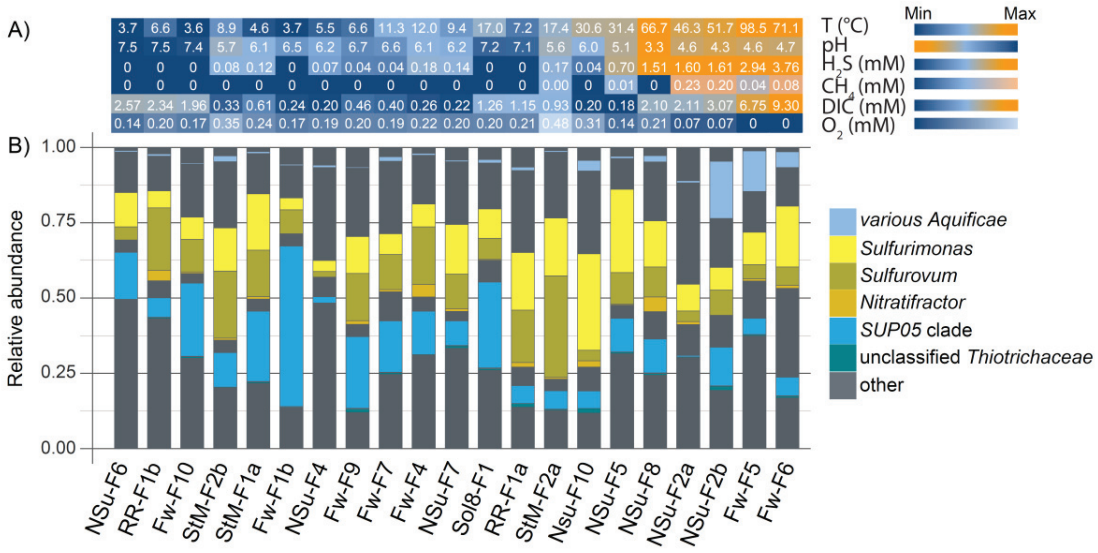

Figure 3: A) *In situ* determined geochemical parameters sorted from cold diluted to more hot and concentrated hydrothermal fluids (left to right) based on sulfide concentration and temperature. B) Distribution of putative SOB genera based on 16S rRNA gene amplicon sequences in diffuse fluid samples.

SOB clade distribution (Fig. 3) along geochemical gradients in the fluid samples revealed that SUP05-clade *Gammaproteobacteria* preferentially inhabit sulfide poor, low temperature fluids. *Aquificae* related sequences were most frequent in hot, sulfide-rich fluids. SMr and SVr *Epsilonproteobacteria* accounted for a substantial fraction of SOP 16S rRNA genes in all fluids, but dominated in the mixing zone, characterized by considerable sulfide concentrations (0.1-1.0 mM), oxygen availability (>0.1 mM) and moderate fluid temperatures (~10-30°C). Distance based redundancy analysis (dbRDA) indicated a response of chemolithotrophic microbial clades such as *Nautiliaceae* and SMr/SVr *Epsilonproteobacteria*, *Aquificae*, and SUP05 to changes of the recorded environmental parameters (Fig. 4). A perMANOVA further confirmed that the position of the sample within the geochemical mixing gradient (temperature as proxy) could explain 15% of the community composition variance on genus level (p=0.0003).

Particularly notable was the difference in response that SVr and SMr microorganisms showed towards oxygen availability (Fig. 4). While the response of SMr to changes in oxygen concentration was only minute, increasing oxygen concentration had a strong positive effect on the relative abundance of SVr microorganisms. A perMANOVA test confirmed the significant impact of oxygen concentration (p=0.02) and showed relative

abundances of SVr species to be the most positively affected by increasing oxygen concentrations. Apart from a correlation to the position in the gradient, we also checked for correlation with Gibbs' free energies available from sulfide oxidation per kg fluid-water mix at a given sampling point (Table S1). However, no significant correlation between community composition at any level and the Gibbs' free energies was observed.

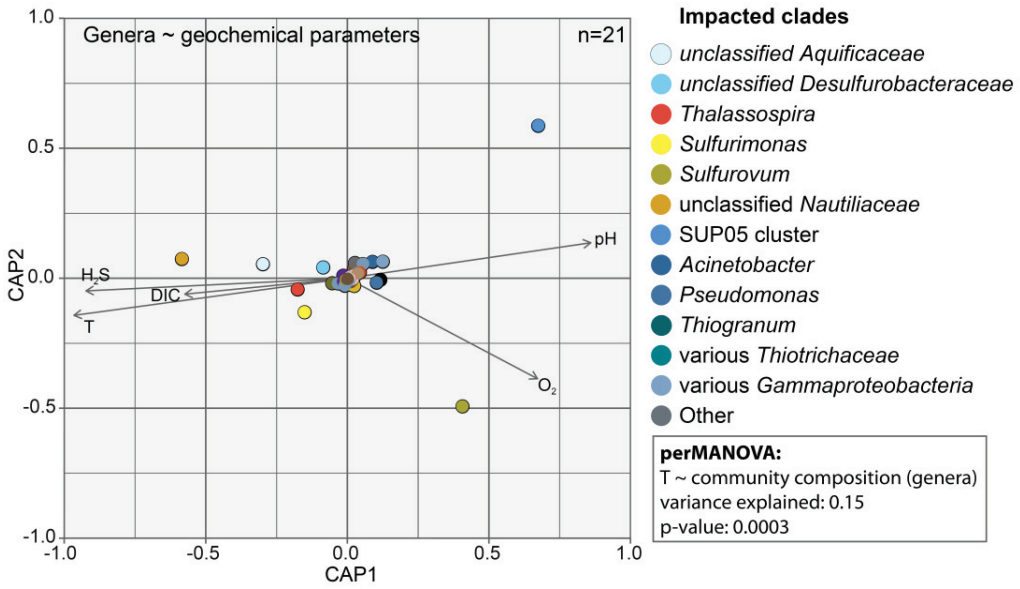

Figure 4: Distance based Redundancy Analysis (dbRDA) calculated based on a Bray-Curtis dissimilarity matrix and standardized, log-normalized geochemical parameters. Distance matrix calculated based on relative abundances of microbial genera in 21 fluid samples with geochemical data. Results of a non-parametric permutational multivariate analysis of variance (perMANOVA) are stated in the frame adjacent to the dbRDA panel. PerMANOVA was calculated using the "adonis" function of the "vegan" package in R (Oksanen et al., 2013).

Genomic variability among hydrothermal vent SOB

We sequenced and analyzed the metagenomes of the samples NSu-F2b, NSu-F5, Fw-F1b, Fw-F3 and RR-F1B (Fig. S4). The NSu-F2b metagenome was obtained from a 52°C hot acidic fluid (pH = 4.3), with high sulfide (1.6 mM H_2S) and low oxygen (0.07 mM) concentrations. The NSu-F5 metagenome originates from a more diffuse fluid sample from the same vent site (T = 31°C, pH = 5.1, 0.7 mM H_2S, 0.14 mM O_2). The Fw-F1b, Fw-F3 and RR-F1B metagenomes originate from diffuse venting sites with high fauna colonization (T = 3.7 - 6.6°C, pH = 6.5 - 7.5, no detectable H_2S and 0.17 - 0.2 mM

O_2). By multi-criteria binning and targeted re-assembly we could obtain 28 bins from the three target groups (11 *Sulfurovum*-related [SV], 5 *Sulfurimonas*-related [SM], 12 SUP05-clade; Fig. S5). Read mapping of the five metagenomes to the bins showed the occurrence and distribution pattern of the epsilonproteobacterial *SOP* to be relatively diverse (Fig. S5). Some SMr and SVr bins appeared only in one sample, others in two or more (e.g. SV-5 only in NSu-F5, SV-4 in NSu-F2b and NSu-F5, SV-9 in NSu-F2b, NSu-F5, Fw-F1b, and Fw-F3). Most of them had the highest read coverage in the NSu-F2b or NSu-F5 samples. The bins of the SUP05-clade bacteria, in contrast, showed a very synchronized distribution pattern. All genomes classified as free-living SUP05-clade bacteria were present in NSu-F5, Fw-F1b, Fw-F3, and RR-F1b and were most abundant in the RR-F1B metagenome, with the exception of SUP05-5 which almost exclusively appeared in the NSu-F5 metagenome. The three SUP05-clade bins classified as sulfur-oxidizing symbionts were all most abundant in the Fw-F3 sample (Fig. S4).

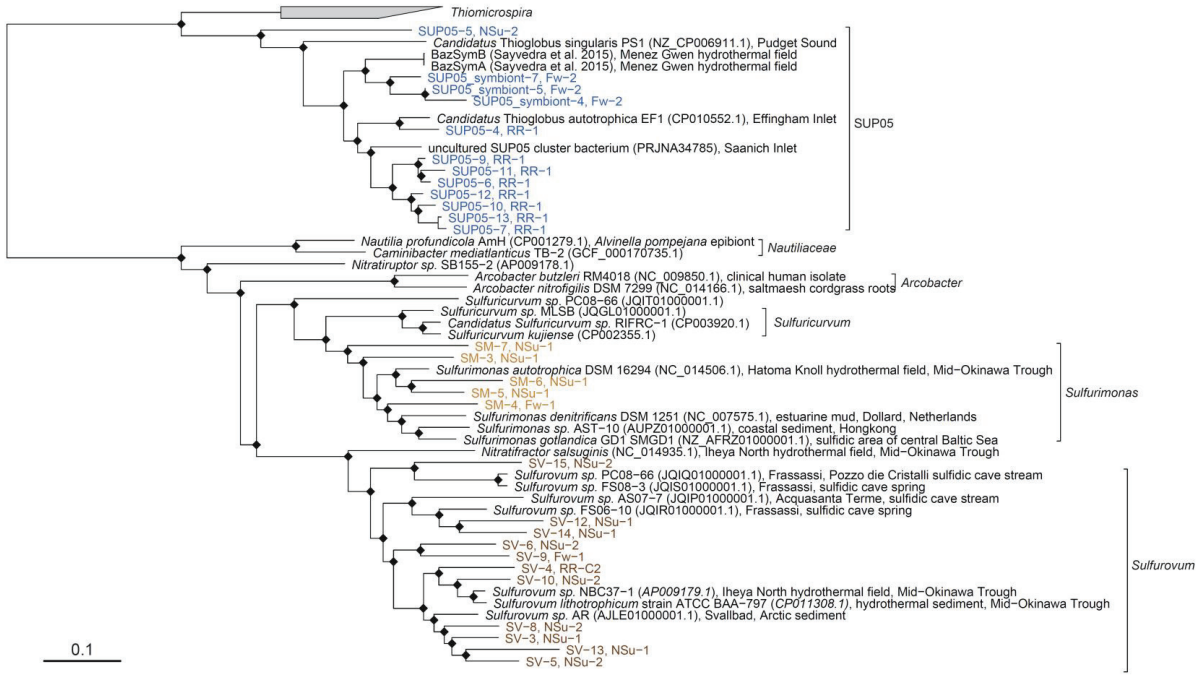

Figure 5: A phylogenomic tree of the retrieved epsilonproteobacterial and gammaproteobacterial bins (in color) together with publicly available genome sequences (in black). The tree was calculated with PhyML, based on a concatenated alignment of 138 conserved single-copy genes (Campbell et al., 2013). Black diamonds indicate Bayesian approximate branch support above 70%.

The patterns of high 16S rRNA gene diversity among SVr/SMr and low diversity within the SUP05-clade were also reflected in the (meta-)genomic data. A phylogenomic tree based on a concatenated alignment of 138 single-copy marker genes (Campbell et al., 2013) from all 28 SOP bins and complete reference genomes showed that the phylogenetic distances between the bins/genomes of different *Sulfurovum* and *Sulfurimonas* species were larger than between the SUP05-clade bins (Fig. 5). We further compared the average nucleotide identities (ANIs) between bins and between the bins and available complete genomes according to thresholds suggested by Goris *et al.* (2007), where ANI above 70% is indicative of the same genus and ANI >95% of the same species. ANI of SVr bins obtained in this study ranged between 66% and 81% (70% on average, Table S3a). SV-10 showed the highest identity to a cultured representative with 78% ANI to *Sulfurovum sp*. NBC37-1 (Nakagawa et al., 2007). The five *Sulfurimonas* bins showed a similar level of diversity, with an average ANI of 72% (68 - 76%; Table S3b). SM-5 and SM-6 exhibited the highest ANI (both 75%) to a cultured representative of the genus, *Sulfurimonas autotrophica (Sikorski et al., 2010)*. Thus, not all of the obtained SVr and SMr bins would belong to one genus. In contrast all retrieved SUP05 bins, except SUP05-5, would belong to the candidate genus Thioglobus (Marshall and Morris, 2013; Shah and Morris, 2015). Furthermore, SUP05-6 and SUP05-9 as well as SUP05-7 and SUP05-13 would represent different strains of the same species.

Finally, we compared the metabolic repertoire of all SOP bins with respect to energy generating pathways, carbon assimilation, and adaptations to environmental stress (Fig. 6). As expected, all SVr and SMr bins contained genes encoding enzymes of the reverse tricarboxylic acid (rTCA) cycle (ATP-citrate lyase, 2-oxoglutarate synthase, and fumarate reductase), while the SUP05-clade genomes harbor marker genes of the Calvin-Benson-Bassham (CBB) cycle (e.g. RUBISCO encoding *cbb* genes). The sulfur oxidation multi-enzyme complex (SOX), terminal cytochrome c oxidases (cbb3-type) for aerobic respiration as well as respiratory nitrate reductase (Nap) genes were found in all three SOP groups. All SUP05 bins were lacking the genes encoding SoxCD. Most of the SVr bins (9/11) also encoded a complete denitrification pathway, while SMr and SUP05-clade bins were consistently lacking the *nos* genes encoding a nitrous-oxide reductase. Only SUP05-clade bins had the ammonia forming nitrite reductase (NirD/B), while SMr and SVr genomes contained the NO-producing NirS and some as well the ammonifying NirA nitrate reductase. A unique feature of the SVr and SMr genomes is the presence of

a membrane bound polysulfide reductase (subunit genes *psrABC*), indicating the potential use of polysulfides as electron acceptor.

Comparing possible adaptations to the environment, we found that SVr and SMr bins harbor a much wider range of heavy metal and oxygen stress resistance genes than SUP05-clade genomes (Fig. 6). The efflux pumps repertoire was also different between SVr and SMr bins. Another feature unique to epsilonproteobacterial SOP bins was the presence of genes for capsular polysaccharide synthesis and export, which were absent in all SUP05 bins. Chemotaxis and flagellar motility was exclusively found in SMr bins, while both SMr and SVr bins encoded genes for aerotaxis and type II and type IV pili, possibly enabling twitching motility. SUP05-clade bins contained neither pili or flagella, nor chemotaxis genes (Fig. 6).

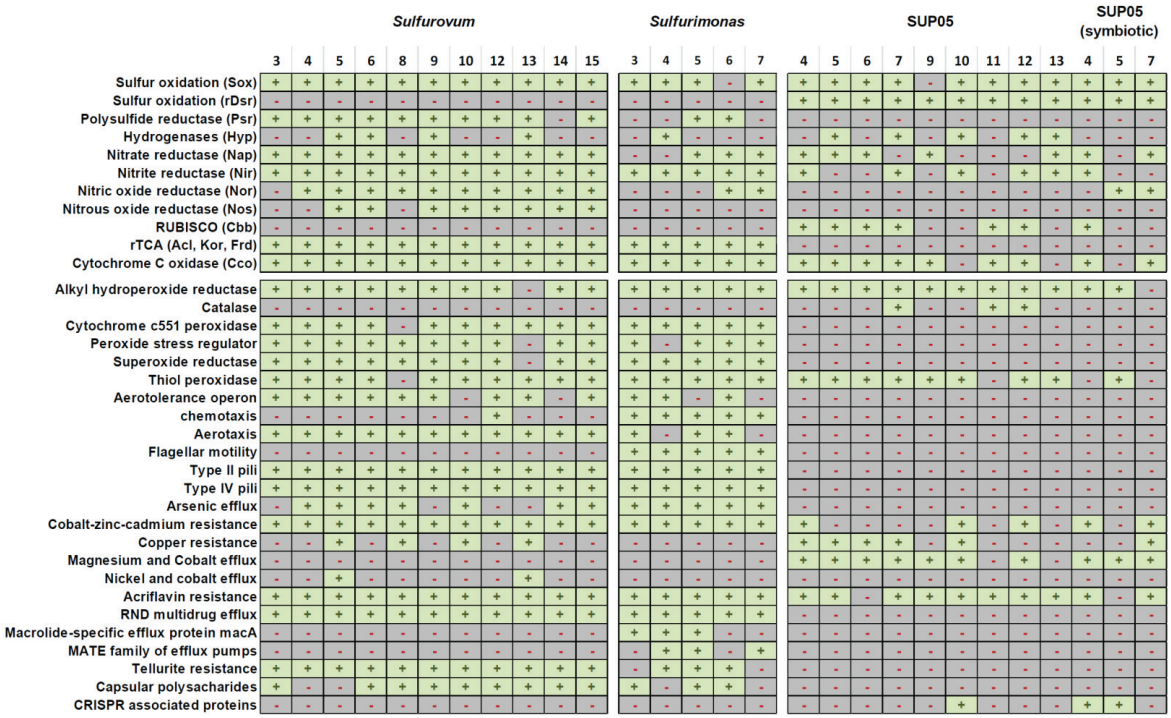

Figure 6: Comparison of metabolic capabilities with respect to energy, carbon metabolism and environmental adaptations on the re-assembled bins. Green plus indicates presence of a pathway / key enzymes, red minus indicates absence of any pathway specific enzymes.

We looked in detail into the diversity of the *soxY* gene, encoding the sulfur anion binding carrier protein essential to the SOX machinery (Friedrich et al., 2000; Quentmeier and Friedrich, 2001). In SVr and SMr bins, genes encoding the SOX system were split into two different loci (Fig. S5), with genes encoding the sulfur anion carrier protein (SoxYZ) present at both loci. A phylogenetic tree of translated SoxY protein sequences revealed that the two different SoxY proteins encoded by the SMr and SVr bins as well as SMr/SVr SoxY sequences from the bulk metagenome assemblies fall into two distinct clusters (Fig. 7). One branch contained the more conserved SoxY proteins (*Sulfurovum*: 52 - 100% similarity, *Sulfurimonas*: 68 - 95% similarity) encoded together with SoxX, Z, A, and B. The other branch contained a more diverse group of SoxY proteins (*Sulfurovum*: 21 - 95% similarity, *Sulfurimonas*: 42 - 100% similarity) encoded together with SoxZ, C, D, and H (Fig. S6). The single loci encoding the *sox* genes in the SUP05-clade bins had a homogeneous structure and all SoxY protein sequences formed a single clade of sequences (similarity: 49 – 100%), closely related to other gammaproteobacterial SoxY proteins.

A detailed analysis of the sulfur anion binding domains of the two epsilonproteobacterial SoxY clusters (Figure S7) showed that sulfur binding cysteine was conserved in all sequences, whereas the other amino acids (AAs) of the sulfur carrying “swinging arm” (Sauve et al., 2007) were only completely conserved in the first, less diverse SMr/SVr SoxY cluster. Compared to the canonical GGCGG sequence in the SoxY “swinging arm” of *Paracoccus panthotrophus* SoxY of SMr/SVr were missing one C-terminal glycine resulting in the amino acid sequence GGCG. In the second, more diverse SoxY cluster of SMr/SVr the sulfur binding cysteine was followed by a variable position most frequently occupied by glutamic acid and a rather conserved glycine (GGCEG). The five AAs preceding the “swinging arm” were completely conserved in the first SoxY cluster and variable in the second (Figure S7).

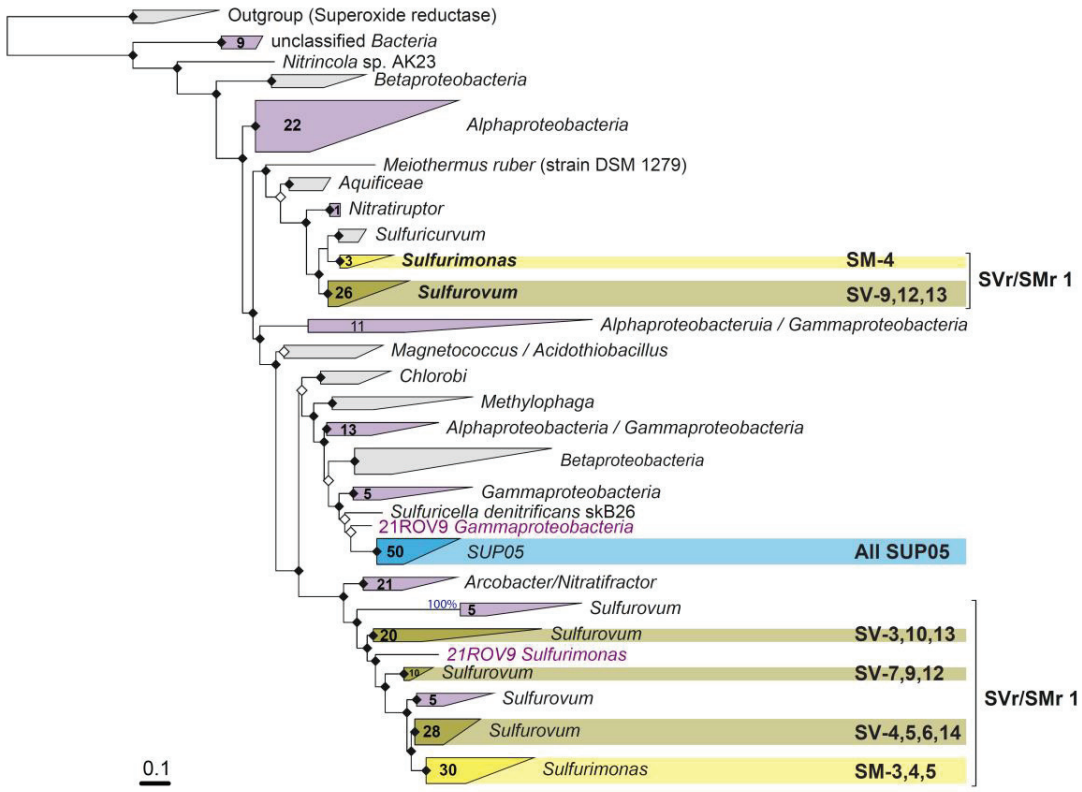

Figure 7: Maximum likelihood tree of SoxY amino acid sequences. In yellow – clusters containing epsilonproteobacterial bins, in blue – SUP05 bins, with respective bins indicated on the right. In purple – clusters containing other sequences encoded in the bulk metagenome assemblies. Numbers on the triangles indicate numbers of SoxY sequences from the bulk metagenomes assemblies contained in the cluster. The tree was calculated with PhyML based on positions conserved in at least 25% of the sequences.

Discussion

The Manus Basin hydrothermal vent system is inhabited by three major clades of SOP with different level of intra-group diversity: low-diversity SUP05-clade *Gammaproteobacteria,* more diverse *Sulfurimonas*-related *Epsilonproteobacteria* (SMr) and highly diverse *Sulfurovum*-related *Epsilonproteobacteria* (SVr). While SUP05-clade was preferentially found at cold, low-sulfide conditions, SVr and SMr SOP dominated in moderate temperature fluids with elevated sulfide concentrations.

For sediment, terrestrial, and limnic sulfidic environments, it has already been reported that succession of different SOP clades mainly occurs along geochemical gradients (Jørgensen and Revsbech, 1983; Jørgensen and Des Marais, 1986; Macalady et al., 2008; Grunke et al., 2011; Headd and Engel, 2013). In particular sulfide and oxygen concentrations have been put forward as determining factors for niche partitioning between gamma- and epsilonproteobacterial SOP (Macalady et al., 2008; Headd and Engel, 2013). In marine environments, SUP05-clade bacteria have often been found co-occurring with sulfur oxidizing *Epsilonproteobacteria* in diffuse hydrothermal venting fluids and oxyclines (Sunamura et al., 2004; Labrenz et al., 2007; Grote et al., 2008; Bourbonnais et al., 2012; Glaubitz et al., 2013; Sheik et al., 2015). However, some insights from studies of anoxic zones and hydrothermal fields suggested that a niche separation based on sulfide and oxygen concentrations may also apply to marine *Epsilonproteobacteria* and the SUP05-clade (Schmidtova et al., 2009; Grote et al., 2012; Anderson et al., 2013). For the first time we are able to statistically confirm the niche separation of SVr/SMr and SUP05-clade according to the dilution grade of the fluid based on correlation of relative sequence read abundances to geochemical data.

Previous studies of SOP at hydrothermal vents have also reported the presence and co-occurrence of multiple *Epsilonproteobacteria* genera at the same venting sites (Nakagawa et al., 2005; Opatkiewicz et al., 2009; Huber et al., 2010; Flores et al., 2012; Akerman et al., 2013). A novel finding of this study is the positive correlation between increasing oxygen concentrations and SVr sequence abundances. Despite employing the oxygen sensitive rTCA cycle for carbon fixation (Hugler et al., 2005; Campbell et al., 2006; Nakagawa and Takai, 2008), SVr species in the Manus Basin seem to thrive best at relatively high oxygen levels (ca. 200 µM, Fig. 3). The detection of genes encoding capsular polysaccharide production and export, genes for aerobic terminal oxidases, various genes involved in reducing oxidative stress, and aerotaxis related genes in

almost all of the reconstructed epsilonproteobacterial bins (Fig. 6) underline the adaptation of the sulfur-oxidizing *Epsilonproteobacteria* in the Manus Basin to oxygenated environments. It is thus intriguing that no clear correlations to oxygen concentration were observed for SMr SOB, despite a genetic repertoire similar to that of SVr. We therefore hypothesize that a different adaptation to increased oxygen concentrations may be an important mechanism for niche partitioning between SVr and SMr SOP. Additionally, the size and nature of our dataset enabled us to reveal a differentiation between surface-attached and planktonic epsilonproteobacterial SOP clades (Fig. 2). However, the few fluid specific epsilonproteobacterial OTUs could also represent subsurface species washed out by the fluids (Akerman et al., 2013; Meyer et al., 2013). The differentiation could then also be viewed as division into “surface” and “subsurface” OTUs.

When considering niche differentiation between clades, the diversity within clades should not be neglected. In this study we observed an impressive level of diversity within the SVr and SMr clades, confirming observations of previous studies (Huber et al., 2007; Huber et al., 2010; Meyer et al., 2013). The convergent results of independent short and long 16S rRNA gene analyses combined with the insights from the metagenomes strongly support that the observed diversity is not a method intrinsic artifact (Quince et al., 2009; Kunin et al., 2010).

Sulfurimonas and *Sulfurovum* are currently classified as genera. Yet, the environmental 16S rRNA gene sequences currently attributed to these genera in databases like SILVA (Quast et al., 2013), as well as SVr and SMr sequences recovered in this study, rather constitute family level clades according to identity-based thresholds suggested by Yarza *et al.* (2014). The average nucleotide identities of here recovered SVr bins support the existence of multiple, closely related genera (Konstantinidis and Tiedje, 2005; Goris et al., 2007). The SUP05-clade sequences recovered in this study, in contrast, look rather uniform. The number of 16S rRNA OTUs is low and their phylogenetic distances limited. Also the ANI of the retrieved bins is rather high. We speculate that the difference in microdiversity levels between the SUP05-clade and SVr/SMr SOP might be explained by their position in “flat” and “steep” part of the geochemical gradients occurring at hydrothermal vents (Fig. 8).

Previously, microdiversity of highly abundant organisms occupying a broad niche has been attributed either to slightly different adaptations to varying environmental conditions, such as light intensity in the case of the phototrophic *Prochlorococcus*

(Moore et al., 1998; Urbach et al., 1998), or to the differentiation of physiological roles, as in the case of hydrothermal *Methanosarcinales* biofilms (Brazelton et al., 2011). In our case, the varying concentrations of reduced sulfur compounds could be the main driver for diversification. Looking at genes encoding the sulfur anion binding SoxY protein (Quentmeier and Friedrich, 2001; Sauve et al., 2007), that was reported to be the most highly expressed gene of the SOX-complex in vent *Epsilonproteobacteria* (Dahle et al., 2013), we found two different versions in SVr and SMr bins, as well as in the bulk metagenome contigs classified as *Sulfurovum* or *Sulfurimonas*. The two different SoxY loci correspond to the ones found in *Sulfurovum* and *Sulfurimonas* isolates (Sievert et al., 2008a; Sievert et al., 2008b). Protein alignments showed that while the sulfur binding domain and five preceding amino acids are highly conserved in one version of SoxY, some of the amino acids surrounding the sulfur binding cysteine were variable in the other SoxY version (Fig. S7). These variations could represent an adaptation to differing sulfur compound concentrations which appear as a spatial gradient within pores of a hydrothermal chimney wall (Tivey, 2004; Flores et al., 2011), or as a temporal variation caused by differences in venting intensity (Fig. 8). A similar hypothesis was raised with respect to SoxB gene diversity correlating to geochemical gradients in a sulfidic spring (Headd and Engel, 2013). However, SoxB diversity reported by Headd and Engel (2013) correlates with the distribution of major sulfur oxidizing bacterial clades. In contrast, we find a high variability of the SoxY gene within the two epsilonproteobacterial clades of *Sulfurimonas* and *Sulfurovum.* Spatial and temporal variation of reduced compounds concentrations makes the habitat of SVr/SMr SOP one of “intermediate disturbance” (Conell, 1978; Huston, 1979; Flöder and Sommer, 1999; Roxburgh et al., 2004). This might be the main cause for the high diversity within the *Sulfurovum* and *Sulfurimonas* populations (Fig. 8).

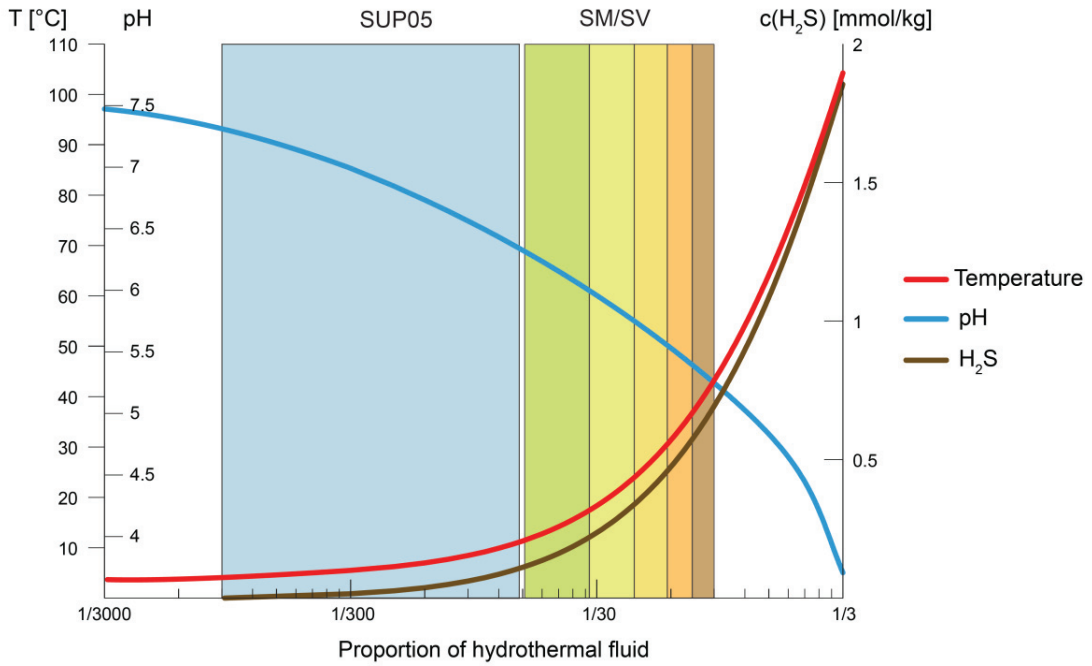

Figure 8: Schematic placement of niches of SUP05 (blue) and *Sulfurimonas* / *Sulfurovum* (SM/SV) (green to brown) in the mixing gradient. Being placed in the steep part of the gradient, *Sulfurimonas* and *Sulfurovum* are exposed to higher amplitudes of variation of environmental parameters, e.g. hydrogen-sulfide concentrations, which leads to diversification into different species, each one with its own micro-niche.

Compared to the diverse SoxY proteins of *Sulfurovum* and *Sulfurimonas*, the SoxY of SUP05-clade SOP are conserved and uniform. In accordance, we found SUP05 to be abundant in the “flat”, highly diluted end of the mixing gradient where environmental conditions show less variation (Fig. 8). We hypothesize that these stable conditions characterized, e.g., by constantly low reduced sulfur concentrations provide only limited niche space. As a consequence the diversity of SUP05 and its SoxY protein are low.

In conclusion, we have used a combination of 16S rRNA gene analyses and metagenomics to analyze the niche partitioning and diversity of SOP at hydrothermal fields. This was done in large depth and on a high number of samples characterized by advanced *in situ* physic-chemical measurements. This resulted in a significant refinement of testable hypotheses on niche adaptation of gammaproteobacterial and epsilonproteobacterial SOP. Further, we hypothesize that the high intrinsic diversity of co-occurring *Sulfurovum* and *Sulfurimonas* related species is attributed to the high variation of environmental parameters, e.g. sulfide concentrations, in the part of the

gradient inhabited by them. Steep mixing curve and temporal alterations of the fluid flow divide the overall niche of *Sulfurimonas* and *Sulfurovum* into constantly changing microniches driving diversification and preventing selection of one sub-type over the other. Systematic investigation of microbial communities in other habitats with a considerable gradient of primary energy source could help elucidate if proposed mode of diversification is universally valid for such environments.

Acknowledgements

We would like to thank officers, crew, shipboard scientific party, and the technical team of the ROV Quest 4000m (MARUM) on R/V Sonne cruise SO216, for their invaluable assistance. The cruise SO216 with R/V Sonne was an integral part of the Cluster of Excellence of the MARUM 'The Ocean in the Earth System, Research Area GB: Geosphere-Biosphere Interactions' funded by the German Research Foundation (DFG). We thank Richard Reinhard, Bruno Huettel and the team of the Max Planck Genome Centre in Cologne for sequencing and Harald Gruber-Vodicka and Hanno Teeling for help with computational analyses. Further, we thank Nicole Krombholz and Kathrin Büttner for assistance in the Molecular Ecology department. This work was supported by the Max Planck Society.

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Supplementary material

Table S1: Sampling sites and environmental parameters of collected samples

Sample name[1]	Sample type	Latitude	Longitude	depth [m]	T [°C][2]	pH[2]	H_2S [mM][3]	CH_4 [mM][3]	DIC [mM][3]	O_2 [mM][3]	$\Delta G(H_2S)$ [4]
CTD-1	water/fluid	S 03°51.480'	E 152°07.990'	1700	2.9	ND	ND	ND	ND	ND	-
CTD-2	water/fluid	S 03°51.510'	E 152°08.000'	1700	2.9	ND	ND	ND	ND	ND	-
CTD-3	water/fluid	S 03°47.002'	E 151°40.450'	1700	2.9	ND	ND	ND	ND	ND	-
CTD-4	water/fluid	S 03°47.002'	E 151°40.450'	1700	2.9	ND	ND	ND	ND	ND	-
Fw-A1	fauna surface	S 03°43.699'	E 151°40.344'	1703	ND	ND	ND	ND	ND	ND	-
Fw-A2	fauna surface	S 03°43.684'	E 151°40.159'	1647	ND	ND	ND	ND	ND	ND	-
Fw-A3	fauna surface	S 03°43.684'	E 151°40.159'	1647	ND	ND	ND	ND	ND	ND	-
Fw-A4	fauna surface	S 03°43.613'	E 151°40.321'	1692	ND	ND	ND	ND	ND	ND	-
Fw-A5	fauna surface	S 03°43.613'	E 151°40.321'	1692	ND	ND	ND	ND	ND	ND	-
Fw-A6	fauna surface	S 03°43.732'	E 151°40.327'	1715	ND	ND	ND	ND	ND	ND	-
Fw-A7	fauna surface	S 03°43.728'	E 151°40.333'	1716	ND	ND	ND	ND	ND	ND	-

Sample name[1]	Sample type	Latitude	Longitude	depth [m]	T [°C][2]	pH[2]	H_2S [mM][3]	CH_4 [mM][3]	DIC [mM][3]	O_2 [mM][3]	$\Delta G(H_2S)$ [4]
Fw-F10	water/fluid	S 03°43.697'	E 151°40.351'	1705	3.6	7.4	0	0	1.96	0.17	0
Fw-F1a	water/fluid	S 03°43.700'	E 151°40.345'	1709	6.3	7.5	ND	ND	ND	ND	-
Fw-F1b	water/fluid	S 03°43.700'	E 151°40.344'	1709	3.7	6.5	0	0	0.24	0.17	0
Fw-F2a	water/fluid	S 03°43.697'	E 151°40.343'	1707	8.2	6.4	ND	ND	ND	ND	-
Fw-F2b	water/fluid	S 03°43.698'	E 151°40.343'	1708	6	6.7	ND	ND	ND	ND	-
Fw-F3	water/fluid	S 03°43.698'	E 151°40.350'	1705	3.2	7.2	ND	ND	ND	ND	-
Fw-F4	water/fluid	S 03°43.73'	E 151°40.331'	1716	12	6.1	0.18	0	0.26	0.22	-85.68
Fw-F5	water/fluid	S 03°43.730'	E 151°40.330'	1715	98.5	4.6	2.94	0.04	6.75	0	0
Fw-F6	water/fluid	S 03°43.727'	E 151°40.329'	1715	71.1	4.7	3.76	0.08	9.3	0	0
Fw-F7	water/fluid	S 03°43.698'	E 151°40.346'	1709	11.3	6.6	0.04	0	0.4	0.19	-34.04
Fw-F8	water/fluid	S 03°43.696'	E 151°40.357'	1705	2.9	7.6	ND	ND	ND	ND	-
Fw-F9	water/fluid	S 03°43.728'	E 151°40.332'	1715	6.6	6.7	0.04	0	0.46	0.2	-35.17
NSu-F1	water/fluid	S 03°48.084'	E 152°06.110'	1225	3.4	7.1	ND	ND	ND	ND	-
Nsu-F10	water/fluid	S 03°48.038'	E 152°06.017'	1218	30.6	6	0.04	0	0.2	0.31	-29.09

Sample name[1]	Sample type	Latitude	Longitude	depth [m]	T [°C][2]	pH[2]	H_2S [mM][3]	CH_4 [mM][3]	DIC [mM][3]	O_2 [mM][3]	$\Delta G(H_2S)$ [4]
NSu-F2a	water/fluid	S 03°47.995'	E 152°06.052'	1154	46.3	4.6	1.6	0.23	2.11	0.07	-26.7
NSu-F2b	water/fluid	S 03°47.995'	E 152°06.052'	1155	51.7	4.3	1.61	0.2	3.07	0.07	-27.91
NSu-F4	water/fluid	S 03°47.996'	E 152°06.052'	1155	5.5	6.2	0.07	0	0.2	0.19	-52.08
NSu-F5	water/fluid	S 03°47.955'	E 152°06.080'	1199	31.4	5.1	0.7	0.01	0.18	0.14	-53.34
NSu-F6	water/fluid	S 03°48.042'	E 152°06.090'	1220	3.7	7.5	0	0	2.57	0.14	0
NSu-F6	water/fluid	S 03°48.042'	E 152°06.090'	1220	3.7	7.5	0	0	2.57	0.14	0
NSu-F7	water/fluid	S 03°47.957'	E 152°06.083'	1200	9.4	6.2	0.14	0	0.22	0.2	-77.65
NSu-F8	water/fluid	S 03°47.998'	E 152°06.056'	1155	66.7	3.3	1.51	0	2.1	0.21	-80.59
NSu-F9	water/fluid	S 03°47.991'	E 152°06.028'	1188	3.9	6.8	ND	ND	ND	ND	-
NSu-R1a	rock/chimney	S 03°47.964'	E 152°06.043'	1207	ND	ND	ND	ND	ND	ND	-
NSu-R1b	rock/chimney	S 03°47.964'	E 152°06.043'	1207	ND	ND	ND	ND	ND	ND	-
NSu-R2a	rock/chimney	S 03°47.964'	E 152°06.043'	1207	ND	ND	ND	ND	ND	ND	-
NSu-R2b	rock/chimney	S 03°47.964'	E 152°06.043'	1207	ND	ND	ND	ND	ND	ND	-
NSu-R3	rock/chimney	S 03°48.114'	E 152°06.376'	1480	ND	ND	ND	ND	ND	ND	-

Sample name[1]	Sample type	Latitude	Longitude	depth [m]	T [°C][2]	pH[2]	H_2S [mM][3]	CH_4 [mM][3]	DIC [mM][3]	O_2 [mM][3]	$\Delta G(H_2S)$ [4]
NSu-R4a	rock/chimney	S 03°47.995'	E 152°06.051'	1155	ND	ND	ND	ND	ND	ND	-
NSu-R4b	rock/chimney	S 03°47.995'	E 152°06.051'	1155	ND	ND	ND	ND	ND	ND	-
NSu-R5	rock/chimney	S 03°48.042'	E 152°06.095'	1220	ND	ND	ND	ND	ND	ND	-
RR-A1	fauna surface	S 03°43.239'	E 151°40.520'	1685	ND	ND	ND	ND	ND	ND	-
RR-A2	fauna surface	S 03°43.239'	E 151°40.51998'	1685	ND	ND	ND	ND	ND	ND	-
RR-F1a	water/fluid	S 03°43.238'	E 151°40.520’	1685	7.2	7.1	0	0	1.15	0.21	0
RR-F1b	water/fluid	S 03°43.238'	E 151°40.519'	1685	6.6	7.5	0	0	2.34	0.2	0
RR-R1	rock/chimney	S 03°43.271'	E 151°40.473'	1680	ND	ND	ND	ND	ND	ND	-
RR-R2	rock/chimney	S 03°43.687'	E 151°40.788'	1766	ND	ND	ND	ND	ND	ND	-
SnC-R1	rock/chimney	S 03°43.685'	E 151°40.160'	1647	ND	ND	ND	ND	ND	ND	-
Sol8-F1	water/fluid	S 03°43.030'	E 151°40.365'	1774	17	7.2	0	0	1.26	0.2	0
Sol8-R1a	rock/chimney	S 03°43.832'	E 151°40.451'	1741	ND	ND	ND	ND	ND	ND	-
Sol8-R1b	rock/chimney	S 03°43.832'	E 151°40.451'	1741	ND	ND	ND	ND	ND	ND	-
StM-F1a	water/fluid	S 03°43.613'	E 151°40.321'	1692	4.6	6.1	0.12	0	0.61	0.24	-93.65

Sample name[1]	Sample type	Latitude	Longitude	depth [m]	T [°C][2]	pH[2]	H_2S [mM][3]	CH_4 [mM][3]	DIC [mM][3]	O_2 [mM][3]	$\Delta G(H_2S)$ [4]
StM-F1b	water/fluid	S 03°43.587'	E 151°40.318'	1687	10.3	6	ND	ND	ND	ND	-
StM-F2a	water/fluid	S 03°43.616'	E 151°40.324'	1690	17.4	5.6	0.17	0	0.93	0.48	-131.84
StM-F2b	water/fluid	S 03°43.616'	E 151°40.325'	1691	8.9	5.7	0.08	0	0.33	0.35	-62.45
StM-R1	rock/chimney	S 03°43.610'	E 151°40.329'	1689	ND	ND	ND	ND	ND	ND	-

[1] Sample names reflect i) collection sites: CTD – CTD plume/background seawater samples; SuSu Knolls field: NSu – North Su; PACMANUS field: Fw – Fenway (PACMANUS), RR – Roman Ruins, SnC – Snowcap, Sol8 – Solwara 8, StM – Satanic Mills and ii) sample type and number: A – fauna surfaces, F – fluids/water, R – rock/chimney; iii) a and b indicate different samples of the same fluid/rock. ND – 'not determined'.

[2] Temperature and pH were determined by in-line probes attached to the KIPS-nozzle

[3] Gas concentrations were measured by *in situ* mass spectrometry parallel to the filtering of fluids.

[4] Gibb's free energy calculations available from sulfide oxidation were done with a conservative sulfate concentration estimate of 28 mM (background sea water value).

Table S2

Table S2a: Pearson correlation indices of the environmental variables

	T	**pH**	**H_2S**	**CH_4**	**DIC**	**O_2**
T	1	-0.84***	0.88***	0.65**	0.43	-0.57**
pH	-0.84***	1	-0.88***	-0.59**	-0.26	0.42
H_2S	0.88***	-0.88***	1	0.77***	0.57**	-0.77***
CH_4	0.65**	-0.59**	0.77***	1	0.54*	-0.68***
DIC	0.43	-0.26	0.57**	0.54*	1	-0.66
O_2	-0.57**	0.42	-0.77***	-0.68***	-0.66	1

Significance code: '***' < 0.001, '**' < 0.01, '*' < 0.05

Table S2b: Spearman's correlation indices of the environmental variables

	T	**pH**	**H_2S**	**CH_4**	**DIC**	**O_2**
T	1	-0.82***	0.78***	0.7***	0.28	-0.21
pH	-0.82***	1	-0.91***	-0.69***	-0.12	0.14
H_2S	0.78***	-0.91***	1	0.75***	0.23	-0.3
CH_4	0.7***	-0.69***	0.75***	1	0.44*	-0.57**
DIC	0.28	-0.12	0.23	0.44*	1	-0.44*
O_2	-0.21	0.14	-0.3	-0.57**	-0.44*	1

Significance code: '***' < 0.001, '**' < 0.01, '*' < 0.05

Table S3

Table S3a: Average nucleotide identities between the *Sulfurovum* draft genomes and sequenced representatives in percent.

	***SV*-3**	***SV*-4**	***SV*-5**	***SV*-6**	***SV*-8**	***SV*-9**	***SV*-10**	***SV*-12**	***SV*-13**	***SV*-14**	***SV*-15**	***S.* sp. NBC37-1**	***S.* sp. FS06-10**	***S.* sp. AR**
***SV*-3**	*	71.1	73.3	69.4	75.4	69.8	71.4	70.4	72.3	71.2	69.2	71.6	70.1	74.0
***SV*-4**	70.8	*	69.2	68.2	72.5	67.1	72.8	68.1	68.1	68.0	66.5	73.1	68.1	71.3
***SV*-5**	73.5	69.4	*	69.2	72.8	71.0	70.2	70.1	71.4	71.0	70.0	70.1	70.3	72.4
***SV*-6**	69.3	68.1	69.0	*	69.1	71.2	67.7	69.9	68.1	68.9	69.7	68.1	67.8	69.2
***SV*-8**	75.2	72.4	72.7	69.0	*	69.1	73.4	69.5	70.8	69.5	68.4	73.6	69.7	74.1
***SV*-9**	69.9	67.2	71.3	71.6	69.1	*	66.9	71.8	70.6	72.0	73.4	67.1	70.4	69.0
***SV*-10**	71.2	72.6	69.9	67.8	73.5	66.7	*	67.5	68.7	67.6	66.3	78.1	68.0	72.1
***SV*-12**	70.5	68.1	70.1	70.0	69.4	71.6	67.9	*	72.6	74.9	70.8	68.0	72.3	69.4
***SV*-13**	72.2	68.1	71.3	68.5	70.4	70.1	68.6	72.1	*	79.7	69.7	68.4	71.8	71.0
***SV*-14**	71.4	68.4	71.3	69.3	69.9	71.9	68.0	75.2	80.9	*	71.2	68.0	75.1	69.9
***SV*-15**	69.4	66.8	70.2	70.1	68.5	73.7	66.6	71.1	70.1	71.2	*	66.8	69.6	68.4
***Sulfurovum* sp. NBC37-1**	71.4	73.2	70.0	68.0	73.5	66.9	77.9	67.7	68.6	67.8	66.5	*	68.0	72.1
***Sulfurovum* sp. FS06-10**	69.8	68.0	69.9	67.9	69.2	69.8	67.9	72.1	71.6	74.1	69.0	68.0	*	69.6
***Sulfurovum* sp. AR**	74.0	71.3	72.0	69.1	74.0	69.0	72.0	69.2	71.1	69.5	68.3	72.1	69.6	*

Thresholds according to (Goris et al., 2007): Genus - >70% ANI, Species > 95% ANI. Accession numbers of the complete genomes in Fig. 5

Table S3b: Average nucleotide identities between the *Sulfurimonas* draft genomes and sequenced representatives in percent.

	***SM*-3**	***SM*-4**	***SM*-5**	***SM*-6**	***SM*-7**	***S. autotrophica* DSM 16294**	***S. gotlandica* GD1**	***S. denitrificans* DSM 1251**	***S.* sp. AST-10**
***SM*-3**	*	71.4	73.2	72.4	74.2	72.3	72.2	71.7	71.7
***SM*-4**	70.7	*	71.8	71.3	68.2	72.1	71.7	70.0	71.0
***SM*-5**	73.1	72.2	*	76.2	70.8	75.2	72.0	71.7	71.3
***SM*-6**	72.0	71.4	76.1	*	70.5	75.0	71.3	70.7	70.8
***SM*-7**	73.0	68.5	70.7	70.7	*	69.5	69.1	69.0	69.2
***Sulfurimonas autotrophica* DSM 16294**	72.0	72.0	75.0	74.9	69.4	*	72.1	71.0	71.4
***Sulfurimonas gotlandica* GD1**	71.2	71.1	71.3	70.8	68.7	71.7	*	72.5	75.8
***Sulfurimonas denitrificans* DSM 1251**	71.3	70.1	71.4	70.5	68.9	71.1	73.0	*	73.3
***Sulfurimonas* sp. AST-10**	71.2	71.0	71.2	70.8	69.3	71.0	76.2	73.1	*

Thresholds according to (Goris et al., 2007): Genus - >70% ANI, Species > 95% ANI. Accession numbers of the complete genomes in Fig. 5

Table S3c: Average nucleotide identities between the SUP05 draft genomes and sequenced representatives in %.

	SUP05-4	SUP05-5	SUP05-6	SUP05-7	SUP05-9	SUP05-10	SUP05-11	SUP05-12	SUP05-13	SUP05 symbiont-4	SUP05 symbiont-5	SUP05 symbiont-7	BazSymA	*Cand.* T. autot. EF1	*Cand.* T. sing. PS1
SUP05-4	*	67.4	74.3	74.3	74.1	73.8	74.8	74.7	73.7	72.0	71.0	71.5	72.5	77.1	67.5
SUP05-5	68.1	*	68.5	68.6	67.9	68.4	68.5	67.9	68.5	68.8	68.2	68.0	68.9	68.1	67.3
SUP05-6	74.4	67.9	*	85.3	96.5	84.1	93.4	86.8	80.2	74.0	73.0	73.4	74.8	74.3	67.8
SUP05-7	74.1	67.7	85.3	*	85.8	93.2	90.6	92.8	92.9	73.9	73.0	73.5	74.6	73.9	68.1
SUP05-9	74.0	67.3	95.8	86.3	*	84.2	93.2	89.1	80.4	73.5	73.4	73.8	74.5	73.8	67.4
SUP05-10	73.7	67.3	83.5	93.0	83.2	*	84.7	90.9	92.4	72.8	72.4	72.5	73.8	73.1	67.5
SUP05-11	74.7	67.7	92.4	91.1	92.3	85.2	*	91.6	81.1	74.2	73.4	74.0	75.2	74.4	68.1
SUP05-12	74.2	66.9	85.8	92.1	88.1	89.8	90.8	*	88.6	73.5	72.8	73.1	74.4	73.7	67.6
SUP05-13	73.8	67.6	80.5	96.5	81.2	94.5	80.7	92.0	*	73.5	73.0	73.1	74.4	73.8	67.9
SUP05 symbiont-4	72.2	68.2	74.2	74.1	73.7	73.5	74.4	74.1	73.7	*	95.0	85.4	76.8	72.4	67.5
SUP05 symbiont-5	71.1	67.0	73.2	73.3	73.1	72.5	73.3	72.9	73.0	93.7	*	89.4	75.9	71.5	66.4
SUP05 symbiont-7	71.7	67.7	73.6	73.8	73.5	72.7	74.7	73.4	73.5	85.0	89.9	*	76.4	72.3	67.1
BazSymA	72.3	68.0	74.8	74.8	74.6	74.0	75.3	74.6	74.3	76.7	76.3	76.4	*	72.7	67.4
***Candidatus* Thioglobus autotrophica EF1**	77.1	67.0	74.4	74.1	74.0	73.4	74.9	74.0	73.7	72.4	71.5	72.0	72.6	*	67.4
***Candidatus* Thioglobus singularis PS1**	67.6	66.6	67.8	68.3	67.7	67.5	68.2	67.7	67.9	67.5	66.8	67.2	67.5	67.3	*

Thresholds according to (Goris et al., 2007): Genus - >70% ANI, Species > 95% ANI. Accession numbers of the complete genomes in Fig. 5

Figure S1

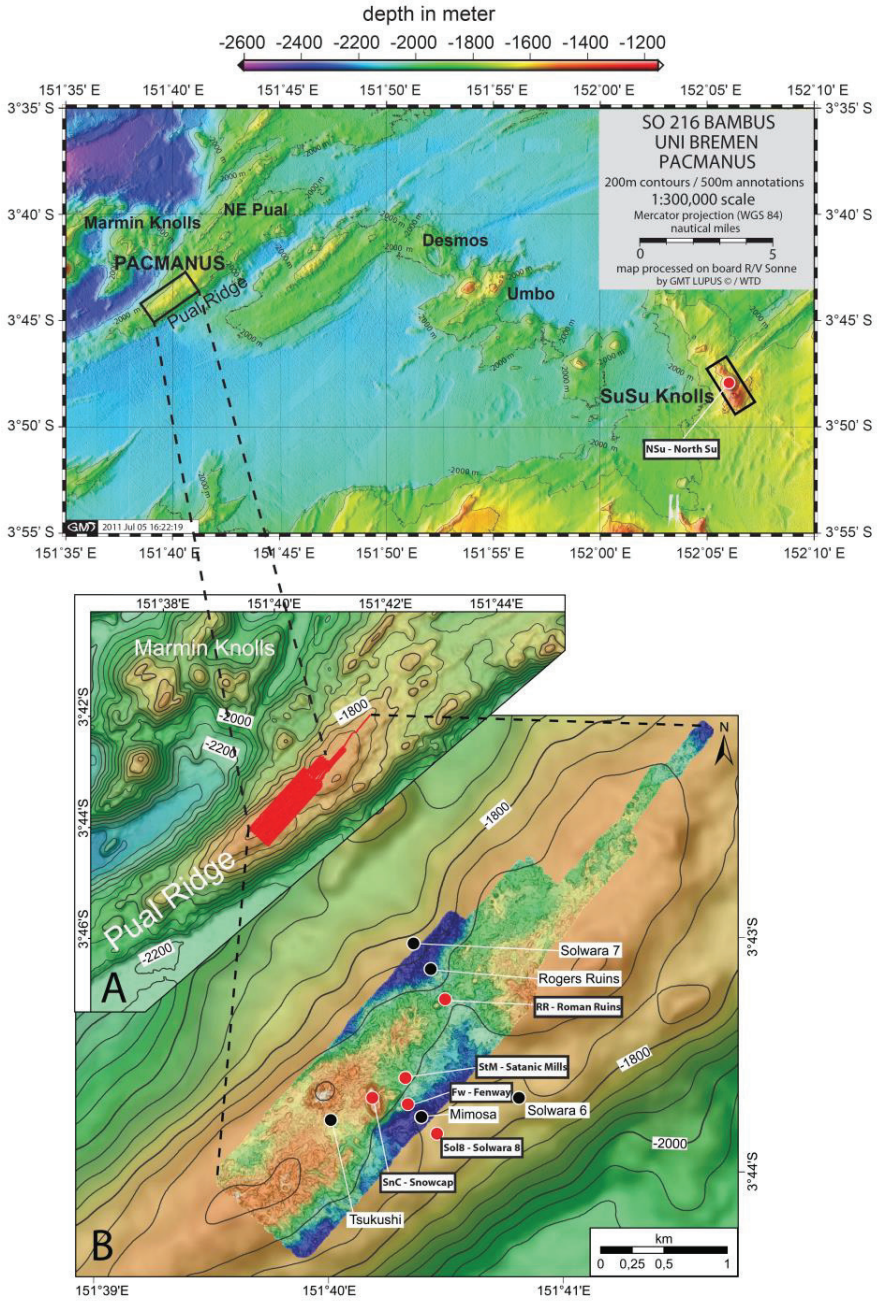


Figure S1: Bathymetric map of the Manus Basin. The upper panel shows the locations of PACManus and SuSu Knolls hydrothermal fields in the Manus Basin. The lower panel is taken from (Thal et al., 2014) and shows a more detailed view of the venting sites within the PACManus hydrothermal field. Venting sites sampled for this study are marked in red.

Figure S2

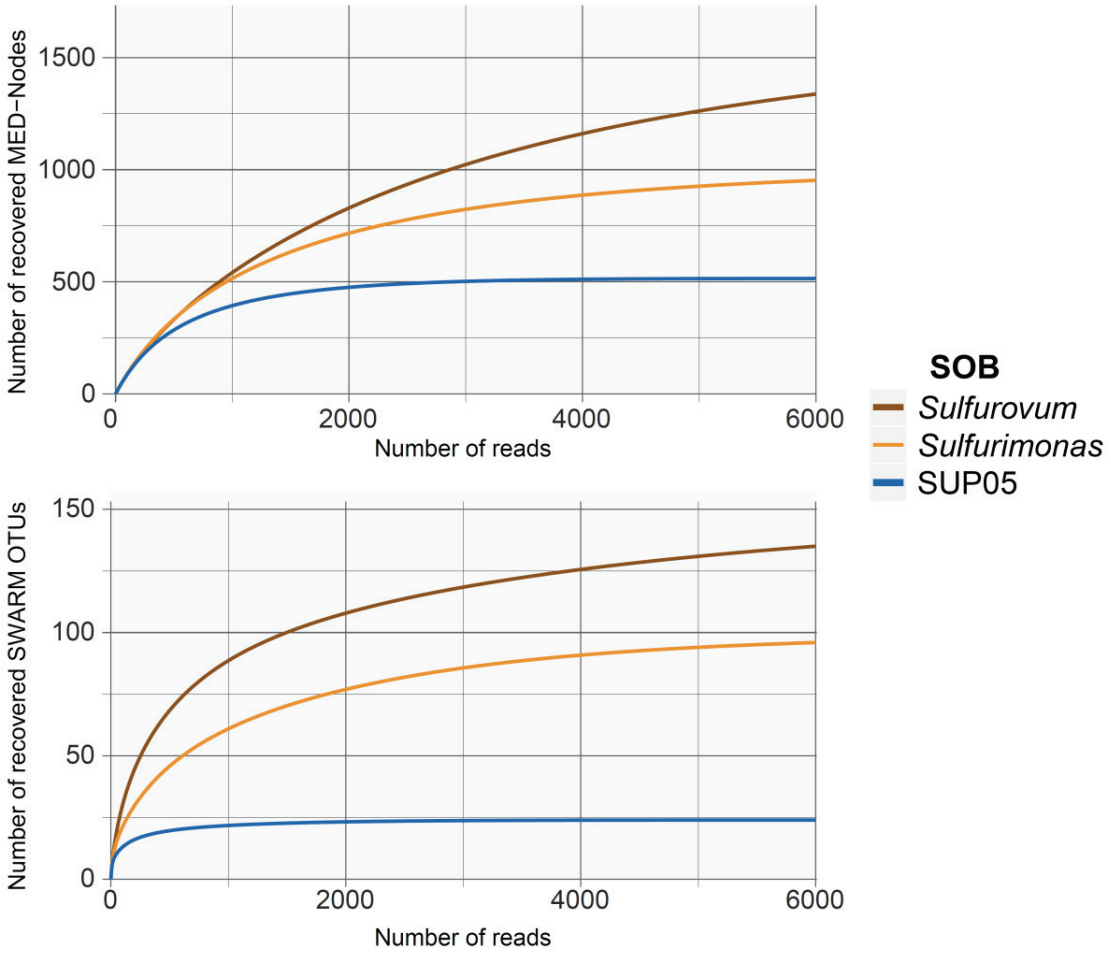


Figure S2: Rarefaction curves of all 16S rRNA amplicon sequences classified as Sulfurovum, Sulfurimonas and SUP05. Upper panel shows the number of total nodes recovered during minimum entropy decomposition (MED). Lower panel shows the total number of OTUs generated by SWARM.

Figure S3

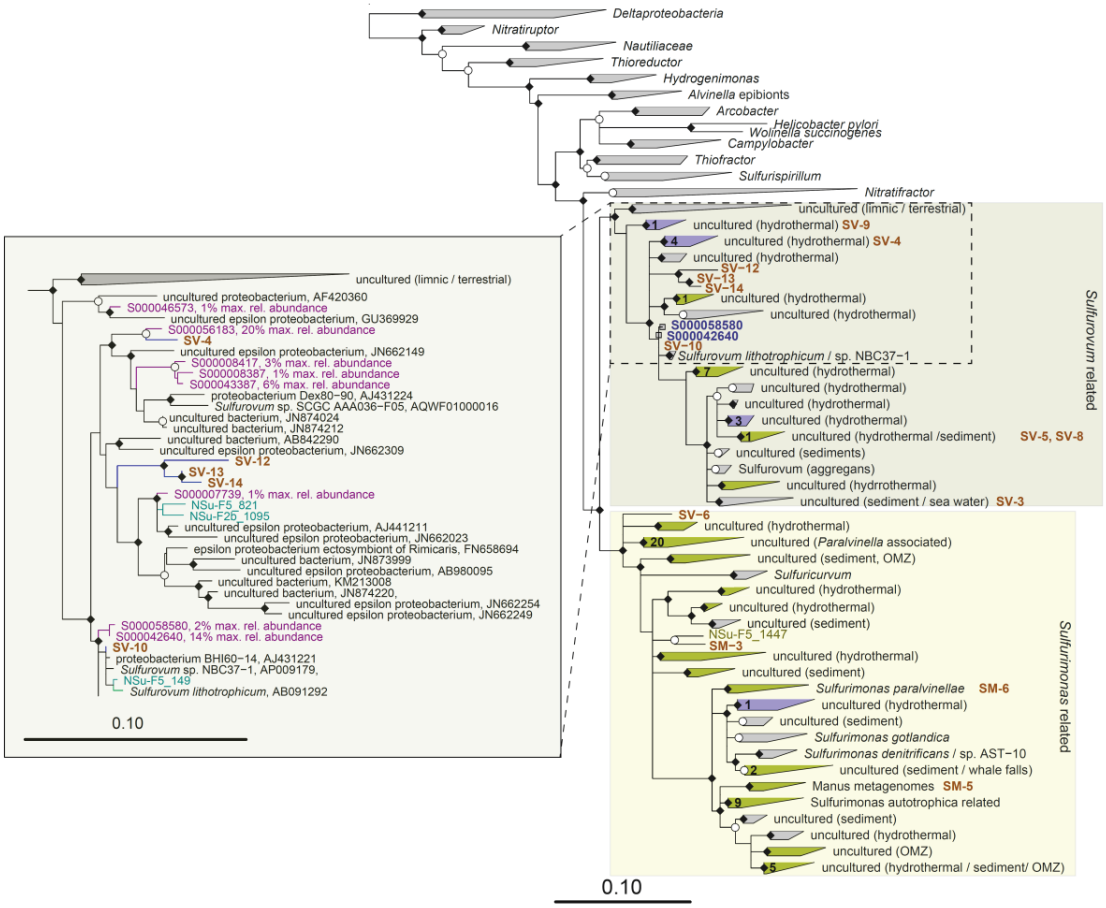
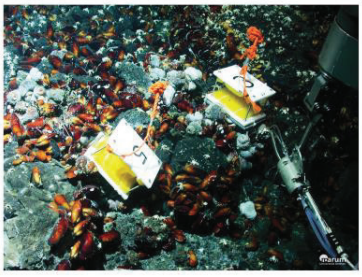


Figure S2: Maximum likelihood phylogenetic of Sulfurimonas and Sulfurovum based on 16S rRNA gene sequences. Filled diamonds indicate branch support > 60%. White circles indicate branches of low significance as indicated by ARB based on support values and branch lengths. Clusters containing long 16S rRNA sequences obtained in this study (via PhyloFlash reconstruction or PacBio sequencing) are colored in yellow. Clusters containing only OTU representative sequences are colored in purple. Numbers indicate OTUs with >1% rel. abundance in at least one sample falling into a cluster. Partial 16S rRNA sequences of the bins are marked in bold orange. Depicted tree was calculated with FastTree based on high quality long sequences obtained in this study and related sequences from the SILVA SSU123 database with a 50% positional conservation filter. Amplicon and partial sequences were added to the tree based on maximum parsimony in Arb. Multifurcations were assigned for branches with less than 50% support or branches shorter than 0.005 changes / base.

Figure S4

NSu-F2b
$T_{average}$=51.7°C (max. 73.6°C)
$pH_{average}$=4.3
collection time: 55 min
H_2S: 1.61 mM
CH_4: 0.2 mM
DIC: 3.07 mM
O_2: 0.07 mM

NSu-F5
$T_{average}$=31.4°C (max. 41.5°C)
$pH_{average}$=5.1
collection time: 30 min
H_2S: 0.7 mM
CH_4: 0.01 mM
DIC: 0.18 mM
O_2: 0.14 mM



Fw-F1b
$T_{average}$=3.7°C (max. 5.3°C)
$pH_{average}$=6.5
collection time: 32 min
H_2S: 0 mM
CH_4: 0 mM
DIC: 0.24 mM
O_2: 0.17 mM

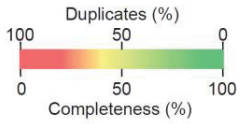
Fw-F3
$T_{average}$=3.2°C (max. 3.7°C)
$pH_{average}$=7.2
collection time: 31 min
H_2S: no data
CH_4: no data
DIC: no data
O_2: no data

RR-F1b
$T_{average}$=6.6°C (max. 15°C)
$pH_{average}$=7.5
collection time: 13 min
H_2S: 0 mM
CH_4: 0 mM
DIC: 2.34 mM
O_2: 0.2 mM

Figure S4: Images of fluid collection events for metagenome analyses and main environmental parameters measured during the time of filtering.

Figure S5

	Comp.	Cont.	Str.het.	NSu-F2b	NSu-F5	Fw-F1b	Fw-F3	RR-F1b
SM-3	78.7	0	0	0.081%	0.016%	0.001%	0.000%	0.000%
SM-4	59.68	0	0	0.000%	0.000%	0.088%	0.002%	0.000%
SM-5	70.92	0	0	0.396%	0.056%	0.013%	0.007%	0.000%
SM-6	71.4	0	0	0.154%	0.019%	0.001%	0.001%	0.000%
SM-7	69.35	0	0	0.052%	0.007%	0.000%	0.000%	0.000%
SV-3	80.34	0.81	0	0.697%	0.004%	0.003%	0.003%	0.000%
SV-4	80.28	0.41	0	0.066%	0.041%	0.000%	0.000%	0.000%
SV-5	82.39	0.41	0	0.000%	0.110%	0.001%	0.002%	0.000%
SV-6	83.73	0.41	0	0.000%	0.110%	0.000%	0.000%	0.000%
SV-8	76.41	0.61	50	0.031%	0.082%	0.001%	0.000%	0.000%
SV-9	81.2	0.41	0	0.043%	0.044%	0.258%	0.114%	0.001%
SV-10	71.92	0.61	50	0.013%	0.070%	0.001%	0.000%	0.000%
SV-12	74.22	0.41	0	0.104%	0.001%	0.003%	0.002%	0.001%
SV-13	70.15	0	0	0.131%	0.005%	0.001%	0.001%	0.000%
SV-14	67.73	1.23	33.33	0.218%	0.059%	0.001%	0.001%	0.000%
SV-15	81.41	0.41	0	0.001%	0.094%	0.003%	0.002%	0.000%
SUP05-4	71.89	0	0	0.042%	0.655%	0.743%	0.890%	1.260%
SUP05-5	78.79	0	0	0.000%	0.050%	0.001%	0.001%	0.001%
SUP05-6	66.29	0	0	0.012%	0.479%	0.470%	0.626%	1.065%
SUP05-7	71.33	0	0	0.004%	0.133%	0.129%	0.165%	0.335%
SUP05-9	46.61	0	0	0.008%	0.337%	0.335%	0.441%	0.762%
SUP05-10	37.81	0	0	0.005%	0.185%	0.172%	0.228%	0.440%
SUP05-11	40.19	0	0	0.003%	0.133%	0.126%	0.167%	0.315%
SUP05-12	64.66	0	0	0.005%	0.206%	0.197%	0.263%	0.471%
SUP05-13	50.41	0	0	0.003%	0.090%	0.085%	0.111%	0.227%
SUP05 (symbiotic)-4	66.42	0	0	0.000%	0.000%	0.280%	0.366%	0.034%
SUP05 (symbiotic)-5	41.23	0	0	0.000%	0.000%	0.151%	0.213%	0.021%
SUP05 (symbiotic)-7	44.35	0	0	0.000%	0.000%	0.170%	0.233%	0.030%



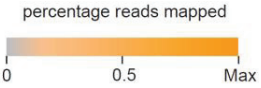


Figure S5: Statistics of bin completeness and relative abundance in the raw read datasets of different metagenomes. The left panel shows completeness, contamination and strain heterogeneity percentages, calculated with CheckM based on amino acid sequences predicted by RAST annotation. The right panel shows the percentages of raw reads mapped to the assembled contigs with minimum sequence identity of 98%.

Figure S6

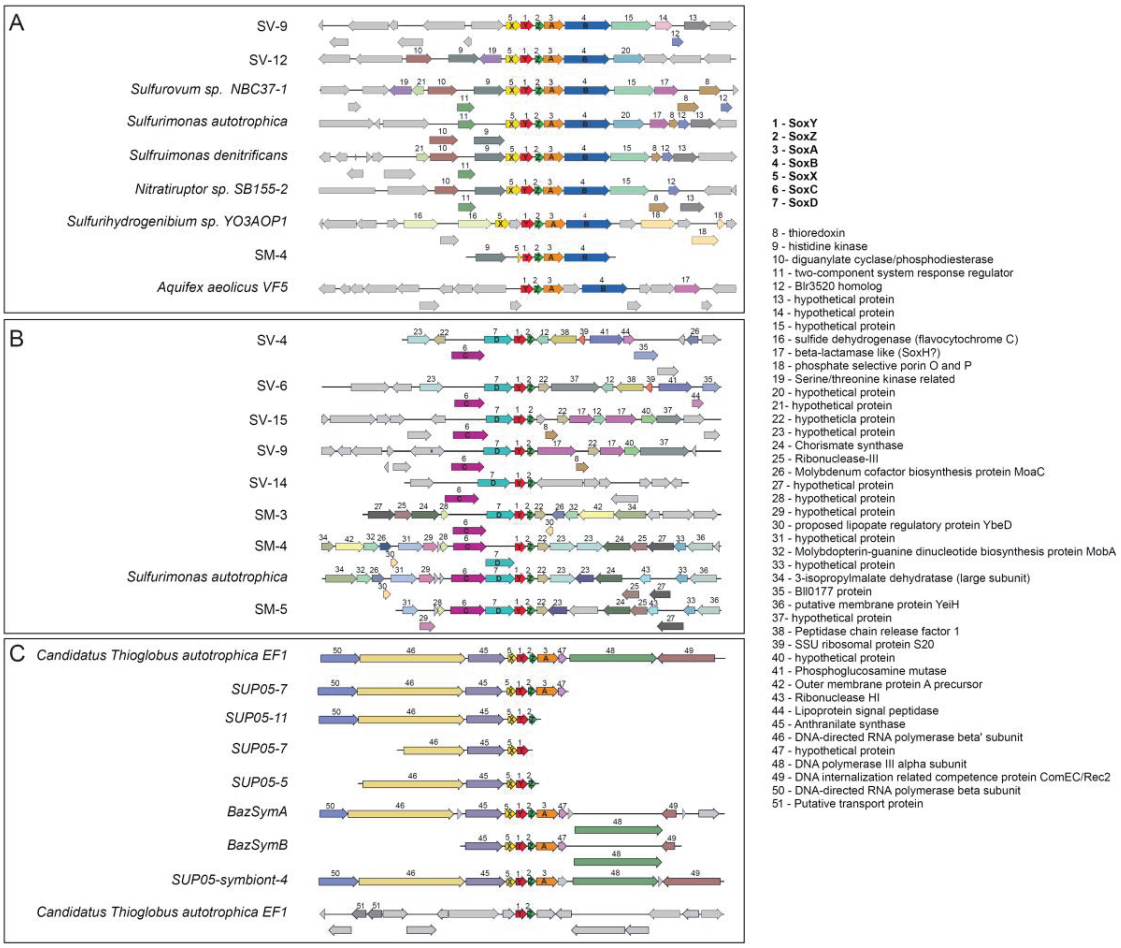


Figure S6: Examples of SoxY loci in different SOB draft genomes and related reference genomes: A) *Epsilonproteobacteria* SoxY locus 1; B) *Epsilonproteobacteria* SoxY locus 2; C) SUP05 and related genomes. Graphs are modified from RAST-generated locus comparison graphs (Aziz et al., 2008). In each graph the sequence similarity of the SoxY protein decreases from top to bottom.

Figure S7

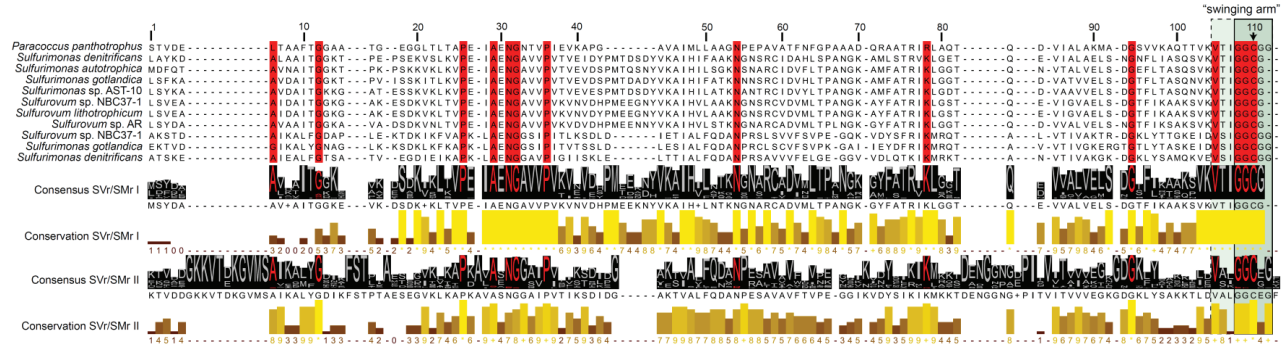


Figure S7: Alignment of the translated *Sulfurimonas* and *Sulfurovum* related (SMr and SVr) SoxY proteins. Upper panel shows aligned SoxY sequences of alphaproteobacterium *Paracoccus panthotrophus* and available complete genomes of *Sulfurimonas* and *Sulfurovum* species. The alignments of the metagenomic SVr/SMr SoxY sequences retrieved in this study are shown as consensus histograms and supplemented with a histogram showing the conservation of residue associated properties (polarity, charge, etc. – yellow bars). In red - residues conserved in all SMr/SVr SoxY sequences. Position numbering refers to the crystallographically resolved *Paracoccus panthotrophus* SoxY protein (Sauve et al., 2007). The "swinging arm" at the C-terminal end is the sulfur anion binding domain. Dashed line indicates a hypothetical extension of the domain (Sauve et al., 2007).

References:

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Sauve, V., Bruno, S., Berks, B.C., and Hemmings, A.M. (2007) The SoxYZ complex carries sulfur cycle intermediates on a peptide swinging arm. *J Biol Chem* **282**: 23194-23204.

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Chapter III

Chemosynthetic microbial communities fueled by oxidation of hydrothermal sulfide minerals

Dimitri Meier, Petra Pjevac, Wolfgang Bach, Rudolf Amann, Anke Meyerdierks

Manuscript in preparation

Contributions:

D.M and A.M. developed concepts and ideas. P.P., A.M. and W.B. collected samples at the PACManus and SuSu Knolls hydrothermal fields. D.M. performed experiments and data analysis, conceived and wrote the manuscript. P.P. performed DNA extraction and sequencing preparations. W.B. analyzed sulfide minerals composition. P.P., W.B., R.A., A.M. conceived and edited the manuscript.

Chemosynthetic microbial communities fueled by oxidation of hydrothermal sulfide minerals

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Running title: Chemosynthetic bacteria on iron-sulfur deposits

Keywords: iron oxidation, sulfur oxidation, carbon fixation, metagenomics, ubiquitous *Gammaproteobacteria*, community shift

Summary

Iron-sulfur minerals such as pyrite are found in many marine benthic habitats. At hydrothermal vent sites they appear as massive deposits referred to as chimneys resulting from the precipitation of minerals from reduced venting fluid upon contact with cold oxygenated sea water. While microorganisms inhabiting actively venting chimneys and utilizing reduced sulfur and iron compounds dissolved in the fluids are rather well studied, only little is known about the microorganisms inhabiting inactive sulfide chimneys. We compared the previously published data from six active and two inactive chimneys to newly analyzed five inactive chimneys sampled during the same cruise in the Manus Basin. The diversity and metabolic potential of the respective microbial communities were investigated by 16S rRNA gene sequencing and metagenome analysis. Inactive iron-sulfide chimneys contained mostly sulfur and iron oxidizing autotrophic bacteria belonging to ubiquitous, but poorly described clades of Gammaproteobacteria.

Introduction

At deep-sea hydrothermal fields the primary energy source for microbial life are often methane and hydrogen, as well as a large variety of reduced sulfur, iron or manganese species (Jannasch and Mottl, 1985; McCollom and Shock, 1997; Bach et al., 2006). These so called “geofuels” can be emitted as hot (up to 400°C) focused venting of hydrothermal fluid or in form of cooler, diffuse fluid already mixed with sea water. Chemolithoautotrophic microorganisms are the only primary producers in the extensive ecosystems covering the hydrothermal fields. Present in form of microbial mats, planktonic cells or as symbionts of vent fauna, they oxidize the gases and other reduced compounds dissolved in the hydrothermal fluids (reviewed in Nakagawa and Takai, 2008; Sievert and Vetriani, 2012).

At most venting systems, reduced sulfur species are the main source of energy for microbial growth (McCollom and Shock, 1997; Amend et al., 2011). However, large parts of the reduced sulfur compounds precipitate with metal ions such as iron, copper or zinc upon contact with cold oxygenated sea water. This results in the formation of massive sulfide deposits referred to as hydrothermal chimney structures (Haymon, 1983; Tivey, 2007). It has been hypothesized that sulfide deposits are oxidized by chemolithoautotrophic microorganisms after the venting has ceased (Sylvan et al., 2012).

Bacteria and *Archaea* capable of oxidizing reduced compounds in mineral deposits are well known from industrial leaching and acid mine drainage studies (Colmer and Hinkle, 1947; Jensen and Webb, 1995; Boon et al., 1998; Bond et al., 2000; Edwards et al., 2000; Johnson, 2001; Lopez-Archilla et al., 2001; Sand et al., 2001; Baker and Banfield, 2003; Gonzalez-Toril et al., 2003). In marine sediments, bacterial oxidation of iron-monosulfide (FeS) with nitrate as electron acceptor was shown by Schippers and Jørgensen (2002). Pyrite (FeS_2), however, seems to be mainly oxidized by abiotic processes, while sediment bacteria are using the dissolved Fe^{2+} and reduced sulfur species (Straub et al., 1996; Benz et al., 1998; Schippers and Jørgensen, 2002; Jørgensen and Nelson, 2004). At hydrothermal vents, the main research focus until now has always been microbes inhabiting active venting sites or vent fauna symbionts (reviewed in Sievert and Vetriani, 2012) oxidizing the dissolved sulfur compounds. Only few studies were conducted on chemolithoautotrophs inhabiting solid sulfide deposits not exposed to active venting. Wirsen and colleagues (1993) were able to show active CO_2

fixation in microbial mats covering inactive hydrothermal chimneys and isolated *Thiomicrospira* strains that grew autotrophically while oxidizing poly-metal sulfides (Eberhard et al., 1995). Edwards and colleagues (2003) isolated several neutrophilic iron oxidizing *Gamma-* and *Alphaproteobacteria* from deep sea hydrothermal deposits. However, the few isolates were only partially characterized and their genomes have not yet been sequenced. Later studies based on comparative 16S rRNA gene analysis documented a microbial community shift between active and inactive hydrothermal chimney structures (Suzuki et al., 2004; Kato et al., 2010; Sylvan et al., 2012; Kato et al., 2015a). It was suggested that mineral-sulfide oxidizing chemolithoautotrophs replace the bacteria oxidizing dissolved sulfur compounds in hydrothermal fluids (Sylvan et al., 2012; Kato et al., 2015a). A recent lipid biomarker based study detected a shift in carbon fixation pathways from reverse tricarboxylic acid (rTCA) cycle in active chimney communities to a Calvin-Benson-Bassham (CBB) cycle-like fingerprint on inactive chimneys, indicating a change of chemolithoautotrophic bacteria (Reeves et al., 2014).

Based on these first valuable insights we conducted a comprehensive study characterizing the microbial communities of inactive iron-sulfide chimneys. In addition to previously published data from two inactive and six active chimneys (Meier *et al.* in prep.), we analyzed the microbial community composition of further five inactive chimneys obtained during the same cruise at the PACManus and SuSu Knolls hydrothermal fields (Manus Basin, off Papua New Guinea). By integrating metagenome analysis of an inactive chimney, for which CO_2 fixation via CBB cycle was suggested by (Reeves et al., 2014), we retrieved first detailed insights into the metabolic potential of the hosted microorganisms. Our results show that after the end of the active venting phase the weathering chimney structures are dominated by discrete bacterial clades which have the potential for sulfur and iron oxidation and carbon dioxide fixation. This confirms that the reductive power stored in the iron-sulfide minerals is fueling chemolithoautotrophic bacterial life in inactive chimneys for extended periods of time.

Table 1: Hydrothermal chimney samples

Name	ROV	Location	Latitude/Longitude	categorization	on board description
Fw-R1	29ROV13	Fenway (PACManus)	03°43.711'S / 151°40.349'E	no visible venting	Polymetallic chimney with porous chalcopyrite filled with dark sphalerite and secondary Cu-sulfides plus barite; Fe-Mn oxyhydroxide crust.
NSu-R1a	12ROV01	North Su (SuSu Knolls)	03°47.946'S / 152°06.043'E	exposed to venting	Fibrous barite-rich chimney next to white smoker, partially coated with yellow native sulfur, oxidized on the outside.
NSu-R1b[2]	12ROV01	North Su (SuSu Knolls)	03°47.946'S / 152°06.043'E	exposed to venting	Fibrous barite-rich chimney next to white smoker, partially coated with yellow native sulfur, oxidized on the outside.
NSu-R2b[2]	12ROV02	North Su (SuSu Knolls)	03°47.946'S / 152°06.043'E	exposed to venting	Barite-rich chimney with an orifice/conduit partially coated with yellow native sulfur.
NSu-R7	47ROV13	North Su (SuSu Knolls)	03°47.992'S / 152°06.029'E	no visible venting	Cu-rich chimney with 0.5 - 1 cm conduit with thin layer of chalcopyrite followed by porous brassy chalcopyrite, rimmed by a thin layer (<0.5 cm) of sphalerite-pyrite with thin outer Fe-Mn oxyhydroxide crust
RR-R1[1,2]	39ROV01	Roman Ruins (PACManus)	03°43.272'S / 151°40.473'E	focused venting	Top of active black smoker chimney measured at 338°C with thin pipe-like dense chalcopyrite conduits
SnC-R1[2]	27ROV06	Snowcap (PACManus)	03°43.685'S / 151°40.159'E	exposed to venting	Fragments of chimney conduits partially lined by pale brassy fine grained chalcopyrite; outer part with sphalerite to tarnished marcasite.
SnC-R2	27ROV08	Snowcap (PACManus)	03°43.686'S / 151°40.160'E	no visible venting	Cu-rich chimney (talus piece) with chalcopyrite showing a purple-bluish outer bornite rim
Sol6-R1	53ROV02	Solwara 6 (PACManus)	03°43.686'S / 151°40.788'E	no visible venting	Dark grey-black chalcocite-bornite chimney. Zones outwards to marcasite-FeMn oxyhydroxide crust with minor occurrences of atacamite
RR-R2[1,2]	53ROV03	Roman Ruins (PACManus)	03°43.252'S / 151°40.499'E	diffuse venting	Weakly shimmering venting chimney, pyrite, sphalerite, chalcopyrite, barite, iron-oxide crusts (Reeves et al., 2014)
Sol8-R1b[2]	49ROV03	Solwara 8 (PACManus)	03°43.831'S / 151°40.451'E	no visible venting	Sphalerite-rich chimney with a Fe-Mn oxyhydroxide crust.

Name	ROV	Location	Latitude/Longitude	categorization	on board description
StM-R1[1,2]	43ROV07	Satanic Mills (PACManus)	03°43.610'S / 151°40.329'E	no visible venting	Weathered inactive chimney: chalcopyrite interior, sphalerite, barite exterior (Reeves et al., 2011a)
StM-R2	31ROV13	Satanic Mills (PACManus)	03°43.614'S / 151°40.321'E	no visible venting	Polymetallic chimney (talus piece) with porous chalcopyrite + sphalerite + bornite

[1] samples published in Reeves *et al.* (2014). [2] samples used in Meier *et al.* (in prep.)

Results

16S rRNA gene amplicon analysis

First we screened the microbial community composition of 13 hydrothermal chimney structures sampled at different vent sites across the SuSu Knolls and PACManus hydrothermal fields (Tab. 1) via comparative 16S rRNA gene analysis.

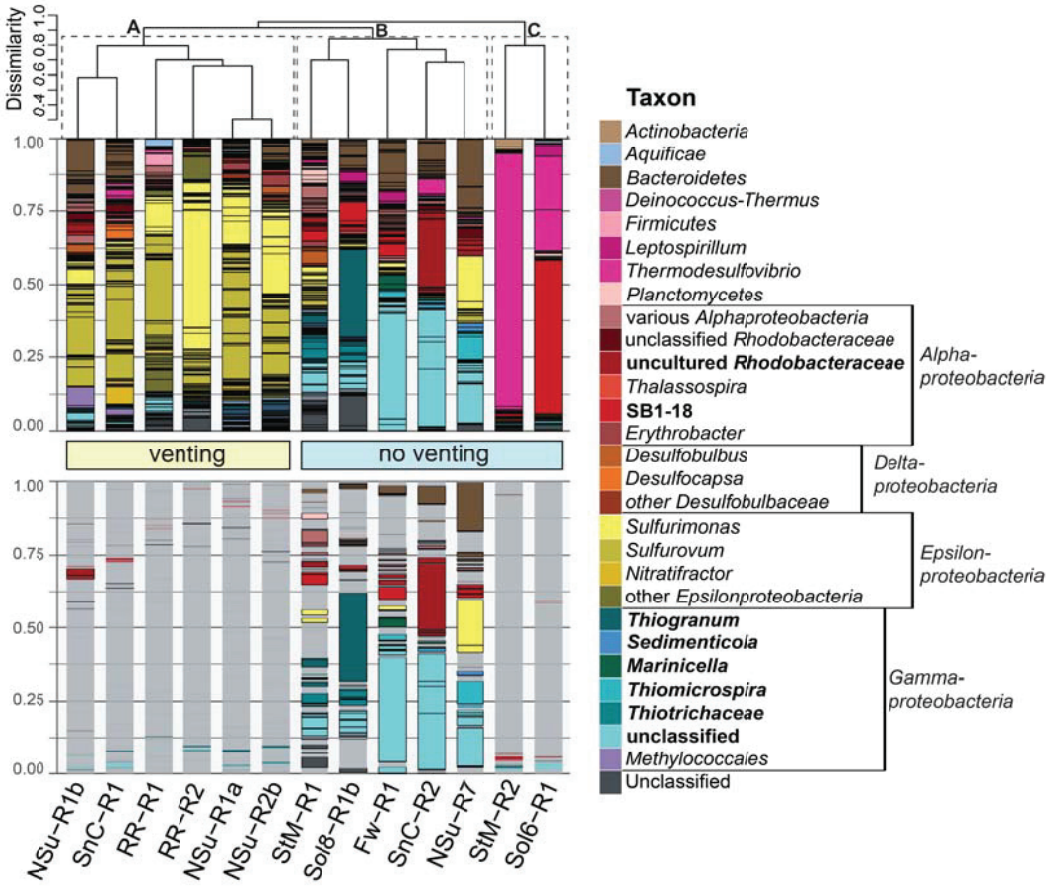

Figure 1: Microbial community composition of active and inactive chimneys as determined by 16S rRNA amplicon sequencing. The clustering was done with the average linkage method based on Bray-Curtis dissimilarity of community structures in R-package “vegan”. A, B, and C designate the three major community types. The upper panel shows the complete community composition. The lower panel shows the distribution of OTUs identified as characteristic for the Type-B communities ($p < 0.05$, except *Thiogranum*: $p = 0.07$) across all samples. Horizontal black lines separate the OTUs and coloring indicates taxonomic classification. Clades containing Type-B characteristic OTUs are shown in bold font.

Retrieved 16S rRNA amplicon sequences were analyzed by minimum entropy decomposition (MED, Eren et al., 2015) and subsequent operational taxonomic unit (OTU) generation with SWARM. The sequences representative for each OTU were classified by lowest common ancestor (LCA) according to the SILVA SSU123 (Quast et al., 2013) taxonomy. Hierarchical clustering of microbial communities revealed three types of characteristic compositions, largely, yet not completely, corresponding to the type of sample with respect to venting activity (Fig. 1). Type-A, comprising communities exposed to venting, was characterized by high relative abundances of *Sulfurovum* and *Sulfurimonas* (*Epsilonproteobacteria*) sequences (58% on average, up to 70%), although *Sulfurimonas* was also present in higher relative abundance in a single Type-B sample (NSu-R7, 20%) (Fig. 1). The communities of inactive chimney structures without any visible exposure to fluid emission clustered in to two completely different branches, further on referred to as Type-B and Type-C. Type-B communities were characterized by high relative abundances of sequences classified as *Gammaproteobacteria*, such as *Thiogranum* (7% on average, 30% max.), uncultured *Thiotrichaceae* (3% on average, 9% max), *Thiomicrospira* (2% on average, 9% max.), and most abundant - gammaproteobacterial sequences classified not further than class level (25% on average, 44% max.) (Fig. 1). The SnC-R2 sample (Type-B) also showed high relative abundance of uncultured *Rhodobacteraceae* sequences (26%). The two Type-C samples showed high relative abundance of *Thermodesulfovibrio* (87% and 32%) and SB1-18 clade *Alphaproteobacteria* (53% in Sol6-R1), which were also present in Type-B samples (5% on average, 10% max.). *Bacteroidetes* sequences were relatively abundant in most Type-A and B samples (Type-A: 8% average, 20% max., Type-B: 15% average, 27% max.).

We conducted a similarity percentages breakdown (SIMPER; Clarke, 1993) in order to reveal OTUs that were highly specific for microbial communities of the Type-B inactive chimneys. The most abundant OTUs significantly contributing to the clustering of Type-B (SIMPER $p < 0.05$) belonged to the unclassified *Gammaproteobacteria* (Fig. 1, Tab 2). Other OTUs characteristic of Type-B samples (SIMPER $p < 0.05$ for all pairwise comparisons) were classified as uncultured *Thiotrichaceae*, *Sedimenticola*, *Marinicella*, and *Thiomicrospira* (*Gammaproteobacteria*), SB1-18 clade (*Alphaproteobacteria*), unclassified *Flavovirgaceae* and *Lutibacter* (*Bacteroidetes),* as well as *Sulfurimonas*

(*Epsilonproteobacteria)* (Fig. 1, Tab 2). Although seemingly Type-B specific, OTUs classified as *Thiogranum* are only assigned a p-value of 0.07 by SIMPER.

Table 2: OTUs characteristic for Type-B communities and their relative abundances

OTU	classification (LCA, SILVA SSU123 taxonomy)	average inactive	average venting	max. inactive	max. venting	p-value
S000036159	unclassified *Gammaproteobacteria*	10.33%	0.24%	35.68%	0.73%	0.005
S000058701	unclassified *Gammaproteobacteria*	7.80%	0.04%	19.47%	0.15%	0.001
S000047296	*Thiogranum (Gammaproteobacteria)*	6.59%	0.48%	30.10%	0.93%	0.072
S000044681	uncultured *Rhodobacteraceae (Alphaproteobacteria)*	5.56%	0.59%	22.94%	2.34%	0.029
S000005228	uncultured *Flammeovirgaceae (Cytophagia, Bacteroidetes)*	5.21%	0.03%	16.26%	0.10%	0.001
S000000654	*Sulfurimonas (Epsilonproteobacteria)*	3.81%	0.09%	15.84%	0.18%	0.027
S000021075	unclassified *Gammaproteobacteria*	2.16%	0.02%	9.17%	0.07%	0.042
S000047127	*Thiomicrospira (Gammaproteobacteria)*	2.03%	0.00%	7.80%	0.00%	0.010
S000058174	SB1-18 *(Alphaproteobacteria)*	1.96%	0.10%	4.07%	0.22%	0.001
S000032918	unclassified *Gammaproteobacteria*	1.28%	0.05%	2.01%	0.22%	0.001
S000044394	*OCS116 clade (Alphaproteobacteria)*	1.20%	0.06%	4.04%	0.20%	0.001
S000058000	*SB1-18 (Alphaproteobacteria)*	1.06%	0.24%	1.34%	0.99%	0.007
S000039391	Unclassified	1.02%	0.03%	3.63%	0.10%	0.100
S000041897	*Sulfurimonas (Epsilonproteobacteria)*	0.84%	0.02%	2.43%	0.07%	0.006
S000021290	uncultured *Thiotrichaceae (Gammaproteobacteria)*	0.79%	0.05%	3.30%	0.16%	0.075
S000003820	*Marinicella (Gammaproteobacteria)*	0.77%	0.00%	2.97%	0.00%	0.044
S000003844	uncultured *Thiotrichaceae (Gammaproteobacteria)*	0.71%	0.08%	2.27%	0.26%	0.057
S000021479	uncultured *Thiotrichaceae (Gammaproteobacteria)*	0.52%	0.05%	2.01%	0.12%	0.099
S000018697	*Planctomyces (Planctomycetacia, Planctomycetes)*	0.49%	0.01%	1.98%	0.05%	0.032
S000047241	unclassified *Gammaproteobacteria*	0.49%	0.04%	2.20%	0.07%	0.082
S000031450	*Lutibacter (Flavobacteriia, Bacteroidetes)*	0.47%	0.00%	1.26%	0.00%	0.023
S000044355	uncultured *Hyphomicrobiaceae (Alphaproteobacteria)*	0.45%	0.00%	1.02%	0.00%	0.002

OTU	classification (LCA, SILVA SSU123 taxonomy)	average inactive	average venting	max. inactive	max. venting	p-value
S000044445	uncultured *Rhodobacteraceae (Alphaproteobacteria)*	0.42%	0.03%	1.83%	0.18%	0.095
S000032955	*unclassified Proteobacteria*	0.42%	0.11%	1.17%	0.45%	0.068
S000021429	uncultured *Piscirickettsiaceae (Gammaproteobacteria)*	0.33%	0.01%	1.12%	0.04%	0.010
S000002047	Unclassified	0.33%	0.01%	1.32%	0.05%	0.008
S000022418	*Sedimenticola (Gammaproteobacteria)*	0.31%	0.00%	1.34%	0.00%	0.099

OTUs with a SIMPER p-value <0.1 for the Type-B vs. Type-A comparison are shown, ordered by decreasing average relative abundance in inactive (Type-B) sulfides samples. In grey, OTUs with p-values > 0.05.

16S rRNA gene reconstruction

We sequenced and analyzed a metagenome of the StM-R1 sample. Long 16S rRNA sequences (over 1200 bp) were reconstructed from raw metagenomic reads. In general, this metagenome contained a much higher proportion of *Gammaproteobacteria* sequences than indicated by amplicon sequencing (53% compared to 28%, Fig S1). We calculated a phylogenetic tree to assess the phylogenetic affiliation of gammaproteobacterial clades inhabiting the inactive chimneys (Fig 2). The tree showed that reconstructed 16S rRNA gene sequences retrieved from the metagenome as well as amplicon sequences representative of abundant OTUs (>1% in at least one sample) affiliated with very divergent clades of "basal" *Gammaproteobacteria* (Williams et al., 2010). Many of these contained only environmental sequences from e.g. coastal marine sediments (Lenk et al., 2011; Acosta-Gonzalez et al., 2013; Dyksma et al., 2016), pyrite colonization experiments (Pjevac et al., 2014), iron hydroxide mats (Sudek et al., 2009), previous studies on active and inactive sulfide chimneys (Voordeckers et al., 2008; Sylvan et al., 2012; Sylvan et al., 2013; Kato et al., 2015a), mud volcanoes (Pachiadaki et al., 2010; Pachiadaki et al., 2011) and tube worm symbionts (Kubota et al., 2007; Losekann et al., 2008; Duperron et al., 2012) (Fig. S2 – S5). Environmental clusters containing the majority of gammaproteobacterial OTUs and reconstructed metagenomic sequences appear to be family level clades related to sulfur oxidizing bacteria of the genera *Thioalkalispira*, *Thiohalophilus*, *Thiogranum* (order *Chromatiales*), *Sedimenticola* (unclassified *Gammaproteobacteria*), and the BD7-8 clade (unclassified *Gammaproteobacteria*) (Sorokin et al., 2002; Narasingarao and Haggblom, 2006; Sorokin et al., 2007; Flood et al., 2015; Mori et al., 2015) (Fig. 2).

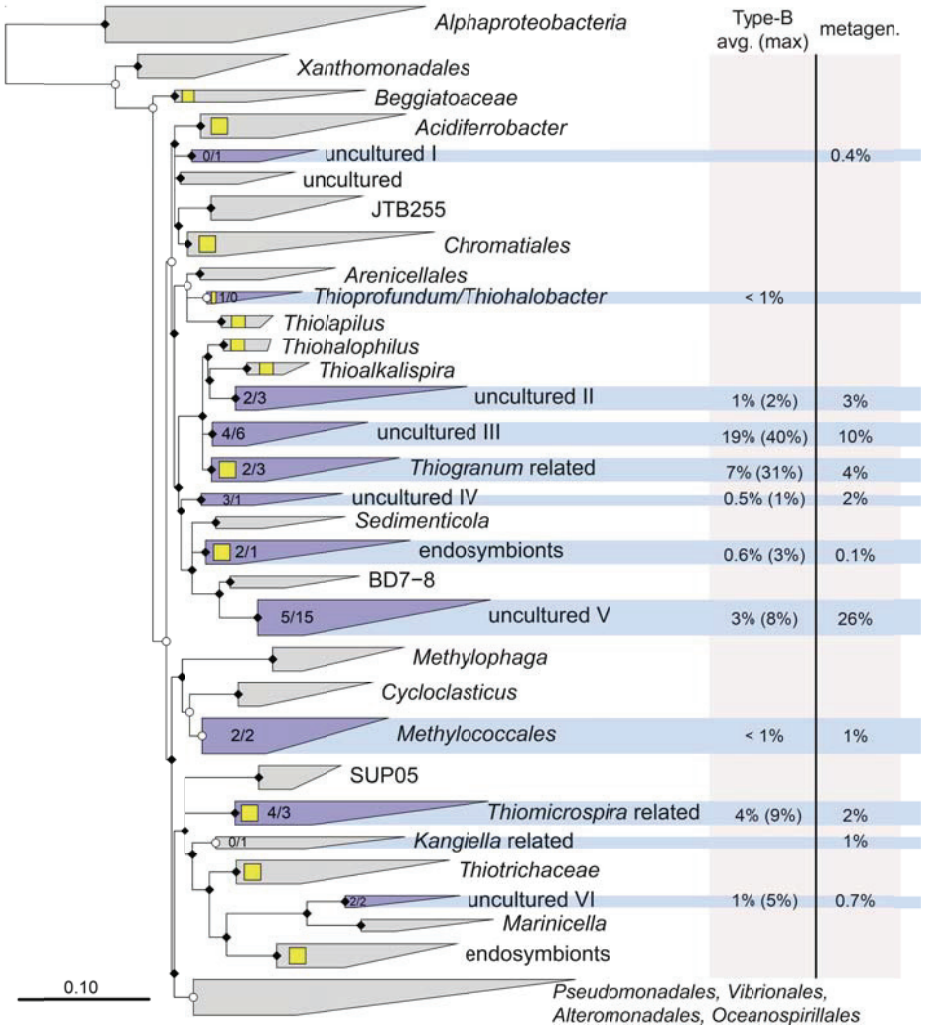

Figure 2: A maximum likelihood tree of gammaproteobacterial 16S rRNA sequences. Filled diamonds indicate branch support > 60%. White circles indicate branches of low significance as indicated by ARB based on support values and branch lengths. In purple – clusters containing sequences obtained in this study. Numbers on the triangles indicate the number of OTUs / number of reconstructed long 16S rRNA sequences contained in a cluster. Yellow squares indicated known sulfur oxidizing species in a cluster. On the right: cumulative relative abundances of OTUs and reconstructed sequences contained in a cluster for Type-B samples and StM-R1 metagenome, respectively. Detailed view of the clusters “uncultured III”, “Thiogranum related”, “uncultured V” and “Thiomicrospira related” can be found in Figure S2 – S5. The tree was constructed with FastTree (Price et al., 2010) using a 30% position conservation filter based on sequences > 1200 bp. Short sequences were added to the tree based on maximum parsimony in ARB.

Metagenomes

Further, we performed an assembly of the metagenome and binned the obtained contigs using MetaWatt (v3.5.2). Most of the obtained bins were classified as *Gammaproteobacteria*, while the second largest taxonomic group among the bins were *Deltaproteobacteria* (Fig. 3). Six *Gammaproteobacteria* bins and five *Deltaproteobacteria* bins were subjected to two rounds of read mapping and targeted de-novo assembly, which led to an improvement of overall assembly metrics (Table S1).

Despite being generated based on a consensus of several criteria (95% tetranucleotide frequency similarity, GC, coverage and connectivity by paired-end read mapping), some of the bins remained incomplete or contained a considerable level of redundancy according to lineage specific single-copy gene analysis. Duplicated genes encoding proteins with less than 90% sequence identity made up 58% of the single copy markers for Gamma-11, 57% for Delta-8, 17% for Delta-6 and 10% for Gamma-10, suggesting that these bins are probably pan-genomes of two closely related species. The rest of the bins had less than 6% of divergent gene duplications.

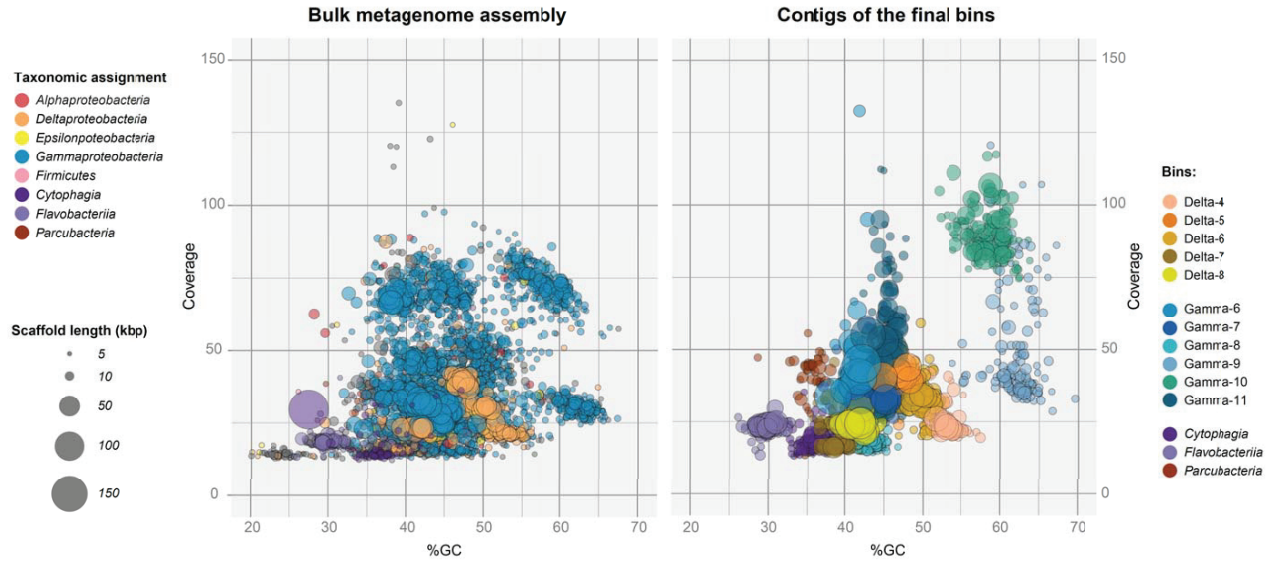

Figure 3: GC coverage plot of assembled contigs (>5000 bp). The left panel shows the first bulk assembly of the metagenome, where coloring indicates contig classification by MetaWatt (Strous et al., 2012). The right panel shows a combined plot of metagenomic bins re-assembled with SPAdes (Bankevich et al., 2012) (representing 25% of the bulk metagenome and 10% of the raw reads), where coloring indicates the assignment of a contig to a bin.

Final bin assemblies were annotated with the RAST automated annotation system (Aziz et al., 2008). Bins were named according to class-level classification and RAST project number: Gamma-6 to Gamma-11, and Delta-4 to Delta-8 (Fig. 4). We calculated a phylogenomic tree based on translations of 43 phylogenetically meaningful single copy genes (Parks et al., 2015) including genomes of all sequenced species indicated as possible relatives either by the affiliation of the reconstructed 16S rRNA sequences or by hits of the MetaWatt classification module (Fig. 5).

The order *Chromatiales* did not appear monophyletic in the protein based tree. Instead it was interrupted by genomes of *Methylococcales*, as already reported by Williams *et al.* (2010), and *Thiotrichales*. None of the bins fell into an existing cluster of genomes confirming the results of the 16S rRNA based analyses. Gamma-6 and Gamma-11 formed a branch on their own next to *Thiotrichaceae* and *Ectothiorhodospiraceae* genomes. Gamma-7, -9, -10 formed another deep branch next to the *Methylococcales* order. Gamma-8 formed a branch on its own next to *Kangiella*. Bins of *Deltaproteobacteria* formed two deep branching clusters on their own. Delta-4, -5 and -6 formed a cluster between *Desulfuromonadales* (*Geobacter*, *Desulfuromonas*) and *Nitrospirae* (phylum) genomes. The *Desulfuromonadales* are classified as *Deltaproteobacteria* but do not form a monophyletic group with the rest of the class. Delta-7 and -8 were placed next to *Leptospirillum ferrooxidans* and *Nitrospina* genomes. Together, *Leptospirillum* (phylum *Nitrospirae*), *Nitrospina* (phylum *Nitrospinae*) and Delta-7 and Delta-8 formed a branch between *Desulfobulbaceae* (class *Deltaproteobacteria*) and *Desulfuromonadales* (currently class *Deltaproteobacteria*).

Analysis of average nucleotide identities (ANIs) between the bins and closest related genomes revealed no ANI over 70% to any present genome, except for 71% between Gamma-9 and *Thioalkalivibrio sulfidophilus* HL-EbGr7 (Tab S2a). Although this value is just above the genus threshold (Goris et al., 2007), short alignment length (18% of *T. sulfidophilus* genome) and placement within the genome tree argue against Gamma-9 belonging to the *Thioalkalivibrio* genus. Among the bins, Gamma-9 and Gamma-10 likely constitute one genus (84% ANI), as well as Delta-4, Delta-5 and Delta-6 (77% - 85% ANI) and Delta-7 and Delta-8 (72% ANI) (Tab S2b).

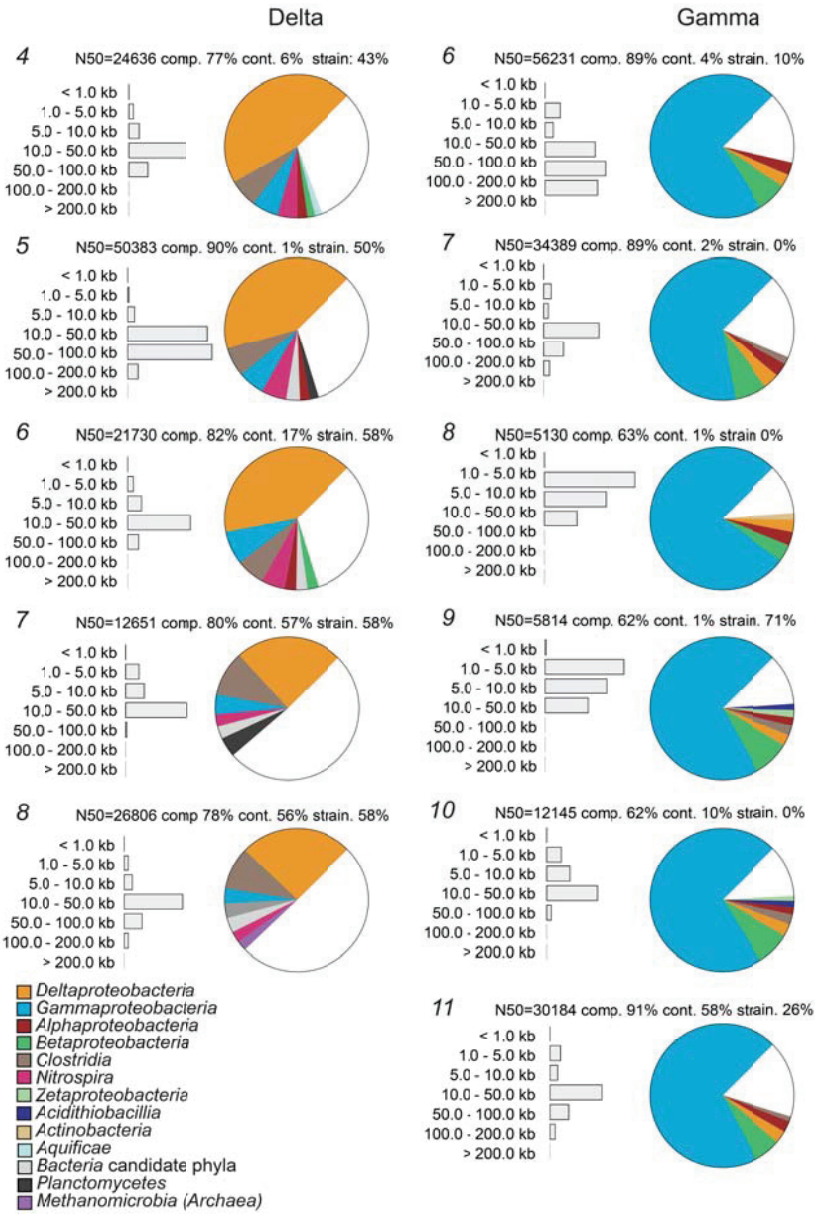

Figure 4: Classification of bins of interest by MetaWatt (Strous et al., 2012) and basic bin metrics. Coloring of the pie charts indicates class-level assignment of diamond blastx hits to reference genomes database. N50 value in bp, completeness in percent, contamination (duplicated genes with amino acid identity <90%) and strain heterogeneity (duplicated genes with amino acid identity >90%) according to lineage specific conserved single genes as determined by CheckM (Parks et al., 2015). Grey horizontal bars indicate contig length distribution.

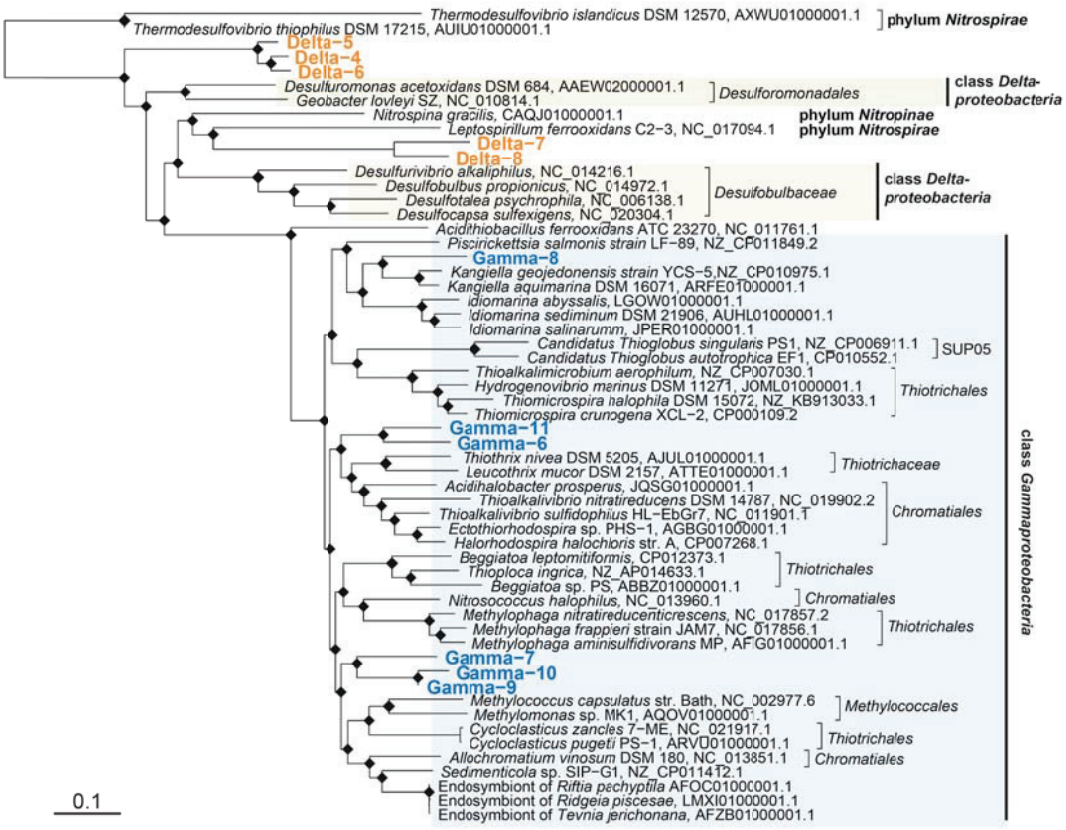

Figure 5: A maximum likelihood tree based on concatenated alignments of 43 phylogenetically meaningful single-copy marker genes (Parks et al., 2015) encoded in the bins and reference genomes. Filled diamonds indicate approximate Bayesian branch support values of >60%. In blue – gammaproteobacterial bins, in orange putative deltaproteobacterial bins. Note the intermixing of *Thiotrichales* and *Chromatiales* orders of *Gammaproteobacteria* as well as the positioning of *Desulfuromonadales* apart from other *Deltaproteobacteria*.

Looking into the metabolic potential encoded on the binned contigs (Fig. 6), we found the main differences being between the overall groups of *Delta-* and *Gammaproteobacteria*, rather than between individual bins. All *Gammaproteobacteria* harbored at least two genes encoding proteins of the sulfur compounds oxidizing Sox complex. All Gamma bins except Gamma-8 and Gamma-11 contained reverse dissimilatory sulfite reductase (rDsr) and adenosine-5′-phosphosulfate reductase (AprAB) genes. Further, most of the *Gammaproteobacteria* bins (8, 9, 10, 11) encoded nickel-iron hydrogenases (Hyd *and* Hyp *proteins*) indicating hydrogen being a further

possible electron donor. No sulfur oxidation related genes were detected in *Deltaproteobacteria* bins. Hydrogenase genes were present in Delta-4, -5 and -6.

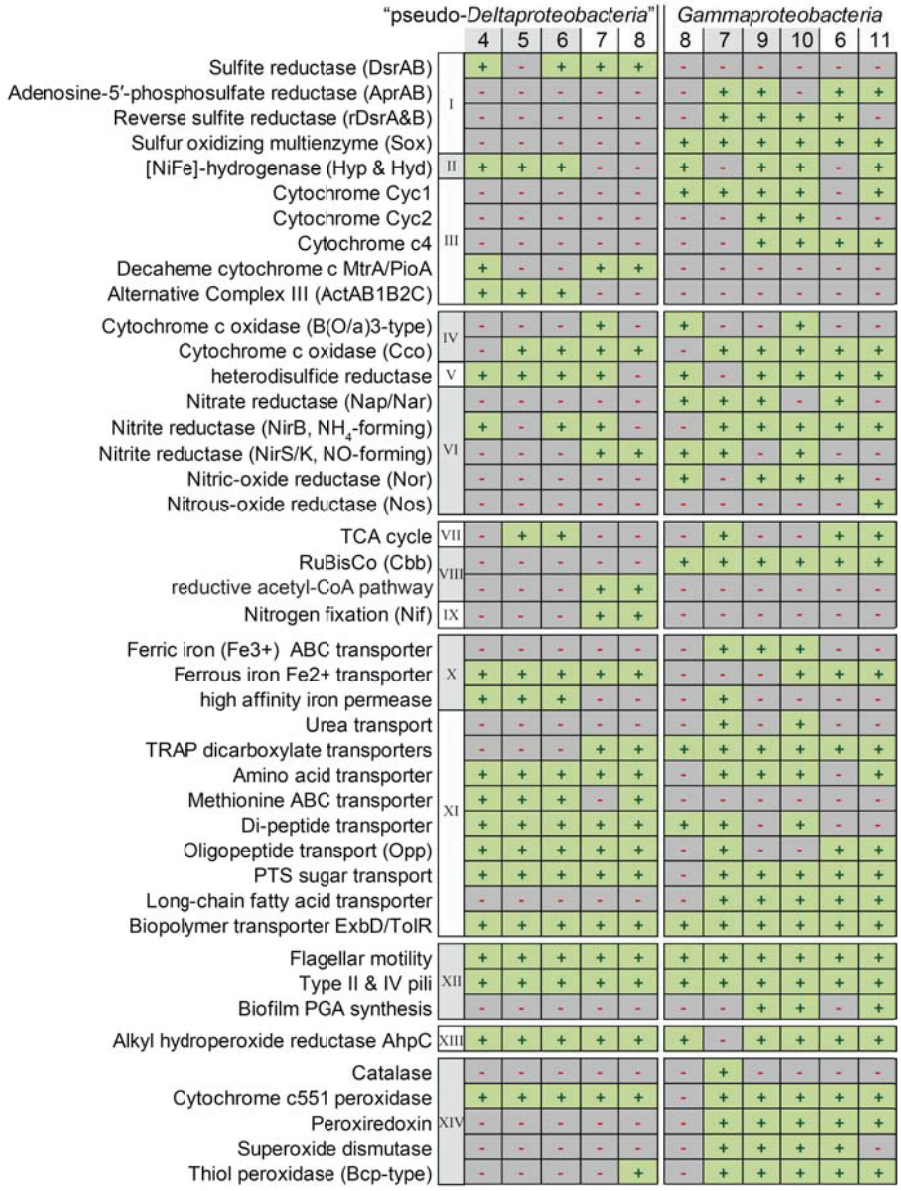

Figure 6: Presence of genes indicative of metabolic pathways and possible lifestyles. Green plus indicates presence of a pathway / key enzyme in the bin. Red minus indicates no pathway specific gene present in the bin. The bins are ordered based on their position in phylogenomic tree. Gene categories: I – sulfur oxidation/reduction; II – hydrogen oxidation; III – putative iron oxidation; IV – aerobic terminal oxidases; V – flavin based electron bifurcation; VI – nitrogen reduction; VII – carbon oxidation; VIII – carbon fixation; IX – N_2-fixation; X – iron transport; XI – organic compounds transport; XII – motility and attachment; XIII – nitrosative stress tolerance; XIV – oxidative stress tolerance.

In addition, we performed a phmmer search for cytochromes recently reported to be involved in iron oxidation, Cyc1 and Cyc2 (Ilbert and Bonnefoy, 2013; Barco et al., 2015; Kato et al., 2015b). While Cyc1 was found in all gammaproteobacterial bins except Gamma-6, Cyc2 was only found in Gamma-9 and 10 (Fig. 6). Unclassified C4-type cytochromes were found in Gamma-6, 9, 10 and 11 by Pfam motif search. Proteins of the alternative complex III (ActA, ActB1, ActB2, and ActC) which might also be involved in electron transport during iron oxidation could not be detected in *Gammaproteobacteria*. On the other side, Delta-4, 5 and 6 contained genes encoding proteins of the alternative complex III. Neither Cyc1 nor Cyc2 was detected in *Deltaproteobacteria* bins. However, genes encoding a decaheme cytochrome c (MtrA/PioA) involved in neutrophilic iron oxidation could be detected in Delta-4, 7 and 8 (Fig. 6).

All gammaproteobacterial bins, except Gamma-8, encoded a CBB3 type cytochrome C oxidase (CBB3-type). Gamma-8 encoded a rather rare B(O/a)3-type cytochrome-c oxidase (Sakamoto et al., 1997) (Fig. 6). Cytochrome C oxidase genes were also found in all deltaproteobacterial bins except Delta-4. Gamma-6, -7, -8, -9 encoded dissimilatory nitrate reductase (Nap/Nar) and all gammaproteobacterial bins, except Gamma-8, contained genes for ammonia forming nitrite reductase (NirB). NO-forming nitrite reductases (NirK/S) genes were present in Gamma-7, 8, 10. Gamma-6, -8, -9 and -10 further contained nitric oxide reductase (Nor) genes, while only Gamma-11 encoded a nitrous oxide reductase (Nos). In *Deltaproteobacteria* bins, the only nitrogen reduction related genes were the ones encoding ammonia forming NirB nitrite reductase (found in Delta-4, 6, and 7). Delta-7 and 8 contained a nitrogenase (NifH) encoding gene and other nitrogen fixation related genes (NifA, NifB, NifN, NifU). We found a dissimilatory sulfite reductase (DsrAB) in almost all Delta*proteobacteria* bins (except Delta-5) (Fig. 6). However, we only found the phosphoadenylyl-sulfate reductase (CysH) known from assimilatory sulfate reduction in *Deltaproteobacteria* bins. Adenosine-5′-phosphosulfate reductase (Apr) was only found in *Gammaproteobacteria* bins (Gamma-6, -7, -9, -11) as mentioned earlier.

All gammaproteobacterial bins contained genes encoding the RuBisCo enzyme (Cbb). The TCA cycle was only complete in Gamma-6, 7 and 11. Delta-4, 5 and 6 contained no genes encoding inorganic carbon fixation, while Delta-7 and 8 encoded key enzymes of the reductive acetyl-CoA pathway, NADP-dependent formate

dehydrogenase (Fdh) and carbon-monoxide dehydrogenase (Acs). A complete TCA cycle was present in Delta-5 and 6.

Transporter proteins involved in amino acid, di- and oligopeptide transport, as well as phosphor-transferase sugar transport systems were present in all *Deltaproteobacteria* bins, whereas some of them were missing in *Gammaproteobacteria* bins. Methionine ABC transporter genes were exclusively found in *Deltaproteobacteria* (all, except Delta-7). Genes for long-chain fatty acid transport proteins were only found in *Gammaproteobacteria* (all, except Gamma-8).

Finally, we searched the metagenome for adaptations to the environment such as motility, attachment or adaptations to oxidative stress. All bins encoded flagellar motility as well as type II and type IV pili. Genes involved in oxidative stress response were more diverse in *Gammaproteobacteria*. Apart from a Bcp-type thiol peroxidase detected in Delta-8, *Deltaproteobacteria* bins encoded only the cytochrome c551 peroxidase. *Gammaproteobacteria* bins also encoded peroxiredoxin (Gamma-6, -7, -9, -10, -11), Bcp-type thiol peroxidase (Gamma-6, -7, -9, -10, -11) and superoxide dismutase (Gamma-6, -7, -9, -10). Gamma-7 also encoded a catalase. Gamma-8 lacked any of those genes (Fig. 6).

Discussion

Comparing microbial community composition of five inactive hydrothermal chimneys to six active and further two inactive chimney samples published earlier (Meier *et al.* in prep.), all collected at various sites of the Manus Basin hydrothermal area, we detected a clear-cut shift. Microbial communities populating sulfide deposits exposed to active venting and communities populating inactive chimney structures not influenced by nearby fluid emissions were significantly different.

The community shift we observed is similar to the one reported for the Southern Mariana Trough or East Pacific Rise (Kato et al., 2010; Sylvan et al., 2012). Our long 16S rRNA gene sequences of dominant *Gammaproteobacteria* fall together with environmental sequences from coastal sediments (Lenk et al., 2011; Dyksma et al., 2016), mud volcanoes (Pachiadaki et al., 2010; Pachiadaki et al., 2011), tubeworm symbionts (Kubota et al., 2007; Losekann et al., 2008; Thornhill et al., 2008) and other studies of active and inactive hydrothermal chimneys (Sylvan et al., 2012; Sylvan et al., 2013) showing the ubiquity of these gammaproteobacterial clades and their potential global importance for the sulfur, iron and carbon cycling. The sequences from tidal flats contained in the “uncultured V” cluster (Fig. 2, Fig. S4), for example, were recently assigned to the globally distributed *Siboglinidae* symbionts related (SSr) clade responsible for a considerable fraction of carbon fixation in the sediments (Dyksma et al., 2016).

Previous 16S rRNA based studies of hydrothermal deposits communities attributed the dominant *Gammaproteobacteria* to the order *Chromatiales* (Sylvan et al., 2012; Kato et al., 2015a). Our phylogenetic analysis of 16S rRNA genes roughly confirms this affiliation, even though the support for the branching is rather weak. Gammaproteobacterial sequences abundant on inactive sulfide deposits of the Manus Basin form well supported family-level clades together with other environmental sequences, not represented by cultured strains. Closest described relatives of these clades are *Thiogranum longum* (*Chromatiales*) (Mori et al., 2015), *Thiohalophilus spp.* (Sorokin et al., 2007) and *Thioalkalispira spp.* (Sorokin et al., 2002) (*Chromatiales*), and *Sedimenticola spp.* (unclassified *Gammaproteobacteria*) (Narasingarao and Haggblom, 2006; Flood et al., 2015). The phylogenetic analysis based on concatenated alignments of translated single-copy marker genes showed that Gamma-6, -7, -9, -10, -11 bins form

novel lineages within the class *Gammaproteobacteria*, loosely affiliated to the *Chromatiales* and *Thiotrichales* orders. In general, it confirms the results of 16S rRNA based analysis showing significant level of divergence of *Gammaproteobacteria* occurring on inactive chimneys from known groups, although the lack of potentially closely related genomes (as indicated by 16S phylogeny) of e.g. *Thiogranum* or *Thiohalophilus* prevent a more precise classification.

Chemolithoautotrophic *Alpha*- and *Gammaproteobacteria* capable of iron oxidation or growth on poly-metal sulfides have been isolated from hydrothermal environments before (Wirsen et al., 1993; Eberhard et al., 1995; Edwards et al., 2003). The 16S rRNA sequences of the isolates retrieved by Edwards and colleagues (2003) were related to heterotrophic genera of *Alpha*- and *Gammaproteobacteria*, such as *Thalassospira*, *Pseudomonas* and *Marinobacter* not observed in our study. The poly-metal sulfide oxidizing strains isolated by Wirsen and colleagues (1993) belong to the genus *Thiomicrospira* which was detected in our samples, although at low relative abundance. However, the genomes of these likely less representative isolates remain unsequenced and no genomic information is available for the clades reported to dominate inactive sulfides by culture independent studies. Here, we provide the first (meta-)genomic insights into these microbial communities. Our metagenome analysis confirms and specifies the 16S rRNA and isolates based assumptions about metabolic potential of bacteria living on inactive sulfide chimneys (Wirsen et al., 1993; Eberhard et al., 1995; Kato et al., 2010; Sylvan et al., 2012). Genes indicating a sulfur oxidation based chemolithoautotrophic lifestyle were found in all *Gammaproteobacteria* bins. Apart from that, ability of hydrogen oxidation seems to be a common trait among the analyzed *Gamma*- and *Deltaproteobacteria*. The presence of RuBisCo genes in all *Gammaproteobacteria* bins is concordant with the lipid biomarker analysis of the same sample (Reeves et al., 2014) suggesting the Calvin-Benson-Bassham cycle as the main carbon fixation pathway in inactive sulfide deposit communities.

No known iron oxidizing organisms like e.g. *Zetaproteobacteria* oxidizing iron contained in the ocean crust (Kato et al., 2009; Forget et al., 2010; Edwards et al., 2011; McAllister et al., 2011; McBeth et al., 2013; Singer et al., 2013; Field et al., 2015) were detected in our samples. Yet the cytochromes reportedly involved in iron oxidation (Castelle et al., 2008; Ilbert and Bonnefoy, 2013; Barco et al., 2015; Kato et al., 2015b) were found in some *Gamma*- and *Deltaproteobacteria* bins giving a possibility of active microbial leaching of the minerals instead of just using the sulfide released via abiotic

oxidation of iron-sulfides (Schippers and Jørgensen, 2002; Jørgensen and Nelson, 2004). According to our phylogenomic analysis, Delta-7 and Delta-8 bins could be related to *Leptospirillum ferrooxidans,* an iron oxidizer well known from bioleaching of sulfide minerals and acid mine drainage (Schrenk et al., 1998; Hippe, 2000; Sand et al., 2001), than to *Deltaproteobacteria*.

Interestingly, two *Sulfurimonas* OTUs and some *Bacteroidetes* OTUs appeared to be characteristic for the Type-B samples. Although in general, *Bacteroidetes* and *Sulfurimonas* sequences appeared in considerable proportions in other samples, there are several OTUs which appeared exclusively on inactive chimneys (Fig. 1). The *Bacteroidetes* species are most likely heterotrophic, since no autotrophs are known in this phylum. In marine environments they are known to specialize on degradation of polysaccharides after an algal bloom (Cottrell and Kirchman, 2000; Pinhassi et al., 2004; Bauer et al., 2006; Gomez-Pereira et al., 2012). Recently *Bacteroidetes* species have also been shown to degrade polysaccharides produced by a hydrothermal epsilonproteobacterial biofilm (Stokke et al., 2015). Substrate specificity might play a role in differentiation of *Bacteroidetes* OTUs on active and inactive chimneys (Gomez-Pereira et al., 2012; Teeling et al., 2012), assuming that *Gammaproteobacteria* dominating on inactive chimneys produce different polysaccharides than *Epsilonproteobacteria*. The *Sulfurimonas* sequences specific for inactive deposits could represent a *Sulfurimonas* species rather specialized on oxidation of solid phase sulfur inclusions in chimneys, than on reduced sulfur compounds contained in the fluids. Supporting this speculation, *Sulfurimonas* and *Sulfurovum* was successfully enriched on sulfur particles in tidal flats (Pjevac et al., 2014).

Another remarkable observation are the Type-C communities, which are dominated by *Thermodesulfovibrio* (phylum *Nitrospirae*) related sequences. While the samples originate from seemingly inactive sulfides not exposed to any visible venting, dominance of a thermophile (Sonne-Hansen and Ahring, 1999; Haouari et al., 2008; Sekiguchi et al., 2008) in hosted microbial communities could indicate either a very recent ceasing of venting or a conductive heating from below. On the other hand, these dominant sequences could represent a cold-adapted relative of *Thermodesulfovibrio* and Type-C samples a different weathering stage of the chimneys. Supporting this hypothesis, *Nitrospirae* sequences have been detected previously in inactive hydrothermal deposits (Suzuki et al., 2004; Kato et al., 2015a).

Differences in microbial community composition between different hydrothermal deposits have so far been explained by difference in mineralogy, such as e.g. change from sulfides to basalts (Sylvan et al., 2013; Toner et al., 2013). In our case, all sampled deposits are sulfides and we did not observe any community structuring depending on the mineral composition of the chimneys. Instead, we detected several co-occurring chemolithoautotrophic organisms with similar genomic repertoire, but belonging to different lineages within the class of *Gammaproteobacteria*. This diversity might be explained by different preferences for sulfur minerals among the different species. For example, a *Thiomicrospira* strain retrieved by Wirsen *et al.* (1993) was shown to grow better on chalcopyrite than on other metal sulfides (Eberhard et al., 1995). Also, a succession of different clades of metal-sulfide oxidizing microorganisms throughout different stages of weathering cannot be excluded. Similar successions have been shown in archaeal biofilms on carbonate chimneys, where different OTUs appear to be abundant dependent on the age of the chimney (Brazelton et al., 2010). Our dataset is based on DNA analysis and therefore rather static. Further research should therefore involve better resolved genomes and activity-based techniques studies applying e.g. transcriptomics or proteomics, but also visualization techniques in order to further deepen our understanding of these fascinating microbial habitats.

Conclusions

With this study we confirm the existence of microbial communities specific for inactive sulfide deposits and show that the dominating gammaproteobacterial clades belong to yet undescribed families most probably belonging to the order *Chromatiales*. Further, we provide the, to the best of our knowledge, first metagenomic insight into the metabolic potential of microbial groups populating inactive hydrothermal deposits. The metagenome reveals that sulfur and iron oxidation are probably the main processes fueling the carbon fixation in these communities. Chemolithoautotrophic gammaproteobacterial clades dominating the inactive hydrothermal chimneys also occur in a wide range of benthic environments and, considering the wide distribution of iron-sulfur minerals on the ocean floor, might contribute significantly to carbon fixation in deep sea habitats other than hydrothermal vent fields.

Acknowledgements

We would like to thank officers, crew, shipboard scientific party, and the technical team of the ROV Quest 4000m (MARUM) on R/V Sonne cruise SO216, for their invaluable assistance. The cruise SO216 with R/V Sonne was an integral part of the Cluster of Excellence of the MARUM 'The Ocean in the Earth System, Research Area GB: Geosphere-Biosphere Interactions' funded by the German Research Foundation (DFG). We thank Richard Reinhard, Bruno Huettel and the team of the Max Planck Genome Centre in Cologne for sequencing and Hanno Teeling for help with computational analyses. Further, we thank Kathrin Büttner for assistance in the Molecular Ecology department. This work was supported by the Max Planck Society.

Experimental procedures

Site description and sample collection

Manus Basin is a fast-spreading back-arc basin with multiple basaltic- to felsic-hosted hydrothermal fields located between New Britain and New Ireland islands (Bismarck Sea, Papua New Guinea). All samples (Tab. 1) were collected during R/V Sonne expedition SO-216 in June/July 2011 from the PACManus and SuSu Knolls hydrothermal vent fields emitting highly sulfidic fluids leading to an abundant poly-metal sulfides deposition (Binns and Scott, 1993; Reeves et al., 2011b; Thal et al., 2014; Yeats et al., 2014). Active and inactive hydrothermal chimney samples were collected with the ROV's hydraulic arm and kept in closed bio-boxes during ROV ascent. Retrieved hydrothermal sulfide structures were subsampled and directly frozen at -20°C for DNA extraction.

Collected chimneys are mainly composed of chalcopyrite ($CuFeS_2$), pyrite/marcasite (FeS_2), sphalerite (Zn(Fe)S), and bornite (Cu_5FeS_4) (Tab. 1). StM-R1, RR-R1 and RR-R2 samples have been used for lipid biomarker analysis and 16S rRNA gene amplicon pyro-sequencing in (Reeves et al., 2014). Illumina sequences of 16S rRNA gene amplicons from Nsu-R1a, NSu-R1b, NSu-R2b, RR-R1, RR-R2, SnC-R1, Sol8-R1b, and StM-R1 were published in Meier *et al.* (in prep).

16S rRNA gene amplicon sequencing and analysis

DNA was extracted from 0.5 – 1 g of crumbled chimney material using the Power Soil kit (MO BIO Laboratories, Carlsbad, CA, USA) with an additional 1 h Proteinase K digestion step at 37°C and a 2 h incubation at 65°C after the addition of SDS containing buffer S1 prior to applying the kit protocol. The V3-V4 region of the 16S rRNA gene was amplified as described in Meier *et al.* (2016) sequenced on an Illumina MiSeq sequencer at the Max Planck Genome Centre (Cologne, Germany). After trimming of 3'-ends with quality below q10, paired-end reads were merged using BBmerge with a minimum overlap of 50 bp.

Merged amplicon reads were de-multiplexed and randomly subsampled to 5000 reads per sample using Mothur v.1.34 (Schloss et al., 2009). Reads of the whole dataset were decomposed into "nodes" by MED v2.0 (Eren et al., 2015) with 4 discriminant locations and minimum substantive abundance (count of the most abundant sequence in a node) of 3. Finally, OTUs were generated based on the MED nodes using SWARM (Mahe et al., 2015) with the "fastidious" option, 20 as the number of sequences in a node for it to be considered "big" and otherwise default parameters. Representative sequences of the OTUs generated by SWARM (centroids of the swarms) were aligned to the SILVA seed database and classified by last common ancestor (LCA) using the SINA online aligner (Pruesse et al., 2012; Quast et al., 2013).

The similarity percentages breakdown (SIMPER, Clarke, 1993) was performed using the implementation in R-package vegan v. 2.2-1 (Oksanen et al., 2013) with 999 permutations and otherwise standard parameters.

Metagenome sequencing and assembly

Genomic DNA was extracted from 7 g of the StM-R1 sample with an SDS-lysis and CTAB protein precipitation based protocol as described in Zhou *et al.* (1996) and shotgun sequenced on an Illumina HiSeq sequencer at the Max Planck Genome Centre (Cologne, Germany).

Bulk assembly of the metagenomes as well as reconstruction of full length 16S rRNA reads was performed as described previously (Meier et al., 2016). K-mer depth for read normalization was adjusted to 40 according to the number of reads obtained. Assembled contigs were binned based on a weighted combination of tetranucleotide frequencies (>95% similarity), GC-content and coverage values, paired reads mappings, presence of

138 conserved single-copy genes (Campbell et al., 2013) and similarity of taxonomic classification using MetaWatt v 3.5.2 (Strous et al., 2012).

Bins of interest were used for two rounds of read mapping, targeted de-novo assembly of mapped reads using SPAdes v3.7 (Bankevich et al., 2012) and binning as described in Meier *et al.* (2016). The improved assemblies of the bins were automatically annotated with the standard RAST annotation pipeline (Aziz et al., 2008) and loaded into the GenDB (Meyer et al., 2003) annotation system for comparative analyses using the JCoast frontend (Richter et al., 2008). Completeness and quality of final assemblies was assessed by CheckM (Parks et al., 2015) using translated protein sequences exported from RAST and a lineage specific set of conserved single copy genes. Average nucleotide identities to the next sequenced relative and between the assemblies were calculated using the JSpeciesWS web service (Richter et al., 2015). The reference genomes were selected based on affiliation of 16S rRNA sequences reconstructed from the metagenome, majority of diamond blastx hits against a database of reference gnomes (one genome per genus) during the MetaWatt contig classification step, and tetranucleotide based relative search in JSpeciesWS.

Gene annotation

The annotation of genes of interest was manually inspected by comparing the results of the RAST annotation to hidden Markov model based HMMER3 (Eddy, 2011) searches against the Pfam-A database (Finn et al., 2014) and BLASTP searches against the NCBI-Nr database. Cytochromes involved in iron oxidation as well as other iron oxidation related proteins were not explicitly annotated as such by RAST. Therefore, search for proteins involved in iron oxidation was performed as follows: Sequences of proteins reported to be involved in iron oxidation, such as Cyc1 and Cyc2 cytochromes, MtrA/PioA decaheme cytochrome and proteins of the Alternative Complex III, were downloaded from UniprotKB (Magrane and Consortium, 2011). Downloaded proteins were used as a query for a phmmer (Eddy, 2011) search against the protein sequences encoded on annotated contigs as a database (p-value cutoff of 1e-15). Subsequently, the hits were searched against the whole UniprotKB database. In case results from both searches were agreeing, the annotation of the gene was changed.

Phylogenetic tree construction

First, PhyloFlash reconstructed sequences longer than 1200 bp together with related high quality sequences from SILVA SSU123 NR99 database (sequence quality >95) were selected. All 16S rRNA sequences were aligned to a curated SILVA SSU123 NR99 database (Quast et al., 2013), where all sequences with a pintail value below 50 and alignment quality below 70 were removed. Only sequences with an alignment quality >95 were kept for tree calculations. Tree calculations were done with neighbor-joining algorithm implemented in ARB (Ludwig et al., 2004), PhyML (Guindon et al., 2010) and FastTree v 2.1.9 (Price et al., 2009, 2010) employing 30% and 50% positional conservation filters and the bacterial positional variability filter of SILVA. Multifurcations were created with ARB for branches with less than 50% support and branches shorter than 0.005 changes per base. OTU-representative Illumina amplicon sequences were added to the calculated trees based on maximum parsimony without changes of the overall tree topology. Representative maximum likelihood tree shown in paper was calculated with FastTree using 30% positional conservation filter. Branch support was calculated by FastTree based on Shimodaira-Hasegawa test with 1000 resamplings.
Concatenated alignment of 43 phylogenetically informative single copy genes (Parks et al., 2015) was generated with HMMER3 (Eddy, 2011) in CheckM (Parks et al., 2015). Concatenated alignments were imported into ARB and maximum likelihood trees were calculated using PhyML and FastTree v 2.1.9 without filters as well as based on positions conserved in 25% of the sequences. The topology of the tree remained largely stable throughout different calculations. The tree shown was calculated with PhyML based with 25% positional conservation filter.

Nucleotide sequence accession numbers

Sequence data will be submitted to the European Nucleotide Archive and made public by the release of this study.

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Supplementary material

Figure S1

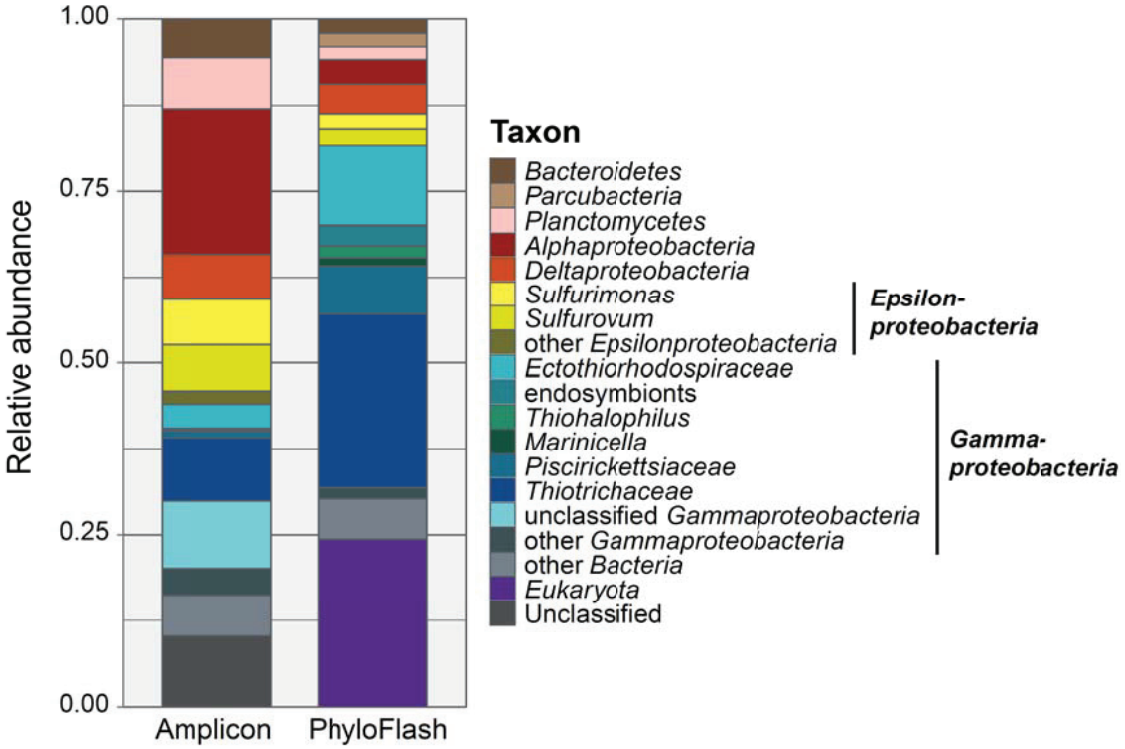

Figure S1: Relative abundances of small subunit rRNA gene sequences in the StM-R1 sample as determined by 16S rRNA gene amplicon sequencing (left) and PhyloFlash 16S/18S rRNA gene reconstruction from metagenomic reads (right)

Figure S2

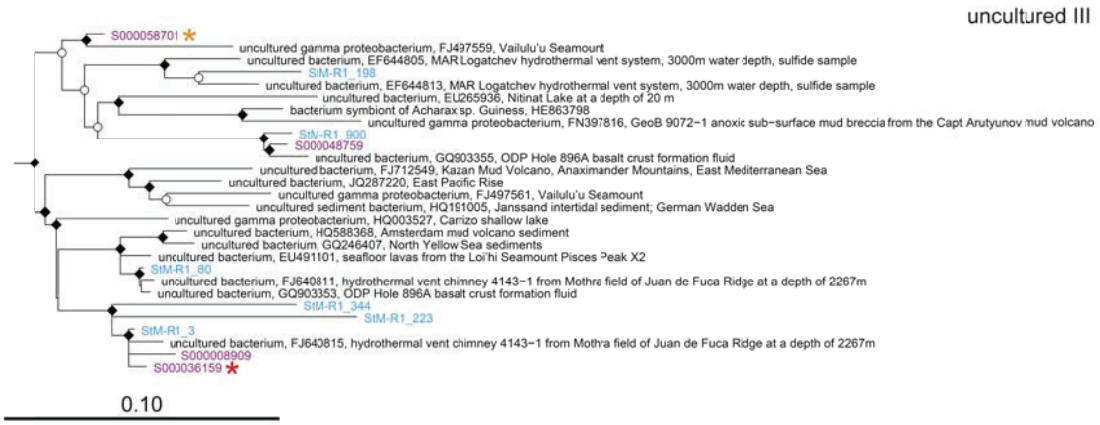

Figure S2: The "uncultured III" *Gammaproteobacteria* cluster as found in the maximum likelihood phylogenetic tree in Figure 2. In blue – 16S rRNA sequences reconstructed from metagenome reads. In purple 16S rRNA gene amplicon sequences representing OTUs generated by SWARM. Red star marks the most abundant gammaproteobacterial OTU (in Type-B samples: 10% on average, 36% max.). Orange star marks the second most abundant OTU (in Type-B samples: 8% on average, 20% max.).

Figure S3

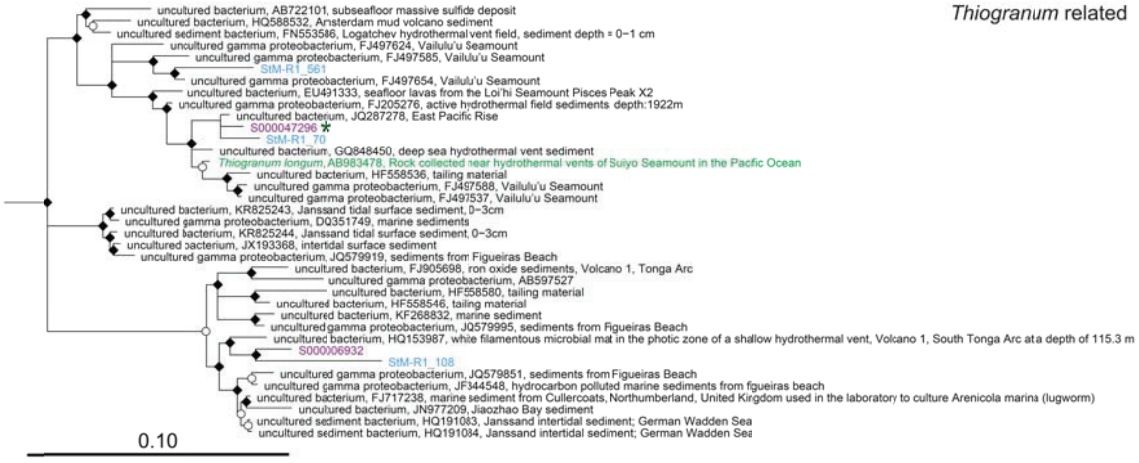

Figure S3: The "Thiogranum related" Gammaproteobacteria cluster as found in the maximum likelihood phylogenetic tree in Figure 2. In blue – 16S rRNA sequences reconstructed from metagenome reads. In purple 16S rRNA gene amplicon sequences representing OTUs generated by SWARM. Green star marks third most abundant gammaproteobacterial OTU (in Type-B samples: 7% on average, 30% max.).

Figure S4

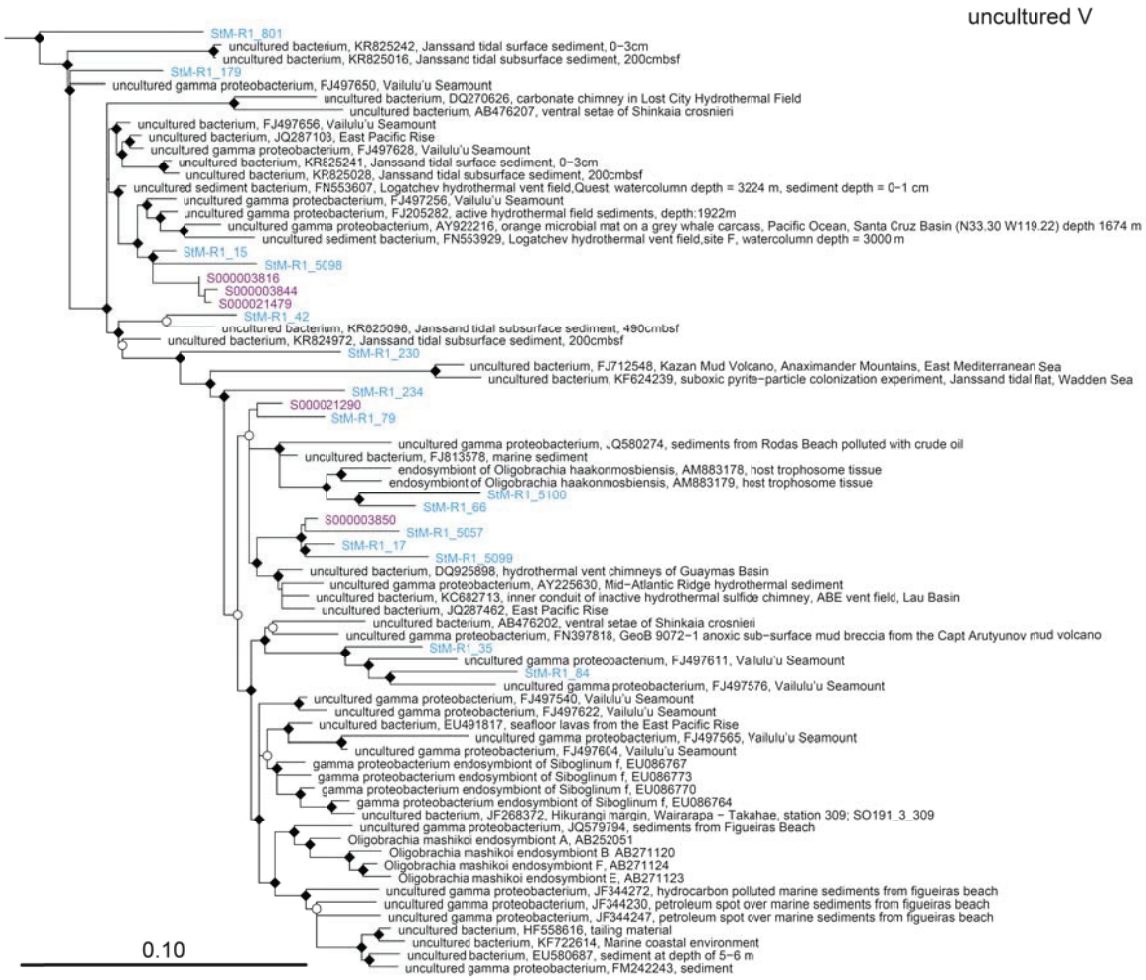

Figure S4: The "uncultured V" *Gammaproteobacteria* cluster as found in the maximum likelihood phylogenetic tree in Figure 2. In blue – 16S rRNA sequences reconstructed from metagenome reads. In purple 16S rRNA gene amplicon sequences representing OTUs generated by SWARM.

Figure S5:

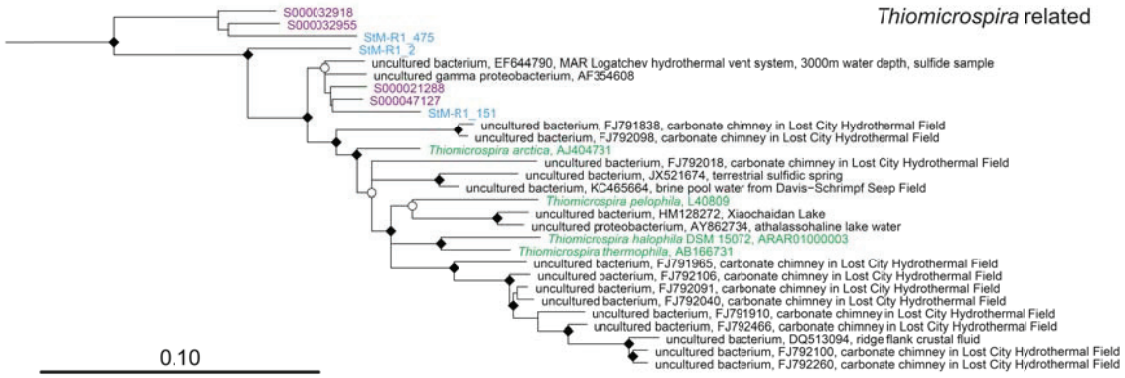

Figure S5: The "*Thiomicrospira* related" *Gammaproteobacteria* cluster as found in the maximum likelihood phylogenetic tree in Figure 2. In blue – 16S rRNA sequences reconstructed from metagenome reads. In purple 16S rRNA gene amplicon sequences representing OTUs generated by SWARM.

Table S1: Metagenome assembly metrics

	bulk assembly	combined bins*
total read pairs (2x100bp)	55,414,317	
assembly size	146.17 Mbp	39.23 Mbp
average coverage	29x	25.4x
reads mapped	38%	9%
number of scaffolds	68,717	4,888
scaffold N50	4,049 bp	18,051
max scaffold	325 kbp	177 kbp
classified scaffolds	47%	93%
unclassified scaffolds	53%	7%

*combined metrics for the scaffolds of 14 bins shown in Figure 3

Table S2a: Average nucleotide identities, *Gammaproteobacteria* bins

	Gamma-6	Gamma-7	Gamma-8	Gamma-9	Gamma-10	Gamma-11	*Piscirickettsia salmonis* strain LF-89	*Kangiella aquimarina* DSM 16071	*Thiomicrospira crunogena* XCL-2	*Acidihalobacter prosperus*	*Thioalkalivibrio sulfidophilus* HL-EbGr7	*Ectothiorhodospira* sp. PHS-1	*Allochromatium vinosum* DSM 180	*Sedimenticola* sp. SIP-G1	endosymbiont of *Riftia pachyptila*
Gamma-6		66	65	65	65	67	65	65	65	64	64	64	64	65	64
Gamma-7	66		64	65	66	66	64	64	64	64	65	64	65	65	65
Gamma-8	65	64		64	64	64	65	65	64	64	63	63	63	64	64
Gamma-9	65	66	64		84	65	63	65	64	69	71	70	70	69	69
Gamma-10	64	66	63	83		66	63	64	63	68	69	68	68	68	68
Gamma-11	67	66	64	65	66		65	65	64	64	65	65	64	65	65
***Piscirickettsia salmonis* strain LF-89**	65	65	64	63	64	64		66	66	65	65	65	65	65	65
***Kangiella aquimarina* DSM 16071**	65	65	66	65	64	64	65		65	64	64	64	64	64	64
***Thiomicrospira crunogena* XCL-2**	65	64	64	64	64	64	65	65		64	64	64	64	64	64
Acidihalobacter prosperus	64	65	63	69	68	65	63	64	63		70	69	69	67	67
***Thioalkalivibrio sulfidophilus* HL-EbGr7**	64	65	63	70	69	65	64	65	63	70		74	69	68	68
***Ectothiorhodospira* sp. PHS-1**	64	65	63	69	68	65	64	64	63	69	74		68	68	68
***Allochromatium vinosum* DSM 180**	64	65	63	69	68	64	64	65	64	69	69	68		69	70
***Sedimenticola* sp. SIP-G1**	64	65	63	68	68	65	64	65	64	67	68	68	68		69
endosymbiont of *Riftia pachyptila* (vent Ph05)	64	65	63	69	68	65	63	64	63	67	68	68	69	70	

In green – ANI > 70% (genus level; Konstantinidis and Tiedje, 2005; Goris et al., 2007)

Table S2b: Average nucleotide identities, *Deltaproteobacteria* bins

	Leptospirillum ferrooxidans C2-3	*Desulfurivibrio alkaliphilus*	*Desulfobulbus propionicus*	*Desulfotalea psychrophila*	*Desulfocapsa sulfexigens*	*Desulfuromonas acetoxidans* DSM 684	*Geobacter lovleyi* SZ	*Geobacter uraniireducens* Rf4	*Geobacter bemidjiensis* Bem	Delta-4	Delta-5	Delta-6	Delta-7	Delta-8
***Leptospirillum ferrooxidans* C2-3**		64	64	64	64	64	64	64	64	61	62	62	62	62
Desulfurivibrio alkaliphilus	63		67	65	65	64	65	65	65	63	63	63	61	62
Desulfobulbus propionicus	62	67		65	65	64	64	65	65	63	62	62	60	61
Desulfotalea psychrophila	65	65	66		67	65	65	65	64	62	62	63	62	62
Desulfocapsa sulfexigens	63	65	65	67		64	64	63	63	62	62	62	62	62
***Desulfuromonas acetoxidans* DSM 684**	62	64	64	64	63		66	65	65	62	63	62	61	62
***Geobacter lovleyi* SZ**	63	65	65	63	63	66		69	68	63	62	63	61	62
***Geobacter uraniireducens* Rf4**	63	64	65	64	63	66	69		71	63	62	63	62	62
***Geobacter bemidjiensis* Bem**	64	65	65	64	64	66	68	71		64	63	63	61	62
Delta-4	62	63	63	63	62	62	63	63	64		78	85	61	62
Delta-5	62	62	62	62	62	63	63	63	62	78		79	62	62
Delta-6	62	63	63	63	62	63	63	63	63	85	79		62	63
Delta-7	62	61	61	62	62	61	62	62	61	61	62	62		72
Delta-8	62	62	61	62	62	62	61	62	62	62	62	62	71	

In green – ANI > 70% (genus level; Konstantinidis and Tiedje, 2005; Goris et al., 2007)

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Chapter IV

Heterotrophic *Proteobacteria* in the vicinity of diffuse hydrothermal venting

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In press, *Environmental Microbiology*

doi:10.1111/1462-2920.13304

Contributions:

D.M and A.M. developed concepts and ideas. A.M. collected samples at the Menez Gwen hydrothermal field. D.M. performed experiments and data analysis, conceived and wrote the manuscript. E.P.R. and C.V. performed geochemical measurements at Menez Gwen and processed the raw data. W.B. assisted in modeling and thermodynamic analysis. H.R.G.-V. and M.R. assisted in bioinformatical analyses. W.B., P.R.G., H.R.G.-V., E.P.R., M.R., C.V., R.A., A.M. conceived and edited the manuscript

environmental microbiology

Environmental Microbiology (2016) **00**(00), 00–00 doi:10.1111/1462-2920.13304

Heterotrophic *Proteobacteria* in the vicinity of diffuse hydrothermal venting

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Summary

Deep-sea hydrothermal vents are highly dynamic habitats characterized by steep temperature and chemical gradients. The oxidation of reduced compounds dissolved in the venting fluids fuels primary production providing the basis for extensive life. Until recently studies of microbial vent communities have focused primarily on chemolithoautotrophic organisms. In our study, we targeted the change of microbial community compositions along mixing gradients, focusing on distribution and capabilities of heterotrophic microorganisms. Samples were retrieved from different venting areas within the Menez Gwen hydrothermal field, taken along mixing gradients, including diffuse fluid discharge points, their immediate surroundings and the buoyant parts of hydrothermal plumes. High throughput 16S rRNA gene amplicon sequencing, fluorescence *in situ* hybridization, and targeted metagenome analysis were combined with geochemical analyses. Close to diffuse venting orifices dominated by chemolithoautotrophic *Epsilonproteobacteria*, in areas where environmental conditions still supported chemolithoautotrophic processes, we detected microbial communities enriched for versatile heterotrophic *Alpha*- and *Gammaproteobacteria*. The potential for alkane degradation could be shown for several genera and yet uncultured clades. We propose that hotspots of chemolithoautotrophic life support a 'belt' of heterotrophic bacteria significantly different from the dominating oligotrophic microbiota of the deep sea.

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Introduction

At deep-sea hydrothermal vents, hot fluids enriched in reduced compounds are released into cold oxygenated sea water. Thereby, mixing generates redox gradients that allow for carbon fixation by chemolithoautotrophic microorganisms (for review: Orcutt *et al.*, 2011; Sievert and Vetriani, 2012), giving rise to extensive microbial and macrofaunal communities. This deep-sea hydrothermal vent microbiota is still poorly investigated, due to limited accessibility and the necessity for elaborate sampling techniques. A systematic understanding of structure and function of microbial communities along the often steep geochemical and physical gradients is missing (for review: Sievert and Vetriani, 2012).

Current insights originate from geochemical, microbial, molecular and ecological approaches. Microbiologists largely focused on the isolation of extremophiles and supposed key chemoautotrophs (Inagaki *et al.*, 2003; Kashefi and Lovley, 2003; Inagaki *et al.*, 2004; Takai *et al.*, 2008; Sievert and Vetriani, 2012) at hydrothermal vents. Culture independent studies detected differences in microbial community composition mostly by comparing samples from different diffuse fluid orifices, focusing on chemolithoautotrophic organisms (Nakagawa *et al.*, 2005; Huber *et al.*, 2010; Lanzen *et al.*, 2011; Roussel *et al.*, 2011; Meyer *et al.*, 2013). Shifts in the dominant bacterial groups have thereby been attributed to changes of geochemical parameters related to the dilution of available geofuels (Perner *et al.*, 2007; Flores *et al.*, 2011). Dramatic differences in microbial community composition have also been observed in diffuse fluids at the same spot, investigated in short time intervals (Perner *et al.*, 2013). A molecular study investigating microbial community structures in diffuse

hydrothermal fluids and plumes indicated a clear differentiation of bacterial communities associated with diffuse fluids and plumes (Anderson *et al*., 2013). Unlike diffuse fluids, hydrothermal plumes seem to have a more stable microbial community composition largely resembling that of the surrounding deep sea water column (Lesniewski *et al*., 2012; Sheik *et al*., 2015). More recent studies of hydrothermal plumes indicate chemolithoautotrophic potential in abundant clades like SUP05 and SAR324 (Anantharaman *et al*., 2014; Sheik *et al*., 2014; Anantharaman *et al*., 2016).

Heterotrophic microorganisms, have largely been neglected in previous studies, although they might play an important role in re-mineralization of carbon fixed by chemolithoautotrophs closing the carbon cycle of vent ecosystems. Cultivation attempts mostly targeted extremophiles, resulting, e.g., in the isolation of fermenting *Archaea*, such as *Thermococci* (Marteinsson *et al*., 1999; Jolivet *et al*., 2003; Gorlas *et al*., 2014; Price *et al*., 2015). Hydrogenotrophic methanogens have been shown to grow in co-culture with hyperthermophilic heterotrophs at vents (Ver Eecke *et al*., 2012). A study combining metagenomic and metatranscriptomic analyses provided evidence for the utilization of diverse organic compounds by deep-sea *Archaea* also detected in hydrothermal plumes (Li *et al*., 2015). Regarding *Bacteria*, genetic and isotope incorporation-based evidence was found for the oxidation of small organic compounds, like short-chain alkanes or organic acids, in plumes and diffuse fluids (Li *et al*., 2014b; Winkel *et al*., 2014) and recently Stokke *et al*. (2015) showed a trophic link between sulphur oxidizing *Sulfurovum* and heterotrophic *Bacteroidetes* in a hydrothermal chimney biofilm. However, the extent and diversity of microbial heterotrophy, the niches and localization of dominant heterotrophic clades within hydrothermally influenced ecosystems is largely unresolved.

In this study, we analysed microbial communities in hydrothermal fluids from different venting sites within the Menez Gwen hydrothermal field (Mid-Atlantic Ridge) as well as in the buoyant stems of hydrothermal plumes. Our main goal was to track the change of microbial community composition with decreasing hydrothermal influence and to identify major factors behind this community change. Since a previous study of Menez Gwen diffuse fluids (Winkel *et al*. 2014) pointed towards a quick response of resident bacteria to organic compounds, we also investigated the presence and potential role of heterotrophic microorganisms in these communities. Apart from 16S rRNA gene tag analysis and fluorescence *in situ* hybridization (FISH) conducted to determine microbial community composition, we used metagenomics to analyze the functional potential of microbial taxa at different points in the mixing gradient. Our study offers valuable insights into the structuring of microbial communities at hydrothermal fields on a small spatial scale (centimeters to meters) providing the evidence for a 'belt' of heterotrophic bacteria surrounding zones of primary production.

Results

Categorization of samples and thermodynamic considerations

Fluid samples were obtained from four different venting sites within the Menez Gwen hydrothermal field: 'White Flames' (WF), 'Cage Site' (CS), 'Woody' (Wd) and 'Milkyway' at the 'Babylon' venting site (Bb-Milky) (Supporting Information Table S1, Fig. 1). Additionally, samples of three rising plumes and one mesopelagic water column sample were collected. The aim was to compare three categories of habitats, further on referred to as Cat A, Cat B and Cat C. Cat A sites comprised orifices of diffuse venting, Cat B sites were located in the immediate vicinity of the orifices (up to 1 m distance), and Cat C sites included the rising plume and the mesopelagic water column.

In a first step, the sites were characterized with respect to physicochemical parameters, determined by online measurement (temperature) or calculated based on the mixing models of the corresponding focused fluids and background water (Bgd) (Table 1). Additionally, Gibbs' free energies were calculated for the oxidation of H_2S, CH_4 and H_2 (Fig. 2), the main energy sources for chemoautotrophic life at hydrothermal vents.

The four Cat A samples retrieved from a venting orifice at Wd (front part: WdCr-f1, WdCr-f2, back part: WdCr-b) and at Bb-Milky differed in their physicochemical characteristics. Fluids obtained from Wd were characterized by higher temperature (91°C at the deepest point, on average 39–46°C at the point of biological sampling), low pH (4.9) and strongly elevated concentrations of reduced species like H_2S (2.7–3.3 mM at sampling point) and CH_4 (1.2–1.4 mM at sampling point). In contrast, a much lower temperature (14°C) and higher pH (6.1) were detected at Bb-Milky indicating a higher dilution grade of the fluid and lower concentration of reduced compounds (e.g. 210 μM H_2S). However, even though the difference in concentration of reduced compounds between the two different Cat A sites was significant, the difference in energy yields from the oxidation of sulfide was less dramatic due to limited availability of oxygen (Fig. 2).

Cat B samples were obtained from Wd, WF and CS. Five were sampled in 10 cm to 1 m horizontal distance from the orifice: ca. 10 cm left of Wd (Wd-10L), over a mussel field next to Wd (Wd-muss), ca. 5 cm from CS (CS-nv), close to a white mat-like patch at CS (CS-mat) and next to mussels ca. 30 cm away from CS (CS-muss). Two additional Cat B samples were collected above diffuse venting sites: ca. 40 cm above Wd (Wd-40UP) and ca. 40 cm above diffuse venting in between mussels at WF (WF-muss). Physicochemical parameters at these seven

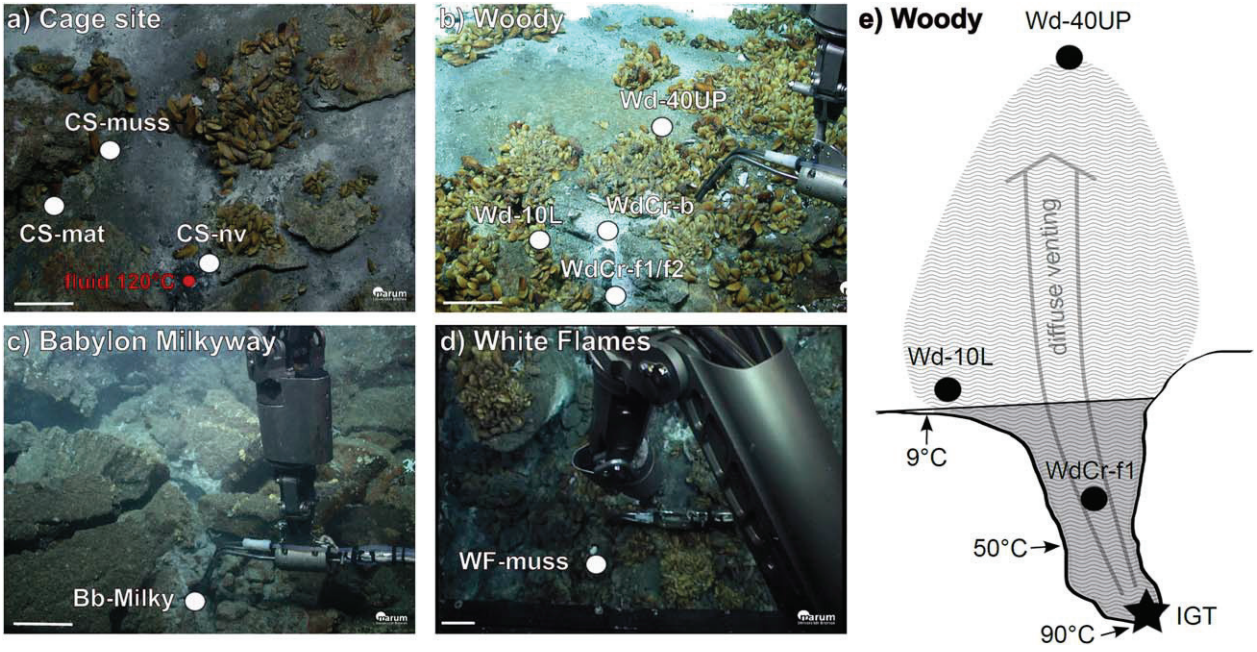

Fig. 1. Overview of the diffuse venting sides. White dots indicate points where samples have been taken. The red dot in image (a) indicates the source of hot fluid ($T = 120$°C) at Cage Site. In image (b), diffuse fluid emerges from a crack, under sampling point 'WdCr-f1/f2'. In image (c), the sample was taken directly from the orifice and in picture (d) diffuse venting occured below the mussel bed. The scale bars represent 10 cm. Image (e) shows the vertical distribution of Woody Crack samples. The star marks the sampling point for the isobaric gas-tight (IGT) sample used for modelling of fluid mixing.

sites exhibited tiny yet distinct differences to background values: temperatures were slightly elevated (on average 8.3–9.8°C with peaks up to 17.5°C) compared to bottom background water (6.7–8.3°C), the pH was slightly lower to equal (6.2–7.7), and a low concentration of reduced compounds (H_2S: 0.8–76 μM; CH_4: 0.4–43 μM) was present.

The three Cat C samples of the rising plume (Plm-1, Plm-2, Plm-3) did not differ from background water values with respect to pH or temperature. Yet, the analysed plumes exhibited elevated methane concentrations (257–426 nM).

The energetically most favourable process at all Cat A and Cat B sites was aerobic sulphide oxidation followed by the aerobic oxidation of methane (Fig. 2).

Microbial diversity

Sequencing of the V3-V4 variable region of the 16S rRNA gene (Supporting Information Table S2) and subsequent analysis of the beta-diversity by hierarchical clustering and non-metric multidimensional scaling (NMDS) based on a Bray-Curtis dissimilarity matrix (Fig. 3) indicated a correlation of microbial diversity and previously proposed habitat categories. Analysis of similarities (ANOSIM) proved the separation of samples into Cat A, Cat B and Cat C to be robust with a *P*-value as low as 0.0001 ($R = 0.72$). Nevertheless, in hierarchical clustering analysis the samples taken not aside, but ca. 40 cm above diffuse venting sites (Wd-40UP and WF-muss) branched together with Cat C samples (Fig. 4) suggesting a stronger plume and background water influence compared to the other Cat B samples (Cat B_{mix}).

Proteobacteria dominated all analysed 16S rRNA gene datasets (44–88%). However, when looking at the relative abundances of 16S rRNA gene tag sequences at higher taxonomic resolution, differences in the community structure became evident (Fig. 4). In Cat A samples, sequences related to *Epsilonproteobacteria* were highly abundant (39% in WdCr-b to 76% in WdCr-f1), compared to Cat B (6–19%) and Cat C samples (0.6–2%). They mainly affiliated with the genera *Sulfurimonas* (8–59%), *Sulfurovum* (9–37%) and *Arcobacter* (3–9%), as well as *Campylobacter* (1–5%). Additionally, *Nautiliaceae*-related sequences were much more prevalent in Cat A samples (3–8%), compared to Cat B (0.1–1%) and Cat C samples (0.03–0.15%). Beyond *Proteobacteria*, in the Wd-Cr-f2 sample, we also found an increased fraction of *Aquificaceae* 16S rRNA gene sequences (11%).

A large fraction of sequence reads from Cat B samples affiliated with *Gammaproteobacteria* (16–60%) and *Alphaproteobacteria* (7–21%). Gammaproteobacterial sequences related to species of the genera *Alcanivorax* (0.3–20%), *Acinetobacter* (0.1–38%), *Thalassolituus* (0.04–8%) *Glaciecola* (0.01–15%), *Vibrio* (0.4–6%) and

Table 1. Main environmental parameters.

Sample name	Short name	Depth [m]	pH	*T* [°C] (average/max.)	Filtering	CO_2 [μmol/kg]	O_2 [mM]	SO_4^{2-} [mM]	H_2 [μM]	CH_4 [μmol/kg]	H_2S [μM]
Mesopelagic background water	Bgd	500	7.7	12	On board	4.8×10^{1}	0.19[b]	28.7	0	1×10^{-2}	0
Plume-1	Plm-1	797	7.7	9.4	On board	4.8×10^{1}	0.19[b]	28.7	0	2.6×10^{-1}	0
Plume-2	Plm-2	819	7.7	9.3	On board	4.8×10^{1}	0.19[b]	28.7	0	4.3×10^{-1}	0
Plume-3	Plm-3	798	7.7	9.5	On board	4.8×10^{1}	0.19[b]	28.7	0	2.7×10^{-1}	0
Woody Crack back	WdCr-b	828	4.8	46/74	*In situ*	2.8×10^{4}[a]	0.15[b]	26.9[a]	4.4×10^{-1}[a]	1.4×10^{3}[a]	3.3×10^{3}[a]
Woody Crack front 1	WdCr-f1	828	4.9	39/49	*In situ*	2.3×10^{4}[a]	0.16[b]	27.2[a]	3.4×10^{-1}[a]	1.2×10^{3}[a]	2.8×10^{3}[a]
Woody Crack front	WdCr-f-B	828	4.9	40/53	On board	2.3×10^{4}[a]	0.16[b]	27.2[a]	3.5×10^{-1}[a]	1.2×10^{3}[a]	2.8×10^{3}[a]
Woody Crack front 2	WdCr-f2	828	4.9	45/53	*In situ*	2.7×10^{4}[a]	0.15[b]	27.0[a]	4.2×10^{-1}[a]	1.4×10^{3}[a]	3.2×10^{3}[a]
Woody Crack (40 cm above)	Wd-40UP	828	6.9	9*	*In situ*	7.2×10^{2}[a]	0.19[b]	28.7[a]	1×10^{-2}[a]	3.4×10^{1}[a]	5.6×10^{1}[a]
Woody Crack (40 cm above)	Wd-40UP-B	828	6.9	9*	On board	7.2×10^{2}[a]	0.19[b]	28.7[a]	1×10^{-2}[a]	3.4×10^{1}[a]	5.6×10^{1}[a]
Woody Crack (10 cm left)	Wd-10L	828	6.9	9*	*In situ*	7.2×10^{2}[a]	0.19[b]	28.7[a]	1×10^{-2}[a]	3.4×10^{1}[a]	5.6×10^{1}[a]
Woody Crack (10 cm left)	Wd-10L-B	828	6.9	9*	On board	7.2×10^{2}[a]	0.19[b]	28.7[a]	1×10^{-2}[a]	3.4×10^{1}[a]	5.6×10^{1}[a]
Woody mussels	Wd-muss	828	6.9	9.2/9.7	On board	9×10^{2}[a]	0.19[b]	28.6[a]	2×10^{-2}[a]	4.3×10^{1}[a]	7.6×10^{1}[a]
Cage Site (near vent)	CS-nv	814	6.2	9.4/17.5	On board	2×10^{2}[a]	0.19[b]	28.7[a]	3.1×10^{-1}[a]	3.7×10^{0}[a]	1.6×10^{1}[a]
Cage Site (mussels)	CS-muss	814	6.8	8.4/8.8	On board	6.2×10^{1}[a]	0.19[b]	28.7[a]	4×10^{-2}[a]	4.1×10^{-1}[a]	9×10^{-1}[a]
Cage Site (near mat)	CS-mat	814	7.7	8.3/8.5	On board	6.2×10^{1}[a]	0.19[b]	28.7[a]	4×10^{-2}[a]	4.1×10^{-1}[a]	8×10^{-1}[a]
White Flames mussels	WF-muss	836	7.2	9.8/10	*In situ*	1×10^{2}[a]	0.19[b]	28.6[a]	2.6×10^{-1}[a]	1.2×10^{0}[a]	8.1×10^{0}[a]
White Flames mussels	WF-muss	836	7.2	9.3/10	On board	7.5×10^{1}[a]	0.19[b]	28.6[a]	1.4×10^{-1}[a]	5.8×10^{-1}	3.2×10^{0}[a]
Babylon Milkyway	Bb-Milky	1,004	6.1	13.9/16.2	On board	2×10^{3}[a]	0.19[b]	28.0[a]	1.2×10^{0}[a]	3.8×10^{1}[a]	2.1×10^{2}[a]

a. Derived from mixing models based on fluid data obtained from isobaric gas tight samples (Reeves *et al.*, 2011).
b. Derived from mixing models based on fluid data measured with an *in situ* mass spectrometer.
*T-probe failed, value taken from a different sampling event at the same position.

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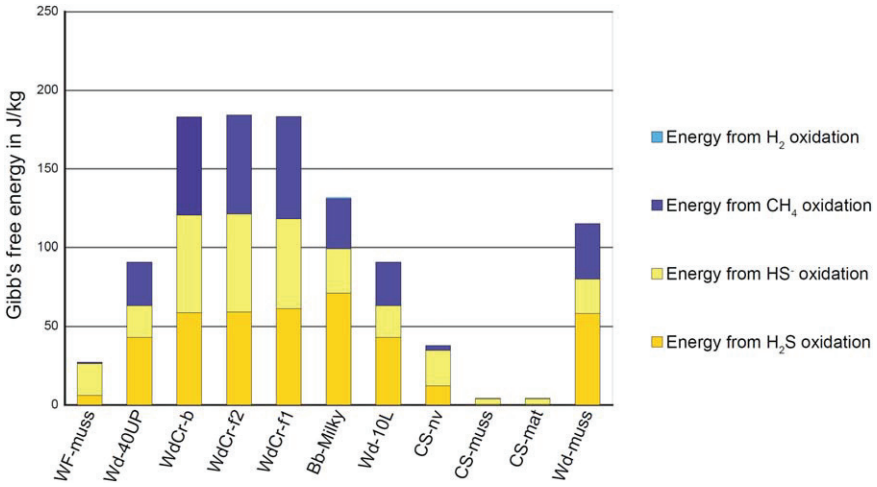

Fig. 2. Gibbs' free energies available from the oxidation of certain fluid species at the sampling sites at Menez Gwen. The values are calculated based on the models for mixing of vent fluids and sea water. All processes are considered to be aerobic as all fluids and bottom water contained oxygen.

Alteromonas (0.7–9%) were highly abundant in Cat B. Also a considerable number of SUP05-cluster 16S rRNA sequences (0.8–8%) was detected. Cat B characteristic alphaproteobacterial sequences were related to various *Rhodobacterales* genera (1–15%) such as *Sulfitobacter* (0.4–8%) and *Hyphomonas* (0.2–4%).

In contrast to samples assigned to Cat B, alphaproteobacterial genera detected in plume and background samples (Cat C) predominantly affiliated with those of the SAR11 clade (10–17% in Cat C as opposed to 2–3% in Cat B samples). Within the *Gammaproteobacteria* present in Cat C samples (13–26%) groups of as yet uncultured bacteria such as the E01-9C-26 marine group (2–6%), SAR86 (1–2%) and others were characteristic, but also a considerable number of *Alteromonas* related sequences (1–6%) was detected. The high abundance of 16S rRNA sequences assigned to the SAR202 clade (*Chloroflexi)* (8–15%), the SAR406 clade (*Marinimicrobia*) (7–10%) and the SAR324 clade within the *Deltaproteobacteria* (2–6%) also distinguished Cat C from Cat A and Cat B.

The primer set used in this study theoretically also targets a decent number of archaeal taxa assuming that one mismatch is tolerated (Klindworth *et al.*, 2013). A high proportion of archaeal sequences was detected in Bgd (22%) and Plm 1-3 (1–7%). Among the reads obtained for Cat A and Cat B the archaeal sequences accounted at maximum for 4% of the reads (WF-muss), with an exception of Wd-40UP (16%). In almost all samples Marine Group I (*Thaumarchaeota)*, II and III (*Euryarchaeota*) sequences were dominant. Only in the Cat A Bb-Milky dataset highest

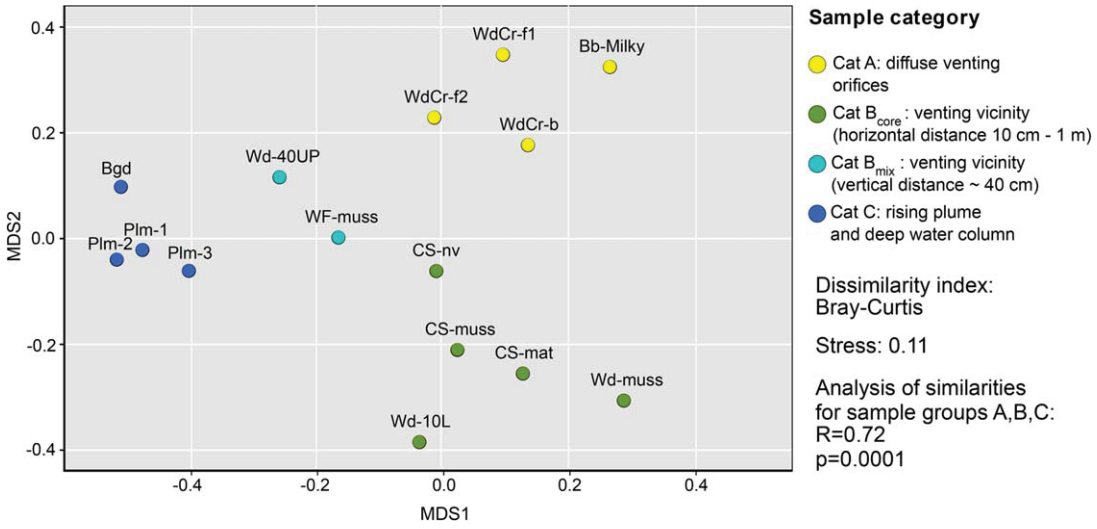

Fig. 3. NMDS ordination plot of microbial community compositions as determined by 16S rRNA gene tag sequencing. The distance matrix was calculated based on the relative abundances of different genera in the samples. The analysis of similarities was performed based on the sample groups A, B and C. The analysis of similarities (ANOSIM) was performed using the 'anosim' function (vegan package) with 9,999 permutations and Bray-Curtis dissimilarity used for the underlying distance matrix.

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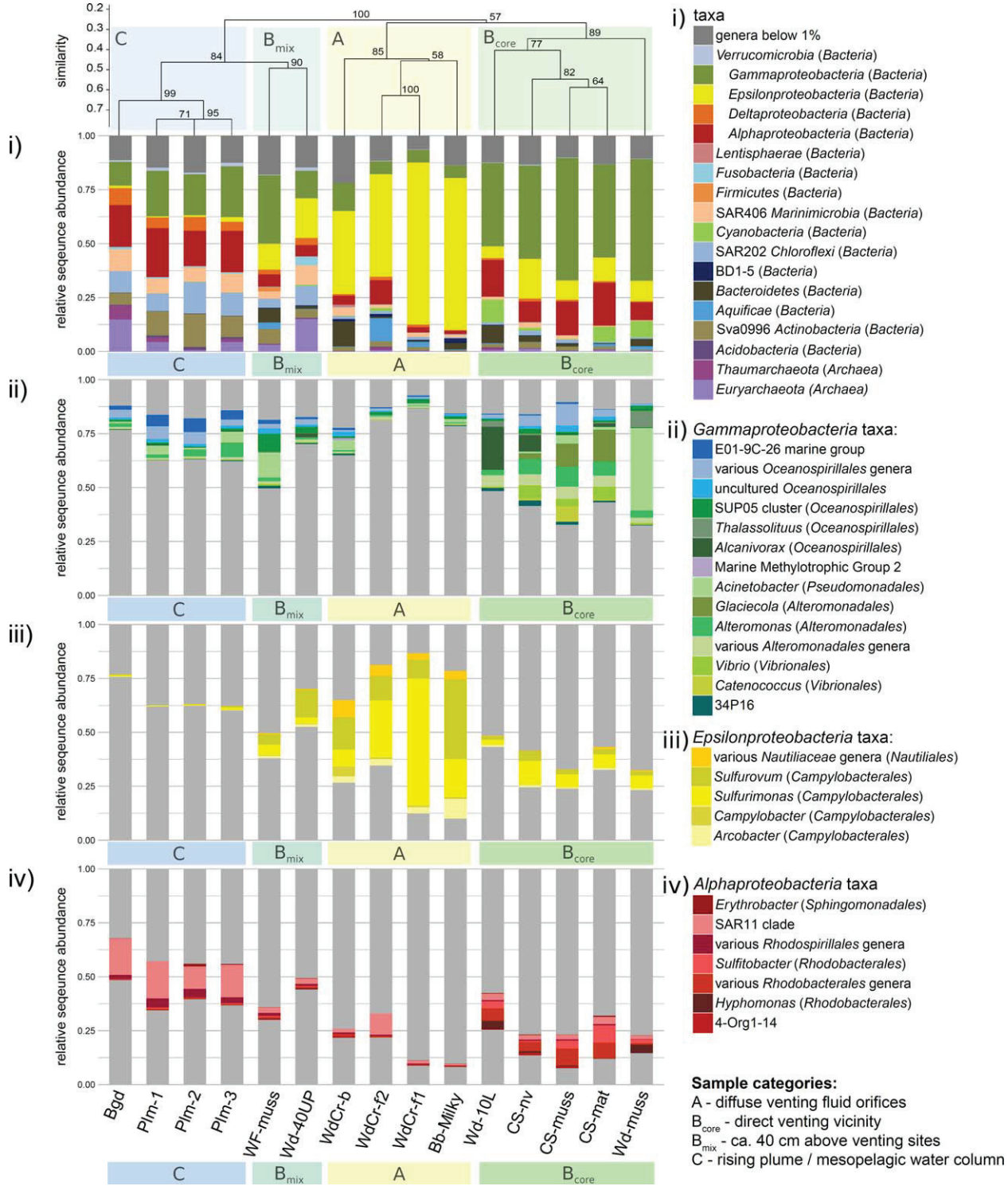

Fig. 4. Relative abundances of 16S rRNA gene amplicon sequences. The sample dendrogram was constructed via hierarchical clustering based on a Bray-Curtis distance matrix using the average linkage method. The process was bootstrapped 1,000 times. The three different sample categories are indicated in capital letters above and in between the bar charts. (i) *Proteobacteria* sequences resolved at class level, the rest of the *Bacteria* – at phylum level; (ii): *Gammaproteobacteria* sequences resolved at genus level; (iii): *Epsilonproteobacteria* sequences resolved at genus level; (iv): *Alphaproteobacteria* sequences resolved at genus level.

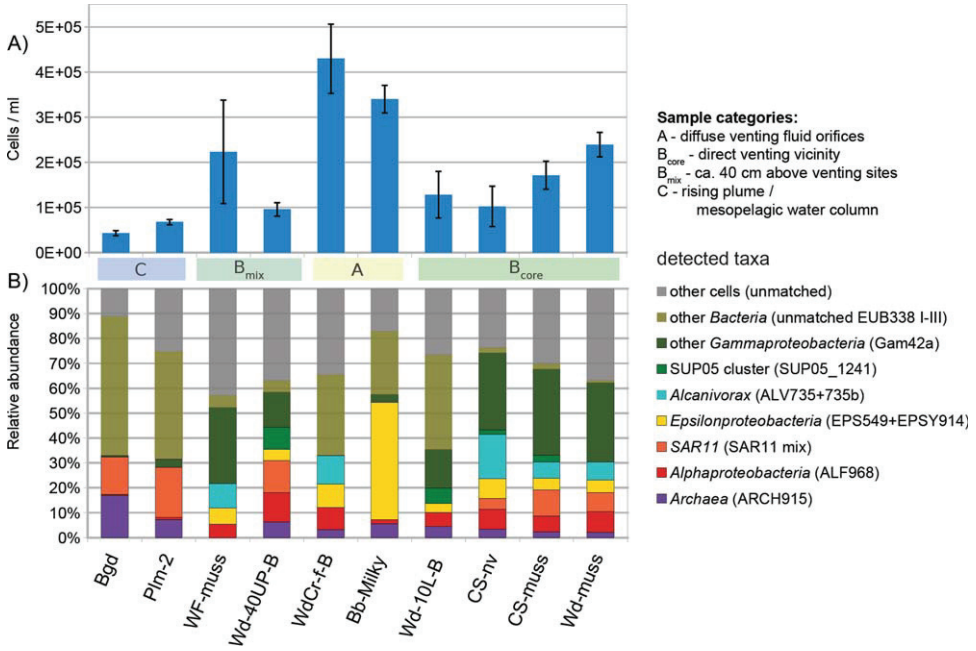

Fig. 5. Total cell counts and relative abundance of selected microbial taxa.
A. Total cell counts as determined by counting of DAPI stained cells prior to CARD-FISH analysis. Exception: for the WdCr-f-B sample SYBR Green I stain was used to obtain total cell counts due to a high content of sediment particles in the sample.
B. Abundance of microbial groups relative to DAPI/SYBR Green I counts. Probes used for the detection are given in brackets.

abundance of *Halobacteriales* (*Euryarchaeota*) (43% of *archaeal*, 0.09% of total reads) assigned reads could be detected (data not shown). The predominance of the *Thaumarchaeota* and *Euryarchaeota* was confirmed by 16S rRNA gene amplicon sequencing using a primer set specific for archaeal 16S rRNA sequences (see Supporting Information Results).

Microbial abundance determined by CARD-FISH

Total cell counts and the abundance of selected microbial groups were determined for representative samples (Fig. 5). Total cell counts were in the range of 4×10^4 to 7×10^4 cells/ml in Bgd and Cat C, and 1×10^5 to 4×10^5 cells/ml in Cat A and B. CARD-FISH confirmed a dominance of *Bacteria* over *Archaea* (EUBI-III: 57–77%, ARCH915: 2–17%) already suggested by 16S rRNA gene tag analysis. The high abundance of *Epsilonproteobacteria* in Cat A was confirmed for Bb-Milky (EPS549 + EPS914: 47%), whereas the WdCr-f1 FISH sample (WdCr-f1-B) exhibited rather low counts for any probe (ALF968: 8.8%, GAM42a + SUP05_1241: 11.6%, EPS549 + EPS914 probe mix: 9.5%), except for EUBI-III (62%). Higher abundance of *Gammaproteobacteria* in Cat B samples was also supported by CARD-FISH (GAM42a + SUP05_1241: 22–51%).

A significant proportion of *Alphaproteobacteria* could be detected with the ALF968 probe (Glöckner *et al.*, 1999) in Cat B (5–12%) and Cat A (2–9%) compared to Cat C (< 1%). However, alphaproteobacterial SAR11 cells, detected with the SAR11 probe mix (Gomez-Pereira *et al.*, 2013), but not with the ALF968 probe, were most abundant in Cat C samples (Bgd: 15% and Plm-2: 20%), compared to Cat B (0-13%) and Cat A (0%).

To evaluate the presence of *Alcanivorax* (*Gammaproteobacteria*), we applied the probes ALV735 and ALV735b (Syutsubo *et al.*, 2001) specific for this genus and other members of the *Alcanivoraceae* family respectively. *Alcanivorax* cells were detected in all Cat B samples in significant numbers (6–18%), except for Wd-40UP and Wd-10L (< 1%), as well as in one of two investigated Cat A samples (WdCr-f-B: 18%). *Alcanivorax* could not be detected in Cat C.

We also designed a probe for the detection of SUP05 clade *Gammaproteobacteria*, which are not covered by the GAM42a probe according to SILVA TestProbe and probe match to SUP05 23S rRNA sequences from metagenomes (Supporting Information Fig. S1). SUP05 clade bacteria were only detected in Cat B. Their abundance ranged from 2% in CS-nv to 9% in Wd-40UP-B.

Bulk metagenome analysis

Metagenome analysis was applied in order to evaluate whether the clear structuring of microbial communities in the mixing zones around venting sites was also reflected in the metabolic capabilities of affected bacterial groups. Three samples from different areas around Woody Crack were chosen (i) WdCr-f2, sampled directly inside Woody Crack (Cat A) (ii) WdCr-10L taken about 10 cm left of the fissure in a less turbulent zone (Cat B_{core}) and (iii) WdCr-40UP taken ca. 40 cm above the fluid emission point (Cat B_{mix}) (Fig. 1e). Between seven and twelve Gbp of sequence information were generated per sample,

resulting in 80–87 Mbp of assembled sequence information longer than 500 bp (Supporting Information Table S4).

The scaffolds of the obtained metagenomes were characterized based on their GC-content, read-coverage, tetranucleotide frequencies and BlastP based taxonomic classification (Supporting Information Fig. S3). Further, we conducted 16S rRNA gene analysis (Supporting Information Fig. S3) as well as a tetranucleotide, coverage and taxonomy-based binning of scaffolds followed by manual curation of the obtained bins and subsequent taxonomic assignment (Fig. 6). Both analyses confirmed the high relative abundance of chemolithoautotrophic *Epsilonproteobacteria* (*Sulfurovum, Sulfurimonas*) and *Aquificae* reads in WdCr-f2 compared to the two Cat B metagenomes. Reads mapping to bins assigned to genera with known hyperthermophilic representatives (e.g. *Aeropyrum*, *Thermococcus*, *Hyperthermus*) (Zillig *et al*., 1990; Marteinsson *et al*., 1999; Nakagawa *et al*., 2004) were exclusively detected in the Cat A metagenome. For both Cat B metagenomes, we observed a dominance of gammaproteobacterial over epsilonproteobacterial sequence information (Fig. 6, Supporting Information Fig. S3). Apart from this, the Wd-40UP metagenome assembly was characterized by highest read mapping to bins classified as SAR202 and SAR324, two clades which were also abundant in 16S rRNA tag analysis (Fig. 6).

Almost exclusively reads of Cat B metagenomes mapped to bins assigned to *Pseudoalteromonas*, *Hyphomonas*, *Thalassolituus* and *Sphingomonas* (Wd-10L: 11.8% Wd-40UP: 2.6%). The presence of these genera especially in Wd-10L is consistent with 16S rRNA gene tag data where sequences of such heterotrophic *Gammaproteobacteria* and *Alphaproteobacteria* genera were found to be more abundant in Cat B compared to Cat A samples.

The bulk metagenome assemblies were automatically annotated with the IMG-MER system (Markowitz *et al*., 2012). Using the 'Function search' module of the IMG interface, we looked for genes indicative of autotrophic and heterotrophic life style (Supporting Information Fig. S4). Key genes of the rTCA cycle were much more frequent in the WdCr-f2 sample (e.g. ATP-citrate lyase: 23× higher frequency compared to Wd-10L, 350× compared to Wd-40UP) and mostly encoded on contigs assigned to *Aquificae* (68%) or *Epsilonproteobacteria* (27%). RuBisCo genes were attributed to *Gammaproteobacteria* (80%) and were more frequent in the Wd-40UP metagenome (1.7× more than in Wd-10L or WdCr-f2). Genes indicative of heterotrophic metabolism showed higher relative abundance in both Cat B metagenomes. Thereby, alkane monooxygenases, as well as xylanases and other glycosyl hydrolases were most frequent in the Wd-10L metagenome (2–40× more than in WdCr-f2, 1.4–5.4× more than in Wd-40UP). Taxonomically they were mostly assigned to *Alphaproteobacteria and Gammaproteobacteria* (both together

Fig. 6. Relative abundance of taxonomically classified metagenomic bins in the initial bulk assemblies. The percentages are representing the proportion of reads mapping to the respective bin in relation to all reads mapping to the complete metagenome with a minimum identity of 95%. Zero value means no bin was generated for the respective taxonomic group from the specific metagenome.

Table 2. Draft composite genomes obtained by iterative reassembly of metagenomic bins.

Bin name	Closest relative (16S gene identity; coverage; accession)	Size [Mb]	N50	Contigs	Features	% Completeness (% of duplicated single copy genes, Campbell *et al.* (2013))	GC	Relative abundance WdCr-f2	Relative abundance Wd-10L	Relative abundance Wd-40UP
Thalassolituus sp.	*Thalassolituus oleivorans* R6-15 (100%; 100%; CP006829.1)	3.32	68193	73	3089	99.3% (1.4% duplicates)	46.80 ± 0.87%	No bin	2.02%	0.02%
Hyphomonas sp.	*Hyphomonas sp.* L-53-1-40 (100%; 100%; NZ_AWFI01000001.1)	2.65	135119	53	2660	97.1% (1.4% duplicates)	58.96 ± 1.97%	No bin	2.63%	No bin
Sphingomonas sp.	*Sphingomonas alpina* (98%; 99%; NR_117230.1)	4.17	13386	413	3915	92.0% (2.2% duplicates)	65.55 ± 1.34%	0.3%	5.53%	2.56%
Sva0996 marine group	*Candidatus* Microthrix parvicella Bio17-1 (90%; 98%; NZ_AMPG01000001.1)	3.17	26769	417	3071	69.6% (35% duplicates)	67.7 ± 1.6%	2.85%	11.39%	6.44%
SAR406 marine group	*Marinimicrobia* bacterium SCGC AAA240-I10 (95%; 56%; HQ675595.1)	3.1	15805	503	2739	42.03% (22% duplicates)	41.00 ± 1.69%	3.27%	9.1%	6.27%
SAR202 clade	Uncultured bacterium clone PSB18 (93%; 97%; KM389602.1)	3.16	16053	478	3079	73% (16% duplicates)	57.24 ± 1.39%	1.35%	6.55%	6.66%
E01-9C-26 marine group	Endosymbiont of *Lamellibrachia satsuma* clone KB45B-H30 (90%; 99%; KJ603241.1)	3.16	79012	141	3065	77% (4.3% duplicates)	52.15 ± 1.34%	1.62%	3.51%	3.91%

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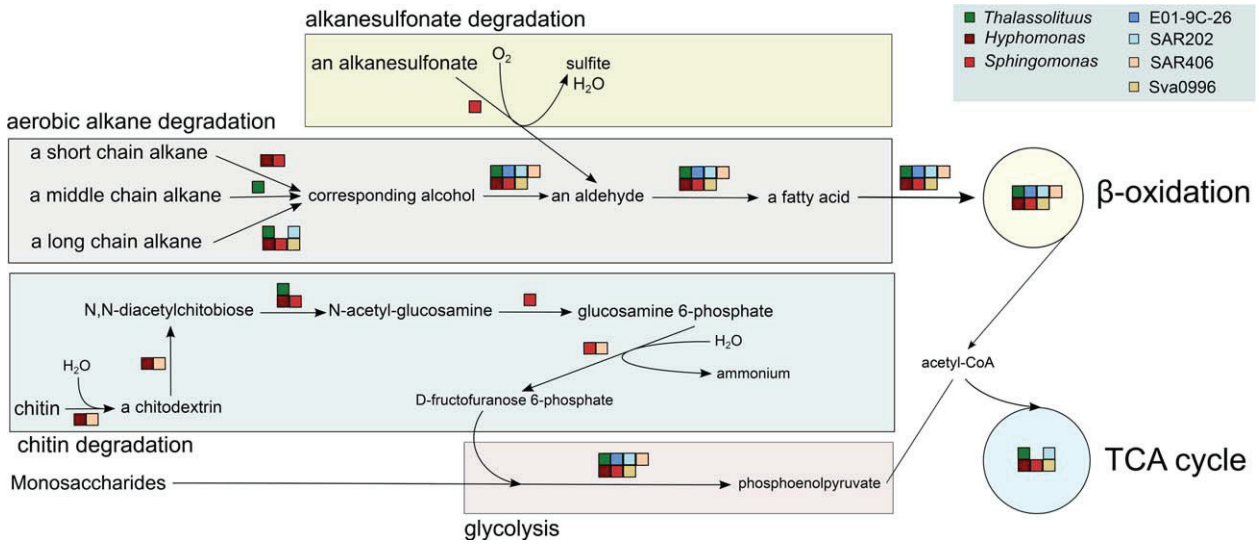

Fig. 7. Differences in main heterotrophic degradation pathways of the obtained draft/population genomes. Coloured squares represent presence of genes coding for the enzymes catalyzing the reaction in the respective draft/population genome as indicated by RAST annotation, Pathway Tools and manual evaluation.

27–76%), to unclassified *Proteobacteria* (2.9–17%) or *Bacteria* (4.3–43%) in general (Supporting Information Fig. S3). To overcome the limitations of bulk metagenome assemblies in terms of reliable gene annotation, taxonomic assignment of short contigs and the linkage of function to taxonomic identity, targeted re-assemblies of selected taxonomic bins were conducted.

Targeted reassembly of population genomes and their metabolic potential

Targeted iterative reassemblies were generated based on the above described datasets and bins, focusing on supposedly heterotrophic species and as yet poorly described clades. We obtained almost complete draft genomes (> 92%) of Cat B characteristic *Thalassolituus*, *Hyphomonas* and *Sphingomonas species* as well as draft population (pan-)genomes (> 42% completeness, 4–35% of single-copy genes redundant) of as yet uncultured bacteria: SAR406, SAR202, Sva0996, E01-9C-26 that were prevalent in the two Cat B metagenomes (Table 2). Based on average nucleotide identities (ANIs) of DNA sequences (Goris et al. 2007, Richter and Rossello-Mora, 2009), only the *Thalassolituus* draft genome was likely representing a known species (ANI > 95% to *Thalassolituus oleivorans* MIL-1 genome).

All of the draft genomes contained an aerobic heterotrophic repertoire of genes including TCA cycle genes, respiratory electron transport chain (except for SAR 202) and degradation pathways for several sugars, amino acids and lipids (Supporting Information Table S5). The TCA cycle was incomplete in the draft population genomes of SAR406 and E01-9C-26 lacking the genes for 2-oxoglutarate dehydrogenase and isocitrate dehydrogenase. The SAR202 and Sva0996 population genomes were only lacking the 2-oxoglutarate dehydrogenase genes. Instead, all four genomes contained genes for the 2-oxoglutarate:-ferredoxin oxidoreductase (alpha- and beta-subunits) known to substitute the 2-oxoglutarate dehydrogenase (Baughn *et al.*, 2009; Zhang and Bryant, 2011). Also, all draft genomes harboured genes for transporter proteins including sugar transporters, peptide and fatty acid transporters and TonB-dependent biopolymer transporters (Teeling *et al.*, 2012). In the population genomes of uncultured clades, genes encoding TonB-dependent receptors were only found on the contigs of the SAR406 clade. In contrast, the E01-9C-26 and SAR202 clade contigs contained more ABC-type amino acid and oligopeptide transporters as well as TRAP dicarboxylate transporter genes.

The *Thalassolituus* draft genome exhibited an average nucleotide identity of 98% to the known oil degrading *Thalassolituus oleivorans* species, and also contained the complete genetic equipment for alkane degradation including AlkB-type (rubredoxin-dependent, encoding middle-size alkane degradation) and AlmA-type (flavin-binding, encoding long-chain alkane degradation) alkane-1 monooxygenases (Supporting Information Table S5) as well as all genes for the subsequent oxidation of the primary alcohol to a fatty acid and its degradation via the beta-oxidation pathway (Rojo, 2010) (Fig. 7). The other genomes encoded proteins similar to monooxygenase genes of various types: AlkB type, cytochrome P450 type proteins (CYPs), Bacluciferase like LadA type and flavin-binding AlmA type (van Beilen and Funhoff, 2005; van Beilen *et al.*, 2006). To

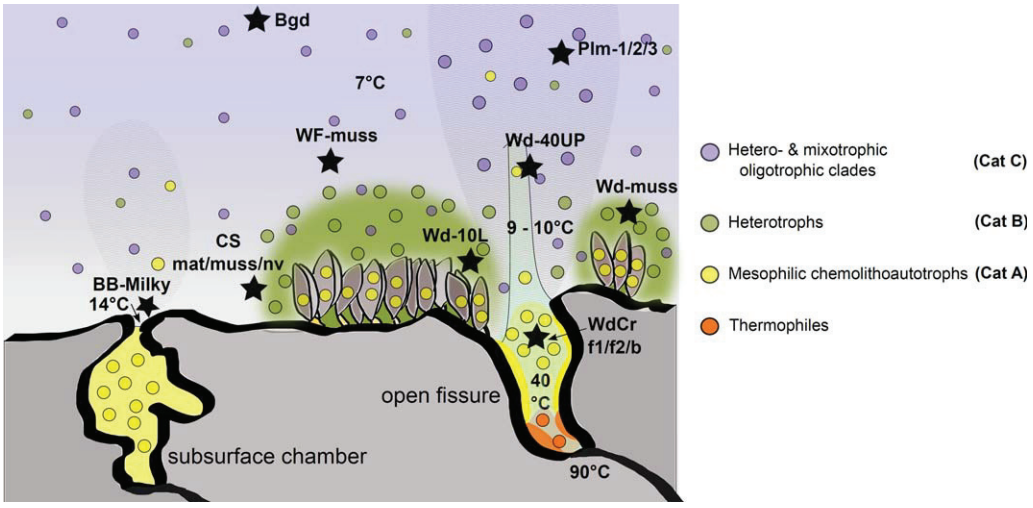

Fig. 8. Suggested schematic distribution of microbial lifestyles at hydrothermal vent fields. In yellow: chemolithoautotrophic microorganisms found at open venting fluid orifices, in subsurface chambers filled with vent fluid, and as symbionts of vent fauna. In green: heterotrophic organisms feeding on organic matter derived from primary production, vent fauna or contained in the fluids. In purple: oligotrophic organisms present in the hydrothermally unaffected deep water column and in the plumes. Stars indicate schematic placing of samples discussed in this study.

confirm and specify the annotation, we calculated phylogenetic trees (Supporting Information Fig. S5a–d). Monooxygenase sequences detected in the population genomes of the uncultured bacterial clades by the presence of Pfam motifs clustered with sequences of as yet experimentally unconfirmed alkane monooxygenases, except for the AlmA type monooxygenase encoded on the Sva0996 draft genome and a LadA type monooxygenase encoded in the SAR202 draft genome. The protein sequences derived from the *Hyphomonas*, *Sphingomonas* and *Thalassolituus* draft genome affiliated with clusters of proteins with confirmed alkane monooxygenase function. The *Hyphomonas* and *Sphingomonas* draft genome contained genes for cytochrome P450 alkane hydroxylases as well as for long-chain alkane monooxygenases of the AlmA (*Hyphomonas, Sphingomonas*) and flavin-binding type (*Sphingomonas*). Apart from this, the *Sphingomonas* draft genome also contained genes for alkane sulfonate monooxygenases (identified by Bac-luciferase like domain), which likely constituted an operon together with alkane sulfonate-binding protein, an alkane sulfonate transport system permease protein and an alkane sulfonate ABC transporter.

The *Hyphomonas* and the SAR406 bins also contained genes for the complete pathway needed for chitin degradation to *N*-acetyl-D-glucosamine (Bassler *et al.*, 1991; Svitil *et al.*, 1997) such as chitinases and beta-hexosaminidases. However, the as yet incomplete genome bins lacked the genes needed for the subsequent transformation step from *N*-acetyl-D-glucosamine to β-D-fructofuranose 6-phosphate, which can enter the glycolysis. Xylanases encoding genes were found on the contigs of the *Sphingomonas* and *Hyphomonas* draft genomes.

In the E01-9C-26 draft genome, we also found sulphur oxidation genes (soxA,B,X,Y,Z,W) as well as genes for RuBisCo activating proteins (CbbO, CbbQ), carboxysome shell proteins (CcmL, CsoS1), and the RuBisCo operon transcriptional regulator (CbbR). All other reassembled draft and population genomes contained no genes indicating chemolithoautotrophy.

Discussion

Until recently studies of hydrothermal vent microbial communities have focused predominantly on chemolithoautotrophic organisms (for review: Sievert and Vetriani 2012). Numerous studies have, however, highlighted the complex origin and fate of dissolved organic matter in these settings, even where no significant source of sedimentary organic matter exists (Brault *et al.*, 1988; Lang *et al.*, 2006; McCollom, 2008; Konn *et al.*, 2009; Lang *et al.*, 2010; Reeves *et al.*, 2014a; Hawkes *et al.*, 2015; McDermott *et al.*, 2015; Rossel *et al.*, 2015). Given the obvious potential for heterotrophy, questions arose about the identity of possible heterotrophic microorganisms present and the extent of their habitats in hydrothermal environments typically assumed to be primarily chemolithoautotrophic (Winkel *et al.*, 2014; Stokke *et al.*, 2015).

In this study, we analysed the structure of microbial communities at the Menez Gwen hydrothermal field on a finer and larger scale based on the comparison of three categories of samples taken at four different venting sites using three complementary molecular methods: 16S rRNA gene tag sequencing, CARD-FISH, and metagenomics including the assembly of draft genomes and draft population

genomes. Although method intrinsic differences and limitations are evident (see Supporting Information Results), CARD-FISH analysis largely supported the sequencing based data and provided important evidence that the main clades discussed in this study were indeed present as active cells. As a result, we have been able to identify microbial communities gaining energy by organic matter degradation, rather than by 'geofuel' oxidation, in areas surrounding zones of primary production like a 'belt' (Fig. 8). These microbial communities detected in fluids in the immediate vicinity of venting sites (Cat B), were distinct from communities in fluids sampled directly at diffuse venting orifices (Cat A), and plumes or mesopelagic background water (Cat C).

Fluids directly at the diffuse vent orifices (Cat A) were characterized by a high abundance of the epsilonproteobacterial genera *Sulfurimonas* and *Sulfurovum* as well as representatives of the *Aquificales* (Fig. 4). The dominance of these groups known to harbour chemoautotrophic species is in line with previous studies, which mainly reported *Epsilonproteobacteria* to be the dominating primary producers at diffuse venting sites and in other highly sulfidic habitats (Lopez-Garcia *et al*., 2003; Nakagawa *et al*., 2005; Campbell *et al*., 2006; Huber *et al*., 2010; Lanzen *et al*., 2011; Perner *et al*., 2013). These bacteria might originate from the subsurface (Sievert *et al*., 2008), including surfaces exposed to the fluid flow (Lopez-Garcia *et al*., 2003) or might have thrived in the fluid. At Milkyway temperatures at the orifice were moderate (14°C), and the sample was taken from a cavity in the basaltic lava (Fig. 1). Therefore, subsurface biosphere may have been sampled in this case. In contrast, at Woody Crack the fluid temperature at the emission point was far above the temperature range tolerated by as yet cultured *Helicobacteraceae* (Inagaki *et al*., 2004; Takai *et al*., 2006) and the abundant epsilonproteobacterial genera are likely to have their niche in the oxic mixing zone right after the fluid emission point. However, detectable frequencies of fast growing mixotrophic *Nautiliales* (Winkel *et al*., 2014) as well as thermophilic *Aquificales* and *Archaea* suggest the presence of subsurface organisms in Woody Crack fluids as well.

When sampling water only few centimetres away from a vent orifice, one may expect a community composition to represent largely a mixture of Cat A and Cat C communities due to the highly dynamic processes at these sites, likely preventing the establishment of distinctly different stable microbial communities. However, in fluids in the close vicinity of diffuse vent orifices, alpha- and gammaproteobacterial groups different from the ones in rising plume and not hydrothermally influenced background water became dominant. Characteristic for Cat B was a high abundance of gammaproteobacterial genera mostly known for heterotrophic species, such as *Glaciecola*, *Acinetobacter*, *Alcanivorax*, *Thalassolituus* and *Vibrio* (Hedlund and Staley, 2001; Yakimov *et al*., 2004; Liu and Shao, 2005; Hunt *et al*., 2008; Klippel *et al*., 2011; Fondi *et al*., 2013), adding up to 51% of all sequences (Fig. 4). This change of community composition was especially impressive in case of Woody Crack, where gradients were steep, samples were taken only few centimetres apart from each other and fluid collection times of up to 1 h (ca. 30L of fluid) likely compensated temporal variations that may have been caused by sampling or natural fluid flow variations. Whereas samples taken inside or in the back part of the crack (Cat A) were dominated by chemolithoautotrophic *Epsilonproteobacteria*, the sample taken just 10 cm aside of the crack ('Wd-10L') exhibited a dominance of the heterotrophic gammaproteobacterial taxa mentioned above. Interestingly, the fraction of typical chemolithoautotrophic *Gammaproteobacteria* like (i) the endosymbionts related SUP05 group, which have often been reported to be the main *Gammaproteobacteria* representatives in diffuse hydrothermal fluids (Crepeau *et al*., 2011; Anderson *et al*., 2013; Glaubitz *et al*., 2013) and (ii) aerobic methanotrophic *Methylococcales* (Niemann *et al*., 2006; Crepeau *et al*., 2011; Ruff *et al*., 2013) was low, despite of the high energy yields available from aerobic sulphide and methane oxidation in the vent's vicinity. For example, SUP05 bacteria accounted for less than 9% in 16S rRNA analysis, metagenomes and FISH.

Apart from the dominance of heterotrophic *Proteobacteria*, all Cat B samples contained mesophilic chemolithoautotrophic *Epsilonproteobacteria* (*Sulfurimonas*, *Sulfurovum*). Two samples taken ca. 40 cm above the ground (Wd-40Up and WF-muss) also showed a clear plume signature with sequences affiliated to SAR11, SAR406, SAR202 and Sva0996. So, Cat B samples carried core fluid and plume signatures because of mixing events close to fluid emission, but they were not a simple mixture of Cat A and Cat C communities. They were specifically enriched in planktonic heterotrophic organisms likely feeding on the biomass resulting from chemolithoautotrophic primary production. Such heterotrophic communities seem to establish best at sites close to geofuel emission, but a little aside from the main fluid flow and, therefore, in areas with less fluctuating conditions and lower temperature. In line with our findings, we could also find the above mentioned heterotrophic genera of *Proteobacteria* in datasets of studies focussing on chemolithoautotrophic microorganisms in typical Cat B, vent vicinity, areas (Meyer *et al*., 2013; Perner *et al*., 2013).

Genera like *Thalassolituus*, *Alcanivorax*, *Hyphomonas* and members of the family *Sphingomonadacae* are ubiquitous in the ocean (Cavicchioli *et al*., 1999; Weiner *et al*., 2000; Yakimov *et al*., 2007; Li *et al*., 2014a). In line with these studies, they were present but below 0.5% in 16S rRNA gene tag data of our background and plume

samples. The abundance was below detection limit for CARD-FISH using a general *Alphaproteobacteria* and *Gammaproteobacteria* probe, respectively, as well as a probe specific for the genus *Alcanivorax*. In contrast, among others, these taxa have been identified to be abundant in and characteristic for the heterotrophic 'belt' around fluid emission sites. So what could lead to an enrichment of these groups in near vent habitats? Some members of the *Gammaproteobacteria* and *Alphaproteobacteria* genera found in the 16S rRNA gene tag datasets, for example some *Vibrio* or *Sulfitobacter* species, might not just be heterotrophs but also mixotrophs with the ability of reduced sulphur compound oxidation (Sorokin, 1992; Durand *et al*., 1994; Pukall *et al*., 1999). In our draft genomes of vent vicinity typical proteobacterial genera, we could not find any evidence for the utilization of reduced sulphur compounds or any other chemolithotrophic energy generation. Instead, draft genome analysis indicated *Thalassolituus*, *Hyphomonas* and Sphingomonas species to be aerobic heterotrophs with complete TCA cycle, aerobic respiration chain, glycolysis and ß-oxidation pathways as known from previous isolates and genome studies (Cavicchioli *et al*., 1999; Weiner *et al*., 2000; Yakimov *et al*., 2004; Maeda *et al*., 2009).

Although detailed data on organic carbon composition of Menez Gwen fluids are scarce, the analysis of dissolved organic carbon (DOC) from Woody fluids conducted by Rossel *et al*. (2015) suggested the presence of organics within the nonextractable DOC pool that could not be fully characterized, and may include low molecular weight carboxylic acids, alcohols or short chain hydrocarbons. Low molecular weight compounds such as amino acids (Lang *et al*. 2013), methanethiol (Reeves *et al*. 2014a,b) and formic acid (McDermott *et al*. 2015) have also been quantified in many diffuse hydrothermal fluids in diverse seafloor settings. Complex hydrocarbons of biotic or abiotic origin were detected and investigated in sulphide deposits of the Rainbow hydrothermal field (Lein *et al*., 2003), and polycyclic organic compounds have been detected at other hydrothermally influenced sites (Simoneit and Fetzer, 1996; Geptner *et al*., 2006). With this information in mind, vent vicinity characteristic taxa identified in this study, such as *Thalassolituus*, *Alcanivorax*, *Acinetobacter, Hyphomonas and Sphingomonas* are also often related to contaminated environments (Cavicchioli *et al*., 1999; Lai *et al*., 2011; Bertrand *et al*., 2013; Fondi *et al*., 2013). Some of these are known to be capable of xenobiotics degradation or even to grow best on alkanes (Weiner *et al*., 2000; Yakimov *et al*., 2004; Maeda *et al*., 2009). Therefore, we checked our metagenomes and genome drafts for alkane degrading capabilities. The key-step in hydrocarbon degradation is the energy demanding initial activation of an alkane by terminal or subterminal oxygenation (Widdel and Musat, 2010). This reaction is performed by different types of alkane monooxygenases (van Beilen and Funhoff, 2005; Austin and Groves, 2011). Alkane monooxygenases encoding genes were most frequent in the Wd-10L metagenome and mostly encoded on contigs assigned to *Alphaproteobacteria* and *Gammaproteobacteria*. We found two genes for alkane monooxygenases in our *Thalassolituus* (AlkB and AlmA) and *Hyphomonas* (CYP153, AlmA) draft genomes, three different ones in the *Sphingomonas* draft genome (CYP153, AlmA and LadA) as well as all genes encoding proteins for the subsequent processing of oxidized alkanes. Automated annotation also indicated some monooxygenase domains in the population genomes of the plume clades SAR406, SAR202, Sva0996 and E01-0C-26 more abundant in Cat C samples, yet these were rather distantly related to alkane monooxygenases with experimentally confirmed function, suggesting that they might target other organic substrates or have a different function.

The capability of hydrocarbon degradation is only one feature out of a range of degradative pathways identified in this study on the contigs of vent vicinity characteristic *Alphaproteobacteria* and *Gammaproteobacteria*. *Gammaproteobacteria* detected as abundant in the 16S rRNA gene dataset in several Cat B samples but rather distantly related to the ones represented in the metagenomes such as *Glaciecola* and *Vibrio* species are, for example, known for sugar polymer degradation. Some species of the genus *Glaciecola*, which makes up to 15% of 16S rRNA gene tags in Cage Site samples, are able to degrade sugar polymers like xylan (Klippel *et al*., 2011) and cellulose. Further, marine *Vibrionaceae* are known for their chitin sensing and degradation capabilities (Svitil *et al*., 1997; Hunt *et al*., 2008). We found genes for xylanases in the draft genomes of *Hyphomonas* and *Sphingomonas* as well as for a chitinase in the *Hyphomonas* and SAR406 draft/population genome. Also in the bulk metagenome assemblies xylanases and other glycosyl hydrolases as well as chitinases were more frequent in the Cat B metagenomes. The source of organic carbon for the vent vicinity characteristic taxa might, therefore, be the polysaccharide sheath of epsilonproteobacterial filaments (Stokke *et al*., 2015) or the extensively present vent fauna. Many of the mentioned *Alphaproteobacteria* and *Gammaproteobacteria* genera, like *Acinetobacter*, *Alcanivorax*, *Vibrio* and *Hyphomonas* have also been isolated from deep sea invertebrate tissue (Sfanos *et al*., 2005) or detected in invertebrate associated communities (Ridley *et al*., 2005; Wegley *et al*., 2007). Hence, the majority of heterotrophic bacteria found in vent vicinity fluids might be fauna associated as symbionts, parasites, commensals or are merely feeding on excretion products (Crepeau *et al*., 2011) and dead biomass derived from animals and microbial mats, whereas microorganisms specialized to survive in extremely oligotrophic conditions prevail in background

waters and plumes (Fig. 8). In our 16S rRNA tag sequencing data we can see that different genera of *Gammaproteobacteria* 'bloom' in different samples from the vent's vicinity, which suggests that they might all have their substrate preferences analogous to different bacterial clades succeeding each other after a phytoplankton bloom (Teeling *et al.*, 2012). However, in contrast to phytoplankton blooms, seasonal availability of substrates does not play a role at deep-sea vents. Here, organic matter is being supplied constantly either by the vent fluids themselves or by chemolithoautotrophy and downstream biomass generating processes, such as symbioses. Therefore, the separation of bacterial clades at vents is rather spatial than temporal due to temperature and chemical gradients. Differences in dominating heterotrophic bacteria at different sites could be related to different organic matter content and composition of different fluids or to different fauna or chemolithoautotrophic microorganisms colonizing the individual venting sites. A more detailed sampling of fauna populated environments and a comprehensive analysis of the diversity and distribution of organic compounds present in low temperature vents could shed more light on these processes and help to further elucidate the role of heterotrophic bacteria in near-vent habitats.

Conclusion

Our data indicate a high spatial variability of vent-associated microbiota. By high throughput 16S rRNA gene sequencing, CARD-FISH and metagenomics we identified versatile heterotrophic *Proteobacteria* inhabiting areas directly adjacent to the hot spots of chemoautotrophy. We show for the first time clear evidence for a 'heterotrophic belt' surrounding diffusely venting orifices and provide data on the genomic potential of this yet poorly described part of deep-sea microbiota. Our findings pose new questions with respect to hydrothermal ecosystem functioning in terms of biological transformation of hydrothermal fluid contents and food web relations.

Experimental procedures

Site description and sample collection

The Menez Gwen hydrothermal field is located in the summit area of a ridge-centered seamount south-west of the Azores on the Mid-Atlantic ridge (37°50′ N, 31°30′ W, in 850 m water depth). It is a basalt-hosted hydrothermal system with moderate concentrations of sulphide and methane and very low hydrogen concentrations in the emitted fluids compared to other known hydrothermal fields at oceanic spreading zones (Charlou *et al.*, 2000; Amend *et al.*, 2011; Reeves *et al.*, 2011). Samples were collected during RV Meteor cruise M82-3 in 2010. Hydrothermal fluids were sampled at four different venting sites within the Menez Gwen field (White Flames, Cage Site, Woody and Babylon) (Fig. 1, Supporting Information Table S1). Sampling sites were selected based on visible venting (e.g. 'shimmering water'), increased temperatures and elevated gas concentrations as indicated by *in situ* mass spectrometry (ISMS) (Wankel *et al.*, 2010) spectra. To target different stages of mixing, we attempted to sample diffuse fluids directly at the orifice as well as in the immediate vent vicinity (10 cm to 1 m). One extensively sampled venting site was a moderate temperature diffusely venting fissure at the 'Woody' site (Marcon *et al.*, 2013), where fluids were characterized by unusually high gas concentrations but major ion concentrations similar to background sea water (Reeves *et al.*, 2011). This is the same site discussed in Winkel *et al.* (2014) and Rossel *et al.* (2015).

Sampling of diffuse venting sites and near vent environments was conducted with the Kiel In Situ Pumping System (KIPS) (Schmidt *et al.*, 2007) mounted on the remotely operated vehicle (ROV) Quest (MARUM, University of Bremen). An in-line temperature probe as well as an inlet of an ISMS was attached to the sampling nozzle in order to measure temperature and gas concentrations directly at the point of sampling. On board samples were immediately split for downstream biological and chemical analyses. For DNA analysis, 400–1,500 ml sample were collected on cellulose-acetate membrane filters (pore size: 0.2 μm; Millipore, Darmstadt, Germany), and stored at −20°C. For total cell count determination and CARD-FISH analyses samples were fixed overnight with formaldehyde (1% final concentration) at 4°C and subsequently filtered through polycarbonate membrane filters (pore size: 0.2 μm; Millipore, Darmstadt, Germany). After drying, filters were stored at −20°C. At several sites, we additionally collected large samples for metagenomic DNA analyses by *in situ* filtration on cellulose-acetate or polyethersulfone membrane filters (diameter 142 mm, 0.2 μm pore size; Millipore, Darmstadt, Germany) using a stainless steel pressure filter holder (142 mm; Sartorius, Göttingen, Germany) directly connected to the KIPS multiport valve. Upon retrieval on board, membrane filters were immediately frozen and stored at −80°C until further use. The buoyant hydrothermal plume was sampled with a CTD-rosette, upon turbidity anomalies detected by multibeam sonar. On board, Niskin bottle contents (10 l) were filtered on 0.2 μm pore-size membrane filters for DNA analysis or fixed for CARD-FISH analysis as described above. For methane concentration measurements in plume samples, water samples were taken from Niskin bottles with gas-tight syringes, and after a headspace extraction, minimum values of methane were measured by gas-chromatography (German *et al.*, 2010).

Three *in situ* filtered samples from the Woody site (WdCr-f2, Wd-10L, Wd-40UP) were chosen for metagenomic analysis.

Thermodynamic calculations

Thermodynamic calculations were conducted as described in Amend *et al.* (2011). Essentially, the chemical composition of the sampled fluid-water mix was calculated with the REACT module of the Geochemist's Workbench software (Aqueous Solutions LLC, Champaign, IL), using the same thermodynamic database as Amend *et al.* (2011). Thereby, data from the *in situ* temperature logger were taken as a proxy for the mixing stage. Gibb's free energies available

from one mol of substrate were calculated according to the following equation:

$$\Delta G_r = \Delta G_r^0 + RTlnQ_r$$

where ΔG_r^0r represents the standard Gibbs energy of reaction, R is the gas constant, T stands for temperature in Kelvin, and Q_r is the reaction quotient. The reaction quotient is defined as:

$$Q_r = \frac{\Pi_j \ a_j^{v_j}}{\Pi_i \ a_i^{v_i}}$$

where a_j are product activities and a_i are educt activities raised to the power of respective stoichiometric factors v_j and v_i.

To determine the energy available per kg of fluid-water mix, calculated Gibbs' free energies were multiplied by concentration of the limiting compound of the reaction.

16S rRNA gene tag sequencing and analysis

DNA was extracted from a ca. 1.5 × 1.5 cm membrane filter pieces and the V3-V4 region of the 16S rRNA gene was amplified using the primer combination Bakt_341F and Bakt_805R (Herlemann *et al*., 2011) in five separate PCR reactions per sample using barcoded primers. Replicate PCRs were pooled and the amplicons were sequenced on a 454 FLX+ sequencer with Titanium chemistry at the Max Planck Genome Centre (Cologne, Germany). For details see Reeves *et al*. (2014b).

Raw sequences were 'denoised' using the implementation of the PyroNoise flowgram clustering algorithm (Quince *et al*., 2009) in Mothur v. 1.32 (Schloss *et al*., 2009). Subsequently, 454 reads of less than 370 bp as well as reads containing any ambiguous bases or homopolymer stretches longer than 8 bp were excluded from further analyses. The remaining reads were analysed with the SILVAngs pipeline (Quast *et al*., 2013) as described in Klindworth *et al*. (2013) (for details see Supporting Information Methods).

CARD-FISH

CARD-FISH analysis was conducted according to Ishii *et al*. (2004). Lysozyme and proteinase K were used in order to permeabilize bacterial and archaeal cells respectively. All hybridizations were conducted for 2.5 h at 46°C. Probes and formamide concentrations used in this study are listed in Supporting Information Table S3. The EUB probes (EUBI-III, Amann *et al*., 1990; Daims *et al*., 1999), the ALV735+ ALV735b probes (Syutsubo *et al*., 2001), as well as different *Epsilonproteobacteria* targeting probes were used as a mix (equimolar concentrations). All probes were tested for sufficient coverage of bacterial groups present in the samples using the Probe-Match function of the ARB software on a SILVA SSU115 database complemented with rRNA sequences from the metagenomes. For the detection of the hydrothermal SUP05 clade, which is not covered by the GAM42a probe (Manz *et al*., 1992) (Supporting Information Fig. S1), we designed probe SUP05_1241 (sequence: 5′-GCA ACC CTT TGU CCT TCC C-3′) based on sequences retrieved through 16S rRNA gene reconstruction from metagenomic reads and closely related sequences present in the SILVA SSU115 database (Quast *et al*., 2013). The designed SUP05 probe was also used in a mix together with the GAM42a probe to obtain total gammaproteobacterial counts.

After CARD-FISH cells were counterstained for 10 min with 1 μg/ml 4′,6-Diamidino-2-phenylindole (DAPI), and subsequently washed with H_2O and 80% ethanol. After air drying filters were mounted on glass slides for microscopy. SybrGreen I staining was conducted on samples with high auto-fluorescent particle contamination, following a protocol modified after Lunau *et al*. (2011): filter pieces were mounted on moviol containing 2.5% ascorbic acid (0.1 g/ml in 1× PBS: 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl) and 2.5% SYBR-Green (1:1,000 diluted in H_2O).

Metagenome sequencing and assembly

High molecular weight genomic DNA for metagenomic analysis was extracted from a quarter of a 142 mm diameter cellulose-acetate or polyethersulfone membrane filter following a protocol modified after Zhou *et al*. (1996). The DNA was paired-end shotgun sequenced on an Illumina MiSeq at the Max Planck Genome Centre (Cologne, Germany) after library construction using the Ovation Ultralow Library system kit (NuGen, San Carlos CA).

Obtained sequence reads were quality checked with FastQC (Babraham Bioinformatics, http://www.bioinformatics.babraham.ac.uk/projects/fastqc) and trimmed off remaining adaptors using BBduk of the BBmap package (version 32.14, http://sourceforge.net/projects/bbmap/). Trimmed reads were normalized using BBnorm (target depth: 18). Overlapping paired-end reads were merged into long single reads (options: 'minoverlap = 50 strict = T') with BBmerge. The remaining reads were treated as paired reads. Assembly was done using a modified version of the IDBA-UD iterative assembler (Peng *et al*., 2012) contained in the A5-Miseq pipeline (Coil *et al*., 2015) (k-mer sizes: 20 to 250 in steps of 10). Bulk assemblies were visualized using the Metawatt binning software (version 2.1) (Strous *et al*., 2012) in order to get an approximate taxonomic affiliation of the contigs. *As reference database all available prokaryotic genomes at NCBI, complemented with relevant draft genomes, such as draft genomes of marine deep water clades SAR202, SAR324, SAR406 were used*. As the assemblies were generated with normalized reads, raw untreated reads were mapped back to the respective assemblies with BBmap (minimum mapping identity set to 95%) in order to generate coverage information. Metagenomes were uploaded to the IMG-MER automatic annotation system for metagenomes (Markowitz *et al*., 2012) for further analyses.

Sequences of 16S rRNA genes were assembled from the raw metagenome reads using PhyloFlash v2.0 (http://github.com/HRGV/phyloFlash), an EMIRGE (Miller *et al*., 2011) and SPAdes (Bankevich *et al*., 2012) based pipeline for targeted assembly of genes based on mapping to a reference database such as the SILVA SSU115 rRNA database.

Bulk metagenome analysis

Genes of interest were identified based on Pfam motifs or KEGG Orthology database terms (Kanehisa and Goto, 2000) assigned by the IMG-MER annotation pipeline. Relative frequencies of the genes were calculated based on the read coverage of the respective contigs normalized by the average coverage of the given metagenome. Genes of the same function encoded on the same contig were counted only once as they were considered to belong to the same organism. Normalized read coverage was also used when comparing the taxonomic affiliations of genes.

Targeted reassembly of metagenomic bins

Our binning and targeted reassembly approach is mainly following the logic presented in Albertsen *et al*. (2013), yet with some adjustments and employing different bioinformatics tools.

Scaffolds generated by IDBA-UD were binned based on multiple criteria such as highly similar tetranucleotide (N4) frequencies (98%), GC-content, coverage and taxonomic classification using Metawatt (version 2.1) (Strous *et al*., 2012). Bins of interest were further inspected and corrected manually. Together with the genomes of closest sequenced relatives, they were used as a reference for mapping of raw unassembled reads from all three metagenomes. The reads that mapped to the references were then assembled de-novo using the SPAdes assembler V3.1.1 (Bankevich *et al*., 2012). Binning of the newly generated assemblies, mapping of raw reads to the new bins and de-novo assembly of these reads were repeated 4–6 times for each bin with increasing mapping stringency, leading to an assembly improvement in each round. From round three on, the completeness of the conserved single-copy gene set (Campbell *et al*., 2013) was used as an additional criterion for the selection of bins in Metawatt. The 16S rRNA genes were detected within each of the reassembled draft genomes, encoded on long contigs ($>$ 10 kbp) confirming their taxonomic classification (Table 2). Average nucleotide identity between coding DNA sequences of the assembled genomes and closest sequenced relatives was calculated using Blast alignments in JSpecies (v.1.2.1) (Richter and Rossello-Mora, 2009).

The generated assemblies were automatically annotated with the standard RAST annotation pipeline (Aziz *et al*., 2008) and analysed with MetaCyc Pathway Tools (Caspi *et al*., 2014) for presence and completeness of metabolic pathways.

The annotation of selected genes, referred to in this study, was manually inspected. Results of BLASTP based searches of translated open reading frames (ORFs) against various protein databases (NCBI-NR, SwissProt, KEGG) were compared to hidden Markov model based HMMER3 (Eddy, 2009) searches against the Pfam-A database (Finn *et al*., 2014).

Phylogenetic tree construction

Sequences of translated ORFs identified on the draft genome contigs together with the top 10 phmmer (Eddy, 2011; Finn *et al*., 2011) hits in the UniprotKB database (Magrane and Consortium, 2011) for each protein sequence and sequences of proteins with experimentally confirmed functions were used to construct phylogenetic trees. Protein sequences were aligned with MAFFT (Katoh and Standley, 2013), using L-INS-I method and the Blosum62 scoring matrix. Trees were calculated with various algorithms implemented in the ARB software: PhyML (Guindon *et al*., 2010), RaxML (Stamatakis, 2014) and Neighbour-joining (Ludwig *et al*., 2004). Main topology and phylogenetic grouping of sequences obtained from metagenomic bins remained stable in all analyses. The final trees presented in this study (Supporting Information Fig. S4a–d) were calculated with maximum likelihood algorithm RaxML based on sequence positions conserved in at least 25% of the sequences, with 100 bootstraps.

Nucleotide sequence accession numbers

All raw sequences and assemblies have been uploaded to the European Nucleotide Archive under project accession number: PRJEB11362. Raw 454 sequences of 16S rRNA gene amplicons can be found under accession numbers ERR1078303 – ERR1078320. The Illumina MiSeq sequence reads of the metagenomes are deposited under accession numbers ERR1078300 – ERR1078302. Assembled draft and population genomes have been uploaded as analyses projects ERZ125827 – ERZ125833.

Acknowledgements

We would like to thank officers, crew, shipboard scientific party, and the technical team of the ROV Quest 4000m (MARUM) on R/V Meteor cruise M83-2, for their invaluable assistance. We thank Xavier Prieto for assistance with plume methane sampling and analysis onboard, and chief scientist Nicole Dubilier for her support. The cruise M82/3 with R/V Meteor was an integral part of the Cluster of Excellence of the MARUM 'The Ocean in the Earth System, Research Area GB: Geosphere-Biosphere Interactions' funded by the German Research Foundation (DFG). We thank Richard Reinhard, Bruno Huettel and the team of the Max Planck Genome Centre in Cologne for sequencing and Christian Quast, Pelin Yilmaz and Hanno Teeling for help with computational analyses. Further, we thank Nicole Krombholz, Rafael Lasso Perez, Christoph Behrens, Philipp Laeseke for assistance in the Molecular Ecology department, as well as Petra Pjevac for productive discussions. This work was supported by the Max Planck Society.

Conflict of interest: All authors have no conflict of interest to declare.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Probe match of the GAM42a probe to SUP05 clade 23S rRNA sequences. The alignment includes SUP05 clade 23S rRNA sequences of the SILVA LSU123 database as well as two SUP05 23S rRNA sequences found on the contigs of the Wd-10L and Wd-40UP bulk metagenome assembly.

Fig. S2. Rarefaction curves were calculated using the Vegan package (v. 2.0.8) based on OTUs clustered at 97% identity level.

Fig. S3. Relative sequence abundances of microbial groups in the three metagenomes as assessed by BLASTP-based classification of all obtained scaffolds $>$ 500 bp via Metawatt considering scaffold coverage (left) and mapping of reads to the SILVA SSU119 database by PhyloFlash (right).

Fig. S4. Normalized gene frequencies and taxonomic affiliation of genes indicative of metabolic pathways. Gene categories indicated by roman numbers: I – alkane oxidation enzymes; II – ß-oxidation of fatty acids; III – carbohydrate active enzymes; IV – transporters for sugars, amino acids and large biopolymer molecules; V – chitin degradation: VI – alkane sulfonate degradation; VII – reverse TCA cycle (CO_2 fixation); VIII – CBB cycle (CO_2-fixation); IX – DNA replication (housekeeping gene). Genes were searched in the annotated metagenome bulk assemblies with the IMG 'search function' interface, based on Pfam motifs, or KEGG Orthologs terms (KO terms) assignment. In order to determine normalized gene frequencies, gene copy numbers were first estimated based on the coverage of the contigs carrying the genes, as conducted by the IMG-MER annotation interface (Markowitz et al., 2012). Gene copy numbers were normalized by dividing through the average coverage of the respective metagenome (total mapped reads/total assembly length) to account for differences in sequencing depth between datasets. Taxonomic assignments were obtained by the IMG-MER annotation system based on the genes encoded on the contigs. Heatmap coloring indicates the fold difference in frequency of a gene between metagenomes.

Fig. S5a. Phylogenetic affiliation of AlkB-like monooxygenases detected in the reassembled genomic bins based on the presence of a FA-desaturase Pfam domain (e-value $>$ e-5). In green sequences of proteins with confirmed alkane monooxygenase activity are shown, and in red proteins with confirmed activity different from those of alkane monooxygenases. In cyan, sequences obtained from the reassembled bins are given. Alignment: MAFFT, treeing algorithm: RaxML (maximum likelihood) with 100 bootstraps and a 25% sequence conservation filter. The bar represents 10% estimated sequence changes.

Fig. S5b. Phylogenetic tree of cytochrome P450 monooxygenases and related sequences. In green are sequences of proteins with confirmed alkane monooxygenase activity, in red proteins with confirmed activity different from those of alkane monooxygenases. In bold cyan, sequences identified in the reassembled bins based on the P450 Pfam domain are given. Alignment: MAFFT, treeing algorithm: RaxML (maximum likelihood) with 100 bootstraps and a 25% sequence conservation filter. The bar represents 10% estimated sequence changes.

Fig. S5c. Phylogenetic tree of flavin-binding AlmA-like monooxygenases and related sequences. In green are sequences of proteins with confirmed alkane monooxygenase activity, in red proteins with confirmed activity different from alkane monooxygenase. In bold cyan, sequences identified in the reassembled bins by the flavin-binding Pfam domain. Alignment: MAFFT, treeing algorithm: RaxML (maximum likelihood) with 100 bootstraps and a 25% sequence conservation filter. The bar represents 10% estimated sequence changes.

Fig. S5d. Phylogenetic tree of luciferase-like flavin-binding monooxygenases and related sequences. In green are sequences of proteins with confirmed alkane monooxygenase activity, in red proteins with confirmed activity different from alkane monooxygenase. In bold cyan, sequences identified in the reassembled genomic bins based on the presence of a luciferase-like Pfam domain are given (original RAST automatic annotation names kept). Alignment: MAFFT, treeing algorithm: RaxML (maximum likelihood) with 100 bootstraps and a 25% sequence conservation filter. The bar represents 10% estimated sequence changes.

Fig. S6. Comparison of relative abundance data assessed by CARD-FISH counts and 16S rRNA tag sequencing of *in situ* filter samples and filters derived from the same KIPS-bottle the CARD-FISH samples were taken from.

Table S1. Samples used in this study.

Table S2. The 16S rRNA gene tag reads: raw and after denoising and quality clipping.

Table S3. HRP-labelled probes used in this study, with formamide concentration for specific binding at 46°C and targeted microbial groups.

Table S4. Metagenome sequencing and bulk assembly.

Table S5. Pathways encoded on assembled draft and population genome contigs.

Supplementary material

Supplementary methods

16S rRNA gene tag sequencing

The DNA was extracted using the Ultra Clean Soil Kit (MoBio, Carlsbad, CA, USA) with slight modifications. Briefly: A filter piece of 1.5 x 1.5 cm (~$2*10^7$ cells) was cut into smaller pieces, pretreated with 200 µg/ml proteinase K in 1x Tris-Cl (pH=8) for 1 h at 37°C and incubated for 2 h at 65°C with the SDS-containing solution "S1" of the Ultra Clean Soil Kit. Subsequent preparation steps followed the "Alternative protocol (maximum yields)" of the manufacturer's instructions.

The V3-V4 variable regions of the 16S rRNA genes were amplified using the primer combination Bakt_341F and Bakt_805R (Herlemann et al., 2011). The forward primer included a six nucleotide barcode at the 5'-prime end. For directional ligation of adaptor A and B for 454 pyrosequencing, distinct, asymmetric Sfi I restrictions sites were present at the 5'-ends of both primers (Bakt_$341F_{454}$: 5´-GAT GGC CAT TAC GGC C - barcode - CC TAC GGG NGG CWG CAG-3´; Bakt_$805R_{454}$: 5´-GGT GGC CGA GGC GGC CGA CTA CHV GGG TAT CTA ATC C-3´). For each sample five independent 50 µl PCR reactions were performed. Each 50 µl PCR reaction mix was composed of 1-3 µl of template DNA in H_2O, 0.4 µM of each primer, 50 µM of each dNTP, and 1 U Phusion DNA polymerase (Thermo Fisher Scientific, Waltham, MA USA) in one fold concentrated Phusion DNA polymerase HF buffer with additional 750 µM $MgCl_2$ and 5% (v/v) dimethyl-sulfoxide. Cycling conditions were as follows: 30 s denaturation at 98°C, followed by 28 cycles of 10 s denaturation at 98°C, 30 s annealing at 55°C and 15 s elongation at 72°C and a 10 min final elongation step at 72°C. The amplification products were purified after agarose gel electrophoresis using the QIAQuick Gel/PCR purification kit (QIAGEN, Venlo, Netherlands). The five replicates of each sample were pooled and the DNA concentration of the PCR products was determined with the Qubit 2.0 fluorometer and the Qubit dsDNA HS Assay KIT (Invitrogen) by fluorometric detection at 260 nm, as described in the accompanying manuals. Amplicons from different samples, each carrying a different barcode, were further pooled at equimolar ratio for sequencing on a 454 FLX+ sequencer with Titanium chemistry conducted at the Max Planck Genome Centre in Cologne (for details see Reeves *et al.* (2014)).

16S rRNA gene tag analysis using the SILVA-NGS pipeline

16S rRNA reads were aligned to the SILVA SSU rRNA SEED database using the SILVA incremental aligner (SINA v1.2.10) (Pruesse et al., 2012). Poorly aligned reads (<50 alignment identity, <40 alignment score), were excluded from the data set. Remaining reads were de-replicated (100% identity) and clustered into operational taxonomic units (OTUs) on 97% sequence identity level with cd-hit-est (version 3.1.2) (Li and Godzik, 2006). The OTU clustering was first performed on a sample by sample level. Subsequently, the OTUs were clustered across all samples. The reference sequence of each OTU was classified by a BLAST search (blastn version 2.2.28+) against the SILVA v115 SSU Ref non-redundant database using standard settings (Camacho et al., 2009). The OTU counts per sample obtained from 16S rRNA gene sequence analysis were further analyzed and visualized with the vegan package (Oksanen et al., 2013) in the R statistics environment.

DNA extraction and metagenome sequencing

High molecular weight genomic DNA for metagenome analysis was extracted from a quarter of a 142 mm diameter cellulose-acetate or polyethersulfone membrane filter (~ $4*10^7$ – $6*10^8$ cells) according to the SDS-based lysis protocol after Zhou *et al.* (1996) with slight modifications. These modifications included an adjustment of all volumes to an initial volume of 550 µl extraction buffer, a sonication step upon the first addition of the extraction buffer (sonication bath for 15 min) and a reduction of the re-extractions from two to one.

40 - 140 ng of high molecular weight genomic DNA were fragmented to an approximate fragment size of 800 bp using a S2 sonicator (Covaris, Woburn, MA USA) using settings recommended in the manufacturers manual. End-repair and ligation of Illumina Tru-Seq adaptors was carried out using the Ovation Ultralow Library system (NuGen, San Carlos CA, USA). The adaptor baring fragments were amplified, applying 15 PCR cycles to obtain sufficient DNA amounts for sequencing (Ovation Ultralow Library system). The fragments were sequenced on a MiSeq sequencer (Illumina, San Diego, CA, USA) in paired-end mode (2 x 250 bp length) at the Max Planck Genome Centre (Cologne, Germany).

Average insert sizes of the resulting libraries were 479 bp for WdCr-f2, 585 bp for Wd-10L and 518 bp for Wd-40UP as determined by BBmap (version 32.14, http://sourceforge.net/projects/bbmap/).

Targeted re-assembly of metagenomic bins

First the scaffolds of the original assembly were binned based on tetranucleotide (N4) frequencies at a high confidence (98%) threshold using MetaWatt (Strous et al., 2012). Subsequently, open reading frames (ORFs) were predicted using Prodigal v 2.6.1 (Hyatt et al., 2010) and scaffolds were taxonomically classified by the BLASTP based module of MetaWatt, which performs a BlastP search of the translated ORFs against the custom made reference database (same as described in "Experimental procedures"). N4 based bins were corrected manually based on GC content, coverage, and consistency of taxonomic classification. Together with the genomes of supposed closest sequenced relatives, selected based on 16S rRNA reconstruction data and taxonomic classification of the bins, they were used as a reference for mapping of raw unassembled reads with BBmap. Reads from all three metagenomes were mapped to the same references. In the first round, the mapping was done at a minimum identity threshold of 80%. The reads that mapped to the references were then assembled de-novo using the SPAdes assembler V3.1.1 (Bankevich et al., 2012). The newly generated contigs were again characterized by GC-content and coverage, binned by N4-frequency and classified with BLASTP in MetaWatt. Again a bin was picked based on the listed criteria and the mapping was repeated applying more stringent settings (minimum sequence identity of 95%). A new SPAdes assembly was done. The binning, mapping and de-novo assembly with SPAdes were repeated 4 to 6 times for each bin, leading to an assembly improvement in each round. From round three on, the completeness of the conserved single-copy gene set (Campbell et al., 2013) was used as an additional criterion for the selection of bins (assessed by HMMER3 (Eddy, 2011) based "Six-frame Pfam" module of MetaWatt). 16S rRNA genes were detected within each of the re-assembled draft genomes, encoded on long contigs (>10 kbp) confirming their taxonomic classification.

Supplementary results

Archaea 16S rRNA gene tag analysis

The amplification of 16S rRNA genes with the archaeal primer pair (Arch20F and Arch958R-mod) (Massana et al., 1997; Pires et al., 2012) succeeded only with 42 PCR cycles. Relative abundances of taxonomically classified sequences did not exhibit a clear pattern. The datasets were either dominated by Marine Group I *Thaumarchaeota* or *Thermoplasmatales* (*Euryarchaeota*) sequences (data not shown). For example, for

two of the plume samples 90% and 95% (Plm-1, Plm-2 respectively) abundance of *Thermoplasmatales* sequences were determined, whereas the dataset for the third plume sample (Plm-3) and the background sample (Bgd) contained 63% and 74% Marine Group I *Thaumarchaeota* sequences.

In contrast, using the bacterial 16S rRNA specific primer pair, which targets also some archaeal 16S rRNA sequences, we could detect the same archaeal groups we already detected with the *Archaea*-specific primer set, but observed clear patterns. For example, we observed similar patterns between similar samples, such as plume and background samples. Therefore, we consider the results obtained with the *Archaea* specific primers to be heavily biased due to the extensive number of PCR cycles that had do be applied to gain enough amplicons for sequencing (42 compared to 28 with the "bacterial" primer pair).

Discrepancies between CARD-FISH and 16S rRNA gene tag analysis

At Woody Crack, we conducted repeated sampling, either in KIPS bottles or on *in situ* filters and found a difference in microbial community composition based on 16S rRNA tags. Analysis of *in situ* filter samples revealed a typical Cat A community, and KIPS bottle analysis revealed rather a Cat B community (Figure S5). This difference was confirmed by FISH for KIPS bottle samples as these samples had also been prepared for FISH analysis on board.

This observed structural difference may have different reasons: i) the variation may be real and caused by a temporal variation of the microbial community as previously reported by Perner *et al.* (2013). ii) Increased entrainment of surrounding water when sampling into KIPS bottles may have led to a co-sampling of Cat A and adjacent Cat B communities, as the lower resistance of bottles compared to filters generally allowed for higher flow rates when sampling into bottles. However, online temperature and pH measurements conducted before and during sampling did not indicate a significant change of parameters at the time of sampling of WdCr-f-B. Further reasons may have been, iii) uneven distribution and occasional breaking off of aggregating bacterial populations attached to surfaces, fauna or pieces of organic matter, and iv) a considerable difference in pumping times (10 min for 3 bottles vs. 1h for an *in situ* filter). During the sampling process, e.g., parts of microbial mats or excretions of vent fauna may have been sampled in this highly dynamic environment. In case of *in situ* filters long

sampling times may have relativized such events. Finally, v) selective growth of populations in bottles in between sampling and further processing cannot be excluded. However, BB-Milky samples taken in three KIPS bottles from a closed chamber under a rock, where entrainment of adjacent water is less likely (Fig 1.), shows a clear Cat A signature with high abundance of *Epsilonproteobacteria* according to both, 16S rRNA gene tag data and CARD-FISH. This indicates that a selected growth in bottles may only have been a side effect.

Cat B samples taken next to Woody Crack, Wd-10L and Wd-40UP, also showed differences in community structure between *in situ* filter and bottle samples. Here the differentially abundant species belonged to heterotrophic *Gammaproteobacteria* (*Alcanivorax*, *Acinetobacter,* 34P16, *Thalassolituus*). Therefore the attribution of these samples to Cat B with a pronounced presence of heterotrophs remained stable.

Although we do not see such clear differences between KIPS bottle and *in situ* filter samples in our on-going studies at other vents (unpublished data), with respect to future studies it has to be noticed that at diverse and dynamic habitats such as hydrothermal fields, sampling precision and awareness of sampling technology limitations are of major importance.

Table S1: Samples used in this study

Sample name	Sample abbreviation	Sample number	Longitude	Latitude	Depth [m]	pH	T [°C]	Filtering
Background	Bgd	716CTD	37°50.667'N	31°31.199'W	500	7.7	8	on board
Plume-1	Plm-1	718CTD	37°50.675'N	31°31.130'W	797	7.7	8	on board
Plume-2	Plm-2	726CTD	37°50.683'N	31°31.141'W	819	7.7	8	on board
Plume-3	Plm-3	727CTD	37°50.649'N	31°31.187'W	806	7.7	8	on board
Woody Crack back filter	WdCr-b	729ROV6	37°50.675'N	31°31.152'W	828	4.8	48	*in situ*
Woody Crack front 1 filter	WdCr-f1	736ROV10	37°50.675'N	31°31.152'W	828	4.9	39	*in situ*
Woody Crack front	WdCr-f-B	736ROV12-16	37°50.675'N	31°31.152'W	828	4.9	39	on board
Woody Crack front 2 filter	WdCr-f2	736ROV11	37°50.675'N	31°31.152'W	828	4.9	45	*in situ*
Woody Crack (40 cm above filter)	Wd-40UP	743ROV5	37°50.675'N	31°31.152'W	828	6.9	9*	*in situ*
Woody Crack (40 cm above)	Wd-40UP-B	743ROV2-4	37°50.675'N	31°31.152'W	828	6.9	9*	on board
Woody Crack (10 cm left filter)	Wd-10L	743ROV9	37°50.675'N	31°31.152'W	828	6.9	9*	*in situ*
Woody Crack (10 cm left)	Wd-10L-B	743ROV6-8	37°50.675'N	31°31.152'W	828	6.9	9*	on board
Woody mussels	Wd-muss	761ROV21-22	37°50.675'N	31°31.155'W	828	6.9	9.2	on board
Cage Site (near vent)	CS-nv	750ROV3-4	37°50.656'N	31°31.184'W	814	6.2	9.4	on board
Cage Site (mussels)	CS-muss	750ROV5	37°50.656'N	31°31.184'W	814	6.8	8.4	on board
Cage Site (near mat)	CS-mat	750ROV6-7	37°50.656'N	31°31.184'W	814	7.7	8.3	on board
White Flames mussels filter	WF-muss	761ROV26	37°50.674'N	31°31.138'W	836	7.2	9.8	*in situ*
White Flames mussels	WF-muss-B	761ROV23-25	37°50.674'N	31°31.138'W	836	7.2	9.3	on board
Babylon Milkyway	Bb-Milky	756ROV8-10	37°48.058'N	31°32.234'W	1004	6.1	14	on board

* T-probe failed, value taken from a different sampling event at the same position

Table S2: 16S rRNA gene tag reads: raw and after denoising and quality clipping

Sample name	Abbreviation	Raw reads	Analyzed quality reads
Background	Bgd	33,871	16,677
Plume-1	Plm-1	42,987	20,501
Plume-2	Plm-2	29,146	13,096
Plume-3	Plm-3	32,514	13,143
Woody Crack back	WdCr-b	5,585	2,704
Woody Crack front	WdCr-f1	17,537	6,706
Woody Crack front 2	WdCr-f2	65,488	30,668
Woody Crack (40 cm above)	Wd-40UP	32,580	22,949
Woody Crack (10 cm left)	Wd-10L	35,362	23,298
Cage Site (near vent)	CS-nv	45,971	17,915
Cage Site (mussels)	CS-muss	29,699	11,667
Cage Site (near mat)	CS-mat	14,607	4,396
Milkyway	BB-Milky	26,955	10,952
Woody mussels	Wd-muss	9,124	3,933
White Flames mussels	WF-muss	6,069	2,709

Table S3: HRP-labeled probes used in this study, with formamide concentration for specific binding at 46°C and targeted microbial groups.

Probe name	% FA	Reference	Targeted groups
EUB338	35%	Amann *et al.* (1990)	most *Bacteria*
EUB338-II	35%	Daims *et al.* (1999)	*Planctomycetales*
EUB338-III	35%	Daims *et al.* (1999)	*Verrucomicrobiales*
ALF968	25%	Glöckner *et al.* (1999)	*Alphaproteobacteria,* except for *Rickettsiales*
ALV735	35%	Syutsubo *et al.* (2001)	*Alcanivorax*
ALV735b	35%	Syutsubo *et al.* (2001)	*Alcanivoraceae*
GAM42a	35%	Manz *et al.* (1992)	*Gammaproteobacteria*
BET42a (unlabelled competitor for GAM42a)	35%	Manz *et al.* (1992)	*Betaproteobacteria*
SUP05_1241	30%	This study	SUP05 cluster
EPS549	35%	Lin *et al.* (2006)	*Epsilonproteobacteria*
EPS914	35%	Grote *et al.* (2008)	*Epsilonproteobacteria*
ARCH915	35%	Stahl and Amann (1991)	*Archaea*
SAR11 probe mix	25%	Gomez-Pereira *et al.* (2013)	SAR 11 clade
NON338	35%	Wallner *et al.* (1993)	control probe reverse complementary to EUB338

Table S4: Metagenome sequencing and bulk assembly

Sample	Raw reads (2x250bp)	Assembly size	N50	Max scaffold	Number of scaffolds (>500 bp)	% GC	Average coverage
WdCr-f2	$24*10^6$	87.5 Mbp	3,135 bp	107.3 kbp	38,135	44.3	32.3x
Wd-10L	$15*10^6$	82 Mbp	4,716 bp	115 kbp	28,272	51.5	20.2x
Wd-40UP	$20*10^6$	80.4 Mbp	4,078 bp	126.6 kbp	29,908	51.2	26.7x

Table S5: Pathways encoded on assembled draft and population genome contigs

EC number	Enzyme name	*Sphing-monas*	*Hyphomonas*	*Thalassolituus*	E01-9C-26	SAR202	SAR406	Sva0996
Respiratory chain								
EC 1.6.5.3	NADH ubiquinone oxidoreductase chain A-N	+	+	-	+	+	+	+
EC 1.3.5.1	Succinate dehydrogenase	+	+	+	+	+	+	+
EC 1.10.2.2	Ubiquinol-cytochrome C reductase	+	+	+	+	-	+	+
EC 1.9.3.1	Cytochrome c oxidase	+	+	+	+ (alternative)	+	+	+
TCA cycle								
EC 4.2.1.2	Fumarate hydratase class I, aerobic	+	+	+	+	+	+	+
EC 1.1.1.37	Malate dehydrogenase	+	+	+	+	+	+	+
EC 1.1.5.4	Malate:quinone oxidoreductase	+	+	+	-	-	-	-
EC 2.3.3.1/ 2.3.3.16	Citrate synthase	+	+	+	+	+	+	+
EC 4.2.1.3	Aconitate hydratase	+	+	+	+	+	+	+
EC 1.1.1.42	Isocitrate dehydrogenase	+	+	+	-	+	-	+
EC 1.2.4.2, EC 2.3.1.61, EC 1.8.1.4	2-oxoglutarate dehydrogenase complex	+	+	+	-	-	-	-
EC 1.2.7.3	2-oxoglutarate oxidoreductase	+	-	-	+	+	+	+
EC 6.2.1.5	Succinyl-CoA ligase [ADP-forming]	+	+	+	+	+	+	+
EC 1.3.5.1	Succinate dehydrogenase	+	+	+	+	+	+	+

EC number	Enzyme name	*Sphingomonas*	*Hyphomonas*	*Thalassolituus*	E01-9C-26	SAR202	SAR406	Sva0996
Beta-oxidation								
EC 5.1.2.3	3-hydroxybutyryl-CoA epimerase	+	+	+	+	-	-	-
EC 6.2.1.3	fatty-acid-CoA ligase	+	+	+	+	+	+	+
EC 5.3.3.8	dodecenoyl-CoA isomerase	-	-	+	+	-	-	-
EC 1.3.8.-	Acyl-CoA dehydrogenase	+	+	+	+	+	+	+
EC 4.2.1.17	Enoyl-CoA hydratase	+	+	+	+	+	+	+
EC 1.1.1.35	3-hydroxyacyl-CoA dehydrogenase	+	+	+	+	-	+	+
EC 2.3.1.16	3-ketoacyl-CoA thiolase	+	+	+	+	+	+	+
Alkane oxidation								
EC 1.18.1.7	Ferredoxin reductase	+	+	+	-	+	-	+
EC 1.14.15.3	Alkane hydroxylase cytochrome P450 (CYP153A)	+	+	-	-	-	-	-
EC 1.14.15.3	LadA-type Alkane monooxygenase	+	-	-	-	+	-	-
EC 1.14.15.3	AlmA-type Alkane monooxygenase	+	+	+	-	-	-	+
EC 1.18.1.1	Rubredoxin-NAD(+) reductase	-	-	+	-	-	-	-
EC 1.14.15.3	Alkane-1 monooxygenase (AlkB)	-	-	+	?	-	?	-
EC 1.1.99.-	Alcohol dehydrogenase	+	+	+	+	+	+	+
EC 1.2.1.3	Aldehyde dehydrogenase	+	+	+	+	+	+	+
EC 6.2.1.3	Long-chain-fatty-acid--CoA ligase	+	+	+	+	+	+	+

EC number	Enzyme name	*Sphingomonas*	*Hyphomonas*	*Thalassolituus*	E01-9C-26	SAR202	SAR406	Sva0996
	Alkane sulfonate degradation							
1.14.14.5	alkanesulfonate monooxygenase	+	-	-	-	-	-	-
1.14.11.17	Alpha-ketoglutarate-dependent taurine dioxygenase	+	-	-	-	+	-	+
	Alkanesulfonates ABC transporter ATP-binding protein SsuB	+	-	-	-	-	-	-
	Alkanesulfonates transport system permease protein	+	-	-	-	-	-	-
	Alkanesulfonates-binding protein	+	-	-	-	-	-	-
3.1.6.1	Arylsulfatase	+	-	-	-	-	-	+
1.5.1.29	FMN reductase	-	-	-	-	+	-	-
	Chitin degradation							
	N-acetylglucosamine-regulated TonB-dependent outer membrane receptor	+	-	-	-	-	-	-
3.5.99.6	Glucosamine-6-phosphate deaminase	+	-	-	-	-	-	-
3.5.1.25	N-acetylglucosamine-6-phosphate deacetylase	+	-	-	-	-	-	-
2.7.1.69	PTS system, N-acetylglucosamine-specific	+	-	-	-	-	-	-
3.2.1.52	Beta-hexosaminidase	+	-	-	-	-	-	-
3.2.1.14	Chitinase	-	+	-	-	-	+	-
	miscellaneous							
	Xylanase	+	+	-	-	-	-	-
3.2.1.8	Endo-1,4-beta-xylanase A precursor	+	+	-	-	-	-	-

Figure S1

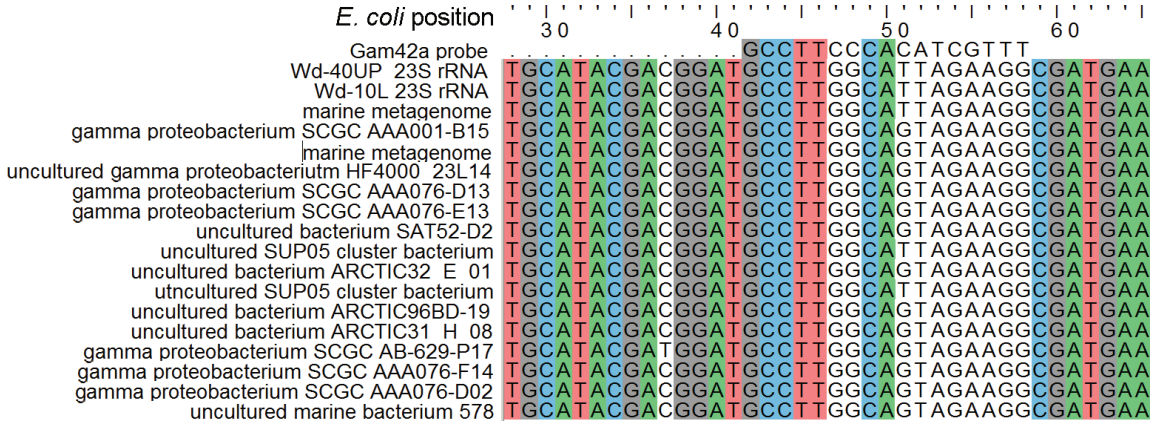

Figure S1: Probe match of the GAM42a probe to SUP05-clade 23S rRNA sequences. The alignment includes SUP05-clade 23S rRNA sequences of the SILVA LSU123 database as well as two SUP05 23S rRNA sequences found on the contigs of the Wd-10L and Wd-40UP bulk metagenome assembly.

Figure S2

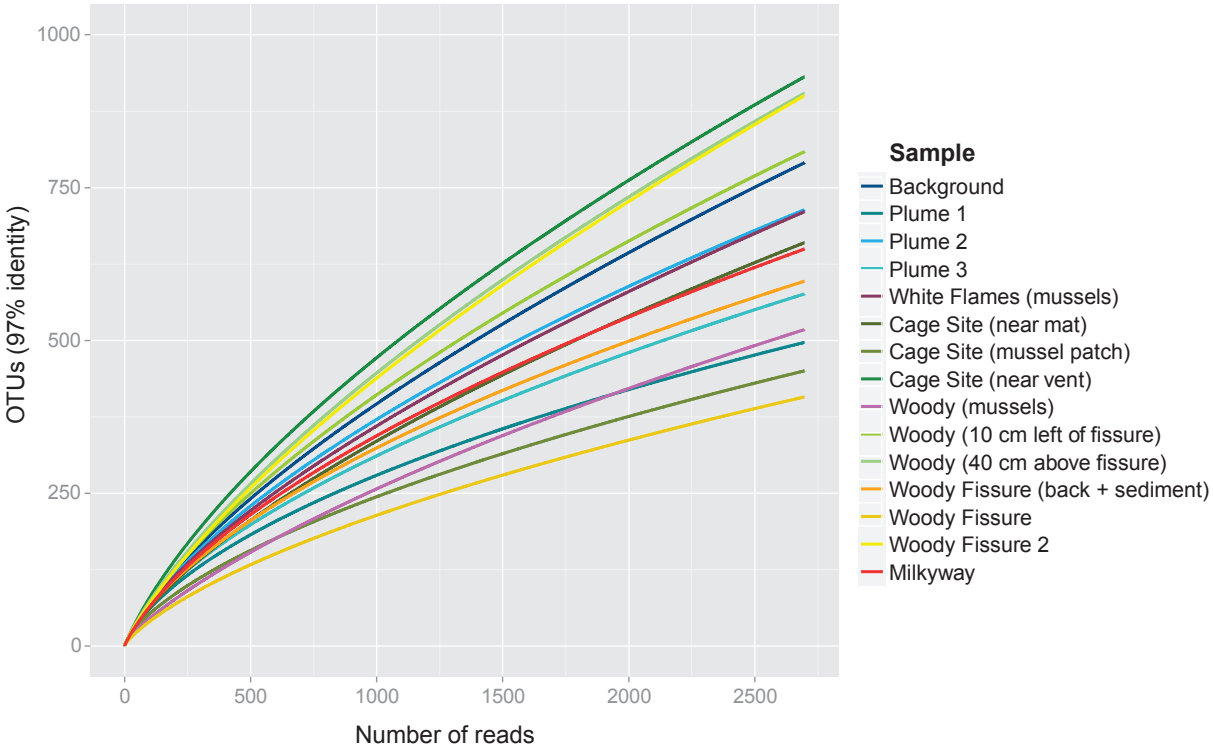

Figure S2: Rarefaction curves were calculated using the Vegan package (v. 2.0.8) based on OTUs clustered at 97% identity level

Figure S3

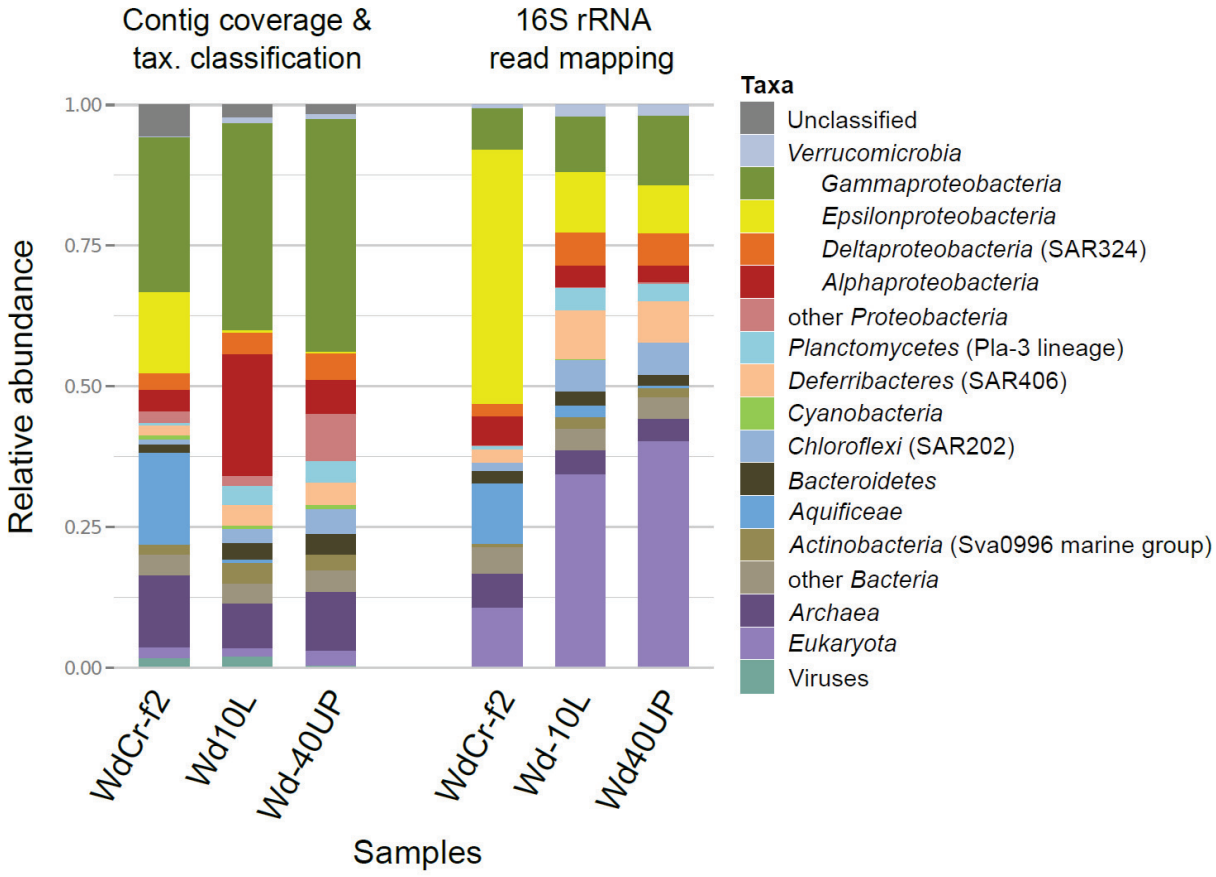

Figure S3: Relative sequence abundances of microbial groups in the three metagenomes as assessed by BLASTP based classification of all obtained scaffolds >500 bp via MetaWatt considering scaffold coverage (left) and mapping of reads to the SILVA SSU119 database by PhyloFlash (right).

Figure S4

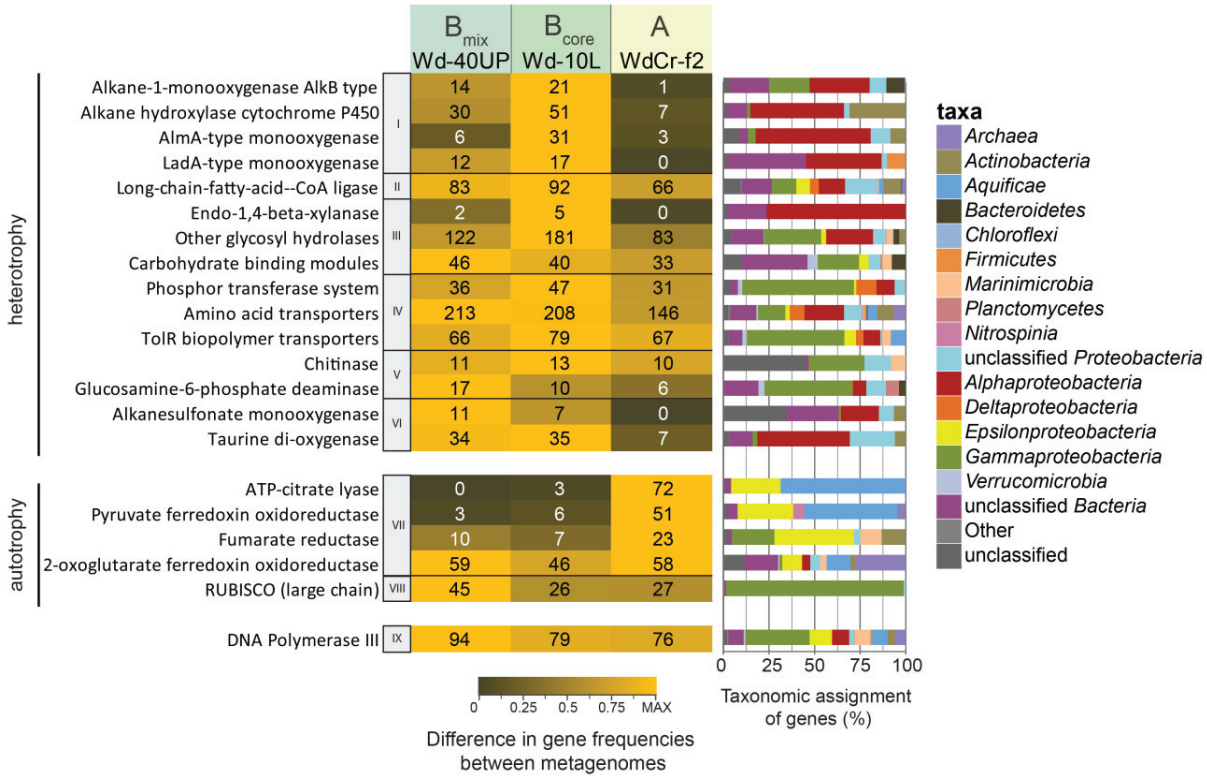

Figure S4: Normalized gene frequencies and taxonomic affiliation of genes indicative of metabolic pathways. Gene categories indicated by roman numbers: I - alkane oxidation enzymes; II - ß-oxidation of fatty acids; III - carbohydrate active enzymes; IV - transporters for sugars, amino acids and large biopolymer molecules; V - chitin degradation: VI - alkane sulfonate degradation; VII - reverse TCA cycle (CO2 fixation); VIII - CBB cycle (CO_2-fixation); IX - DNA replication (housekeeping gene). Genes were searched in the annotated metagenome bulk assemblies with the IMG “search function” interface, based on Pfam motifs, or KEGG Orthologs terms (KO terms) assignment. In order to determine normalized gene frequencies, gene copy numbers were first estimated based on the coverage of the contigs carrying the genes, as conducted by the IMG-MER annotation interface (Markowitz et al., 2012). Gene copy numbers were normalized by dividing through the average coverage of the respective metagenome (total mapped reads / total assembly length) to account for differences in sequencing depth between datasets. Taxonomic assignments were obtained by the IMG-MER annotation system based on the genes encoded on the contigs. Heatmap coloring indicates the fold difference in frequency of a gene between metagenomes.

Figure S5

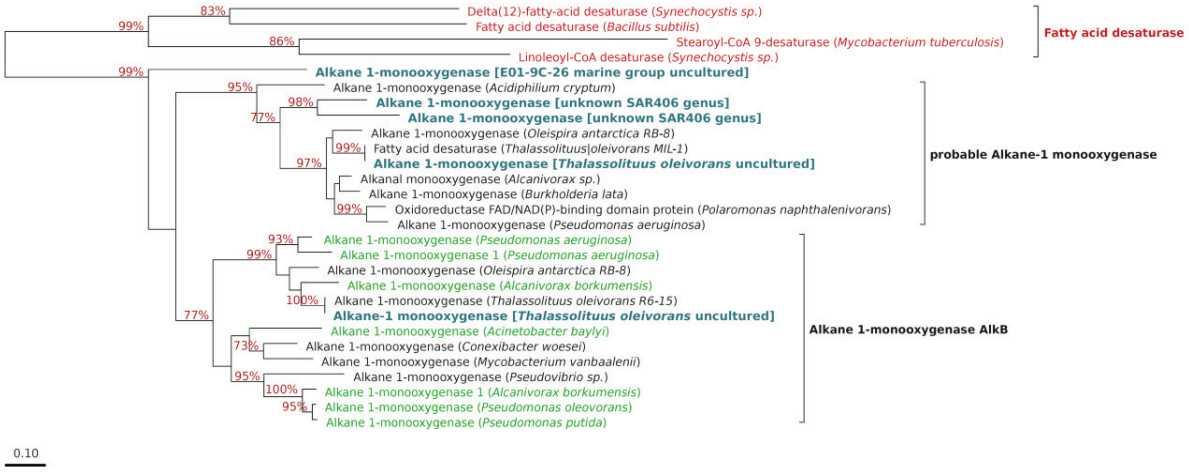

Figure S5a: Phylogenetic affiliation of AlkB-like monooxygenases detected in the re-assembled genomic bins based on the presence of a FA-desaturase Pfam domain (e-value > e-5). In green sequences of proteins with confirmed alkane monooxygenase activity are shown, and in red proteins with confirmed activity different from those of alkane monooxygenases. In cyan, sequences obtained from the re-assembled bins are given. Alignment: MAFFT, treeing algorithm: RaxML (maximum likelihood) with 100 bootstraps and a 25% sequence conservation filter. The bar represents 10% estimated sequence changes.

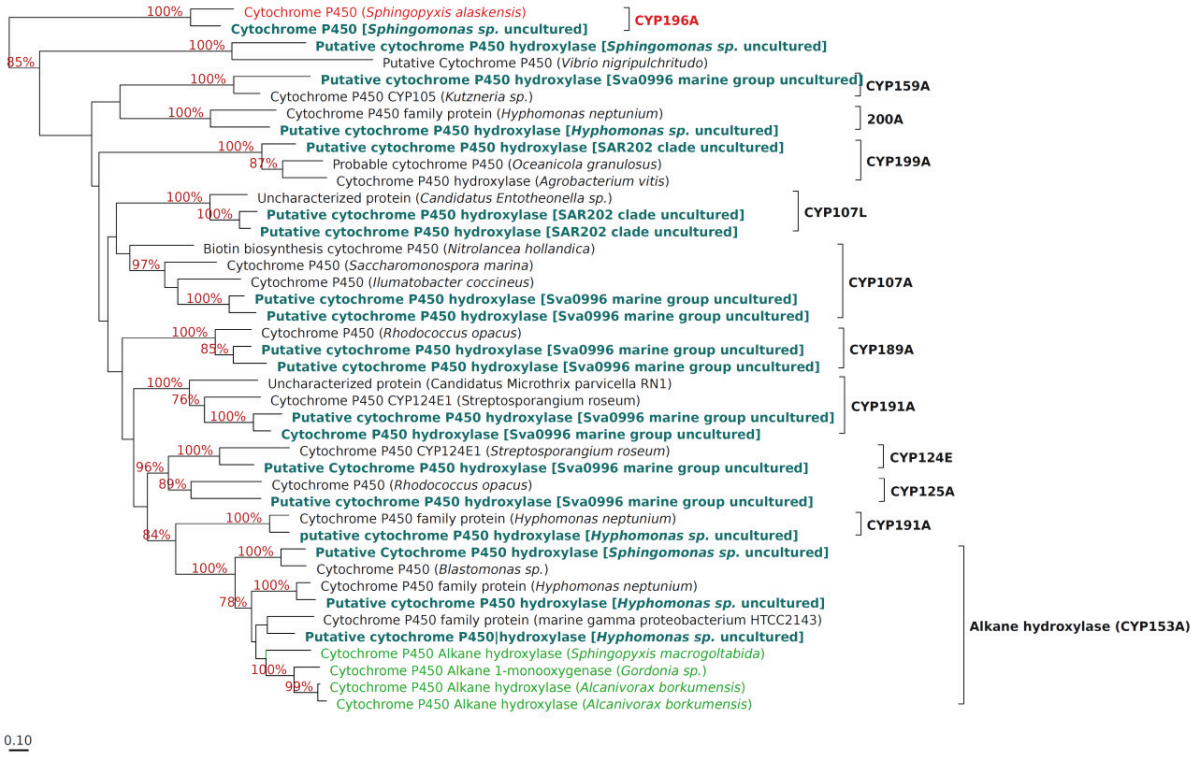

Figure S5b: Phylogenetic tree of cytochrome P450 monooxygenases and related sequences. In green are sequences of proteins with confirmed alkane monooxygenase activity, in red proteins with confirmed activity different from those of alkane monooxygenases. In bold cyan, sequences identified in the re-assembled bins based on the P450 Pfam domain are given. Alignment: MAFFT, treeing algorithm: RaxML (maximum likelihood) with 100 bootstraps and a 25% sequence conservation filter. The bar represents 10% estimated sequence changes.

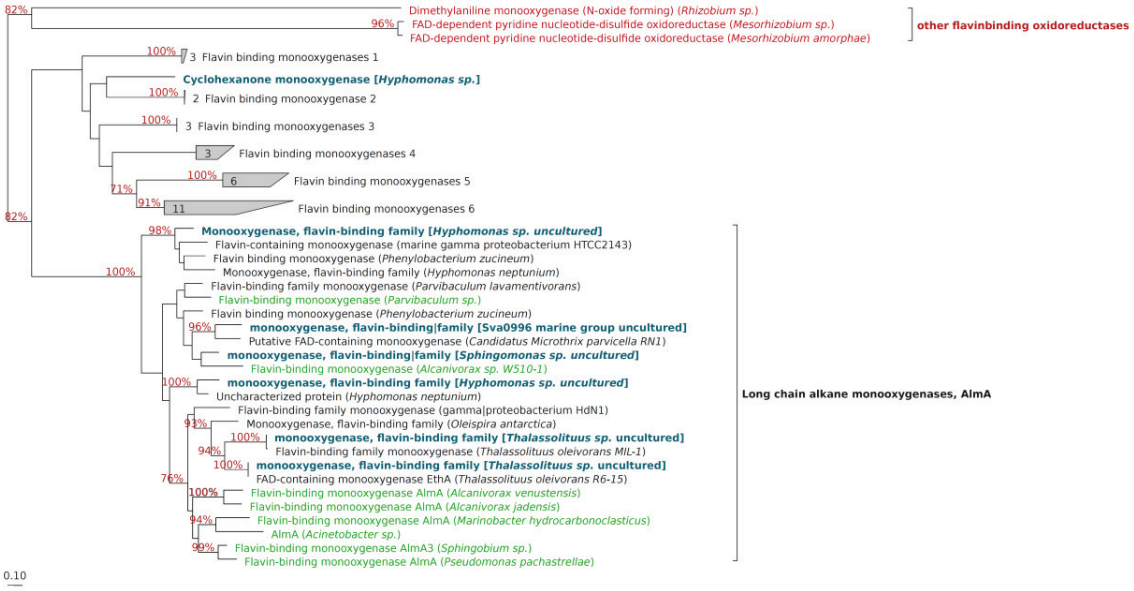

Figure S5c: Phylogenetic tree of flavin-binding AlmA-like monooxygenases and related sequences. In green are sequences of proteins with confirmed alkane monooxygenase activity, in red proteins with confirmed activity different from alkane monooxygenase. In bold cyan, sequences identified in the re-assembled bins by the flavin-binding Pfam domain. Alignment: MAFFT, treeing algorithm: RaxML (maximum likelihood) with 100 bootstraps and a 25% sequence conservation filter. The bar represents 10% estimated sequence changes.

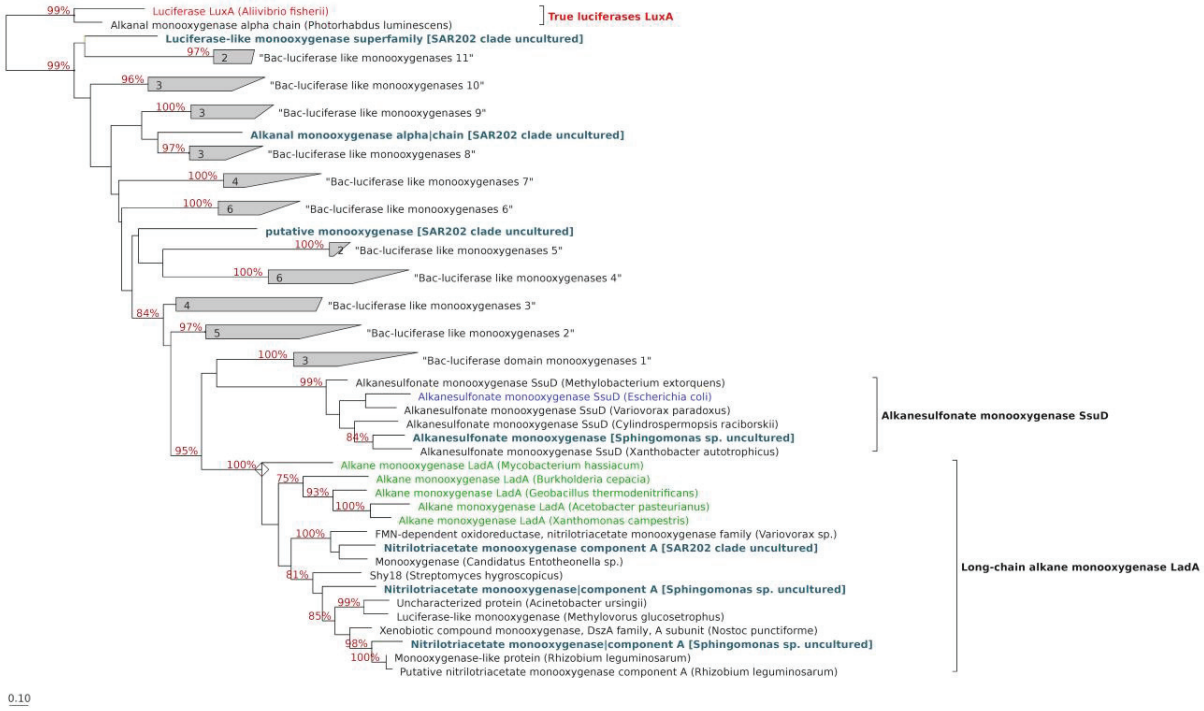

Figure S5d: Phylogenetic tree of luciferase-like flavin-binding monooxygenases and related sequences. In green are sequences of proteins with confirmed alkane monooxygenase activity, in red proteins with confirmed activity different from alkane monooxygenase. In bold cyan, sequences identified in the re-assembled genomic bins based on the presence of a luciferase-like Pfam domain are given (original RAST automatic annotation names kept). Alignment: MAFFT, treeing algorithm: RaxML (maximum likelihood) with 100 bootstraps and a 25% sequence conservation filter. The bar represents 10% estimated sequence changes.

Figure S6

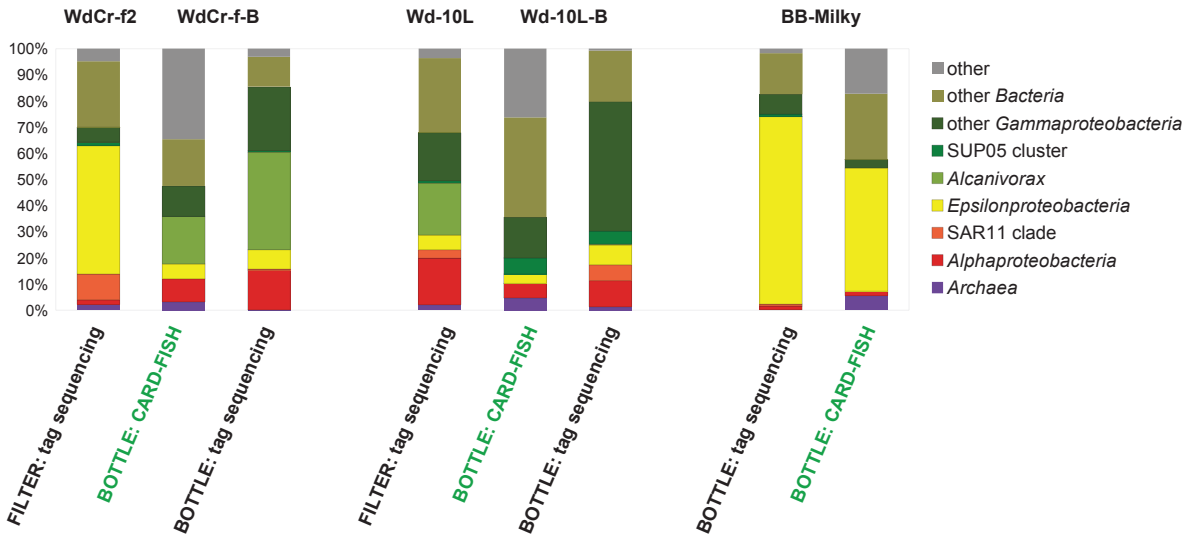

Figure S6: Comparison of relative abundance data assessed by CARD-FISH counts and 16S rRNA tag sequencing of *in situ* filter samples and filters derived from the same KIPS-bottle the CARD-FISH samples were taken from.

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V General discussion

5.1 Discussion

Since the discovery of deep sea hydrothermal vent systems in the late 1970s a large body of research was done on the organisms inhabiting them. A basic understanding of this unique ecosystem relying almost entirely on chemolithotrophic carbon fixation by microorganisms is now well established (Weiss et al., 1977; Karl et al., 1980; Baross and Hoffman, 1985; Jannasch and Mottl, 1985; Orcutt et al., 2011; Sievert and Vetriani, 2012). Representatives of the majority of hydrothermal microbial clades have been isolated and their metabolisms have been studied (Emerson and Moyer, 1997; Finster et al., 1998; L'Haridon et al., 1998; Marteinsson et al., 1999; Alain et al., 2003; Inagaki et al., 2003; Jolivet et al., 2003; Takai et al., 2003; Inagaki et al., 2004; Miroshnichenko et al., 2004; Takai et al., 2006). Yet, the ecological architecture and dynamics of vent microbial communities remain largely unresolved. Due to technical difficulties of sampling in the deep sea, ecologically oriented studies of microbial communities at hydrothermal vents often suffer from small sample sizes and lack of spatial or temporal resolution in a highly dynamic environment (Brazelton and Baross, 2010; Forget et al., 2010; Flores et al., 2011; Roussel et al., 2011; Xie et al., 2011; Perner et al., 2013).

In my doctoral thesis, I performed three comprehensive and, for hydrothermal environments, fine scaled studies on the structuring of microbial communities at hydrothermal vents, highlighting niche separation of chemolithoautotrophic and heterotrophic microorganisms, diversified and uniform sulfur oxidizing bacteria, surface colonizing and diffuse fluids inhabiting bacteria as well as chemolithoautotrophs inhabiting active and inactive sulfide deposits inhabiting chemolithoautotrophs (Fig. 6).

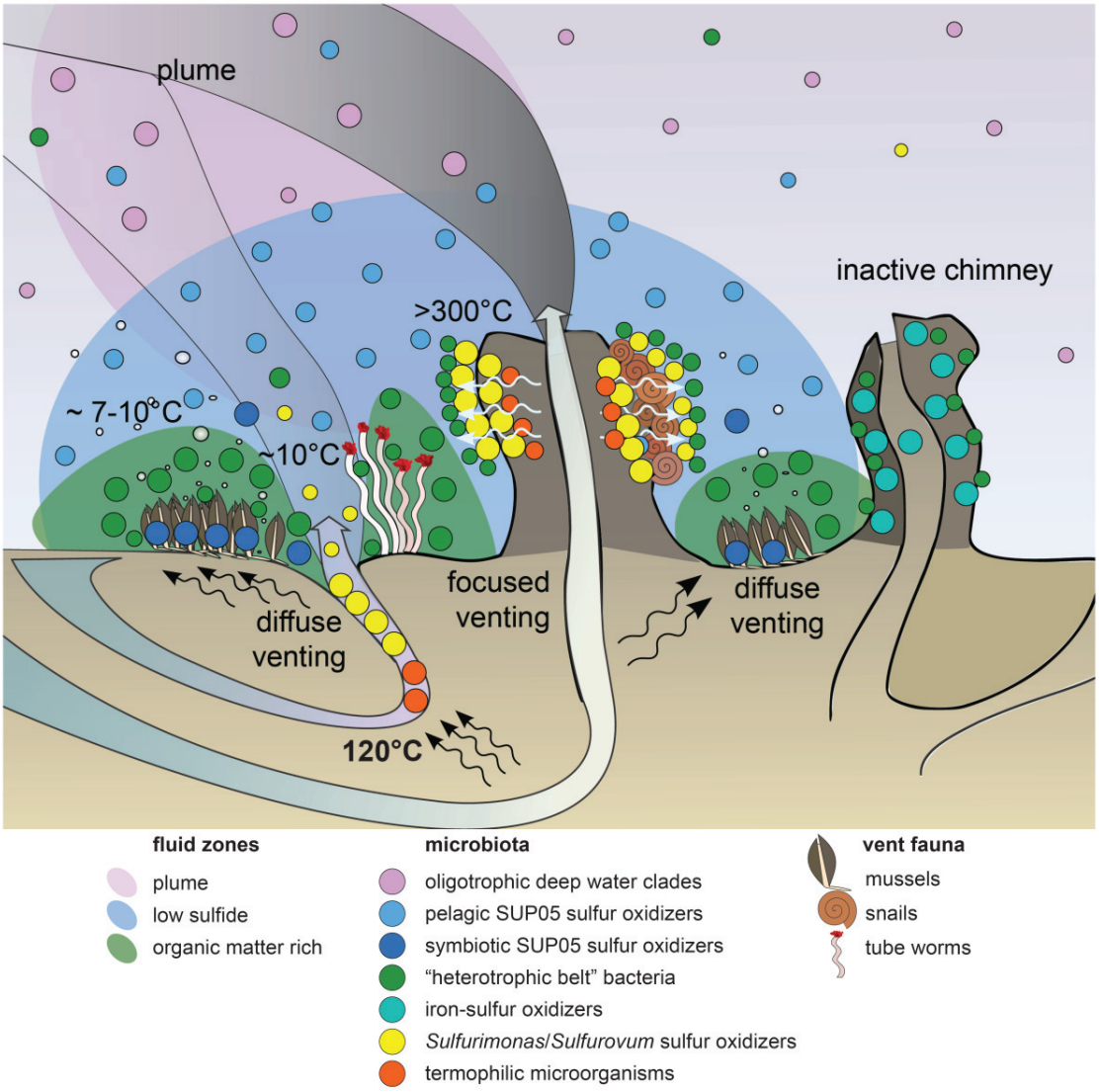

Figure 6: A schematic overview over the placement of microbial niches within the hydrothermally influenced habitats. Arrows indicate the supply of reduced compounds by hydrothermal fluids.

The first two studies presented in this thesis are based on samples taken along different stages of fluid mixing gradients at a small spatial scale. The study of diffuse venting fluids from the Manus Basin comprehensively illustrates the shift in dominating sulfur oxidizers from *Sulfurovum* and *Sulfurimonas* to SUP05-clade bacteria which coincides with increasing dilution of hydrothermal fluid. The study on the Menez Gwen hydrothermal field mainly shows the transition from chemolithoautotrophy dominated areas to adjacent areas dominated by heterotrophic microorganisms across the mixing gradient.

Several recent studies have addressed the influence of environmental gradients on niches of microorganisms in hydrothermal environments (Akerman et al., 2013; Anderson et al., 2013; Perner et al., 2013; Sheik et al., 2015). A detailed study following the microbial community change from the venting orifice into the plume (Sheik et al., 2015) revealed the relative homogeneity of a plume mainly dominated by background sea water microorganisms confirming metagenomic and metatranscriptomic insights from earlier studies (Lesniewski et al., 2012; Anderson et al., 2013). Sheik and colleagues (2015) sampled the venting orifices and nearby bottom sea water but did not resolve the gradient between them as the main focus of the study was the hydrothermal plume. The main differences detected by Sheik and colleagues (2015) were between the orifice microbial community, seafloor community and the plume, but not along the plume itself. Stable isotope probing based incubation experiments at the Axial Seamount observatory showed activity of different chemolithoautotrophic microorganisms at different temperatures in a thermophilic to mesophilic range (Fortunato and Huber, 2016). Our two studies of diffuse hydrothermal venting environments (Chapter II and IV) cover the mesophilic to psychrophilic gradient part between the diffuse venting orifices and the adjacent sea water not covered by Fortunato and Huber (2016) and not resolved by (Sheik et al., 2015), therewith completing the picture of microbial clades distribution in hydrothermally influenced habitats.

To the best of our knowledge, our study is the first one showing a statistically supported correlation between *in situ* measured geochemical parameters and distributions of major clades of sulfur oxidizing microorganisms at hydrothermal vents. This enables us to assign rough niches to the gammaproteobacterial SUP05-clade and *Sulfurovum* & *Sulfurimonas Epsilonproteobacteria* regarding their position in the mixing gradient and their lifestyle as planktonic and attached cells, respectively.

5.1.1 Niche partitioning between *Epsilonproteobacteria* and the SUP05-clade in hydrothermal habitats

Combined with *in situ* measured geochemical parameters, our 16S rRNA amplicon data corroborates niche separation between gammaproteobacterial sulfur oxidizers of the SUP05-clade and epsilonproteobacterial sulfur oxidizers affiliated with *Sulfurovum* and *Sulfurimonas* genera depending on the dilution of hydrothermal fluids (Chapter II, Fig. 3). While SUP05 seems to prefer cold, diluted parts of the mixing gradient, the *Epsilonproteobacteria* are found closer to the emission points. In other environments such as sulfidic caves, fresh water springs, and cold seeps have shown a strong dependence of sulfur oxidizers on sulfide concentration gradients (Jørgensen and Revsbech, 1983; Macalady et al., 2008; Grunke et al., 2011; Headd and Engel, 2013). In our case, it is impossible to relate read frequencies of OTUs to a single chemical parameter, as the mixing gradient is composed of multiple correlated concentration gradients, pH gradient, and temperature gradient. However, our analysis of genomic information of sulfur oxidizing bacteria also points at a driving role of reduced sulfur compounds concentrations (see 7.1.3). It further suggests that the highly fluctuating sulfide concentrations within the *Sulfurovum*/*Sulfurimonas* niche and the constantly low sulfide concentrations in the SUP05 niche result in a high level of intra-clade diversity of *Sulfurovum* and *Sulfurimonas* and low diversity of SUP05.

The likely preference for different sulfur concentrations is only one aspect of the niche partitioning between *Epsilonproteobacteria* and SUP05. We found their niche separation to be also reflected in their lifestyle and environmental stress adaptations. SUP05 bacteria were almost exclusive to the fluids (Chapter II, Fig.1) while the *Sulfurovum* species seem to prefer the attached life-style. This confirms observations of previous studies often reporting *Sulfurovum* as filamentous mats (Engel et al., 2003; Dahle et al., 2013; Meyer et al., 2013; Gulmann et al., 2015; Stokke et al., 2015) and SUP05 as a fluid or plume specific clade (Sunamura et al., 2004; Kato et al., 2009; Lesniewski et al., 2012; Akerman et al., 2013; Anderson et al., 2013; Sheik et al., 2015).

In case of hydrothermal vent sulfur oxidizers the attachment ability and stress tolerance are possibly interrelated with adaptation to sulfide concentrations. The mixing zone of oxygen and sulfide is energetically the most favorable habitat for a sulfur oxidizing microorganism (McCollom and Shock, 1997; Amend et al., 2011). In stratified marine environments like anoxic Fjords and oxyclines of the Black and Baltic Seas, both *Epsilonproteobacteria* and SUP05 sulfur oxidizers were found in high abundances in

such transition zone (Labrenz et al., 2007; Grote et al., 2008; Schmidtova et al., 2009; Grote et al., 2012; Glaubitz et al., 2013). At hydrothermal vents this transition zone lies within the walls of a chimney (Tivey, 2004) or at a turbulent venting orifice. Moreover, apart from sulfide and other reduced electron donors, hydrothermal fluid contains toxic heavy metals. *Sulfurovum* and *Sulfurimonas* have the full genomic potential to access this niche. Just as in previously sequenced genomes (Nakagawa et al., 2007; Sievert et al., 2008c; Sikorski et al., 2010; Yamamoto et al., 2010), we found genes for heavy metal efflux, exopolysaccharides, pili and flagella production as well as chemotaxis and aerotaxis in the epsilonproteobacterial bins. The SUP05 bins lacked these adaptations. Unable to attach to the surfaces and being immotile SUP05 are bound to be flushed out from the high sulfide zone by the hydrothermal fluid flow. In the surrounding water or at very weakly venting sites, SUP05 can still utilize the remaining highly diluted sulfide. The adaptive pressure resulting from constantly low sulfide concentrations selects for most competitive strains and, consequently, we find SUP05 bacteria being represented by only few OTUs. The question arises, if hydrothermal SUP05 became adapted to low sulfide environments because they are incapable to position themselves in high sulfide zones, or if they do not “need” the attachment and detoxification machinery because they are specialized on low sulfide concentrations anyway. This hypothesis could be tested in a highly sulfidic planktonic environment which would exclude the necessity of attachment to access high sulfide areas.

Interestingly, the relative abundances of SUP05-clade bacteria at Menez Gwen are rather low in comparison to the Manus Basin (Fig. 7). Hypothetically, this could be explained by lower flux rates of the sampled diffuse venting orifices at Menez Gwen leading to a steeper mixing gradient and a smaller spatial niche for the SUP05-clade sulfur oxidizers. Unfortunately, flow rates are still a rarely measured parameter in hydrothermal ecology studies and we don’t have such data for the studied systems. Additionally, the low sulfide zone is likely overlapping with the fauna colonization area. Thus, remaining diluted sulfide might be preferentially used up by symbiotic SUP05-clade bacteria residing in the abundant *Bathymodiolus* mussels (Nelson et al., 1995; Duperron et al., 2005; Petersen et al., 2011; Marcon et al., 2013) further suppressing free-living SUP05.

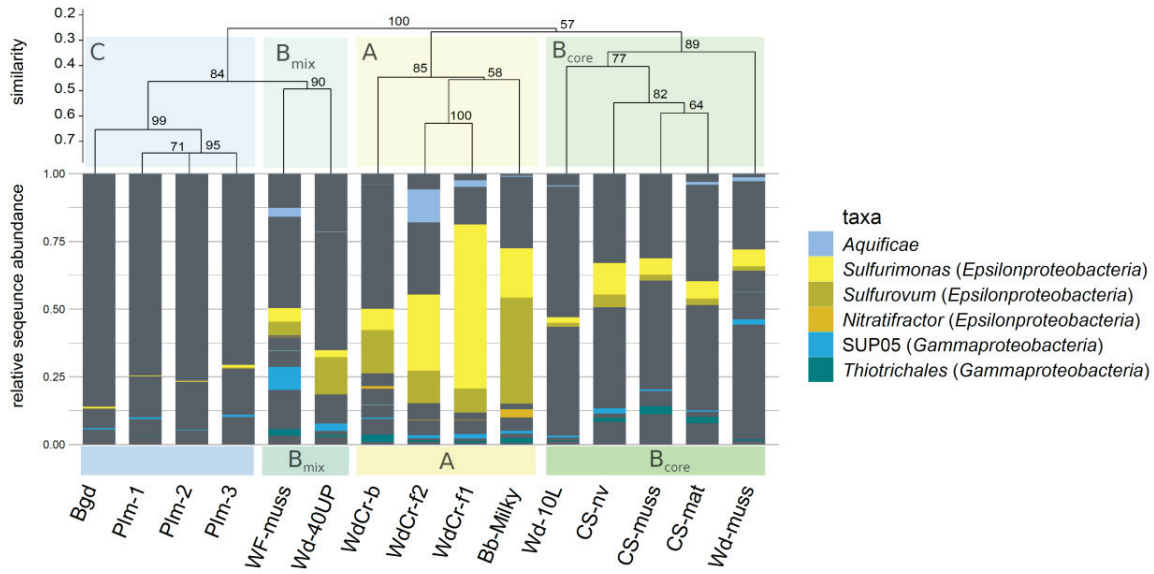

Figure 7: Distribution of 16S rRNA sequence reads of sulfur oxidizing bacterial clades in the Menez Gwen fluid samples. Particularly notable: the absence of SUP05 dominated samples. Clustering and division into categories as in Chapter IV, Fig. 4.

5.1.2 *Epsilonproteobacteria* on solid surfaces and in hydrothermal fluids

Comparison of *Sulfurovum* and *Sulfurimonas* OTUs between fluid and solid surface samples (Chapter II, Fig. 2) showed that OTUs in the fluids are composed of surface specific OTUs, fluid specific OTUs, and OTUs which appear only in few samples and cannot be attributed to neither of these two categories. The solid surface samples are dominated by surface-specific *Sulfurovum* and *Sulfurimonas* OTUs. The heterogeneity within the fluid samples could be interpreted as the result of entrainment of cells from different environments into the fluid sample. The OTUs specific for the fluid samples most likely represent the flushed out shallow subsurface microbiota (Huber et al., 2007; Huber et al., 2010; Akerman et al., 2013; Meyer et al., 2013). The possibility of planktonic fluid specific bacteria seems highly unlikely, considering that the estimated diffuse venting flow velocities are still over an order of magnitude higher than velocities known for fast-moving marine bacteria (Magariyama et al., 1994; Mitchell et al., 1995; Sarrazin et al., 2009). At the same time, the presence of OTUs specific for solid surfaces shows that the *Epsilonproteobacteria* detected in the fluid samples are not only originating from the anoxic subsurface (Huber et al., 2003; Perner et al., 2007;

Opatkiewicz et al., 2009; Brazelton et al., 2012; Akerman et al., 2013; Meyer et al., 2013; Fortunato and Huber, 2016), but could also be washed off from chimney surfaces or orifice biofilms. Could these *Epsilonproteobacteria* species exist in a subsurface-like anoxic niche at the ocean floor surface? Although anoxic micro-niches exist within the porous chimney walls (Tivey, 2004; Flores et al., 2011), high abundance of the same *Sulfurovum* OTUs on shells of vent fauna is a strong sign for their adaptation to oxic environments. The observation of a positive correlation between relative abundances of *Sulfurovum* related sequences and oxygen content of the fluid is supporting the hypothesis of an oxic surface niche for these bacteria. This is further supported by high relative abundances of *Sulfurimonas* and *Sulfurovum* related sequences in Woody Crack samples from Menez Gwen hydrothermal field (Fig. 7). At this site we know that no mesophilic subsurface exists below the orifice since the temperature measured at the deepest point of the crack was ~90°C (Chapter II, Fig. 1). So, the cells of mesophilic *Epsilonproteobacteria* must have been attached somewhere further up in the oxic mixing area of the crack. A strong argument against their aerobic adaptation is the oxygen sensitive reverse TCA cycle employed by sulfur oxidizing *Epsilonproteobacteria* for carbon fixation (Hugler et al., 2005; Campbell et al., 2006; Hugler et al., 2007; Sievert et al., 2008b). However, they could still gain access to an oxic or micro-oxic niche by growth in thick filaments creating anoxic microenvironments for carbon fixation within the filaments.

5.1.3 Microdiversity of sulfur oxidizing *Epsilonproteobacteria*

Another interesting observation was the high level of diversity within *Sulfurovum* and *Sulfurimonas* clades as compared to SUP05 bacteria. This is not only evident in short amplicon based OTUs, but is also confirmed by full length 16S rRNA sequencing and metagenome analysis. Combined, results of these techniques provide robust evidence for co-existence of over a hundred different species of both *Sulfurovum* and *Sulfurimonas* possibly constituting several genera as opposed to only about 20 - 30 closely related SUP05-clade species belonging mostly to one genus (*Candidatus* Thioglobus, Shah and Morris, 2015) (Chapter II, Fig. S2, Tab. S3).

Microdiversity is a phenomenon which has been observed before in different environments. Different explanations have been put forward: i) adaptation to a gradient environment or ii) differentiation of metabolic roles in one confined environment. In case

of *Prochlorococcus Cyanobacteria* occupying a broad niche of phototrophic CO_2-fixation in the pelagic ocean the presence of multiple ecotypes was attributed to their adaptation to different light intensities according to the water depth (Moore et al., 1998; Urbach et al., 1998). The diversity within SAR11 and Marine Group I *Archaea* was as well shown to be largely related to water depth (Garcia-Martinez and Rodriguez-Valera, 2000). On the other hand, a study of *Brevundimonas alba* isolates with identical 16S rRNA sequences showed that the strains were differing in their substrate spectrum and growth rates (Jaspers and Overmann, 2004). Also closely related strains of *Methylomonas* showed differentiation in their metabolic response to increased nitrogen stress (Hoefman et al., 2014). Co-occurring strains of *Salinibacter ruber* dominating hypersaline lakes were shown to differ in their metabolomics profile (Anton et al., 2013). In hydrothermal environments, diversification into different metabolic roles was observed in an archaeal biofilm (Brazelton et al., 2010).

In the bins of co-occurring *Sulfurovum* and *Sulfurimonas* species we could not find any clear evidence for diversification based on metabolic differences. Instead, the diversification seems to be driven by the steepness of the mixing gradient creating dramatically different environmental conditions, such as sulfide concentrations, on a small spatial scale. Supporting this we found high sequence dissimilarities between the SoxY proteins detected in the individual bins and in the bulk metagenomes. Phylogenetically, SoxY amino acid sequences of *Epsilonproteobacteria* clustered into two distant clusters, of which one had a diversity level comparable to SoxY sequence clusters of other phylogenetic groups, and the other one contained the highly diverse sequences (Chapter II, Fig. 7). Not all of the *Sulfurovum* and *Sulfurimonas* bins contained a second, conserved copy of *soxY*, since they are derived from metagenome bins and are therefore incomplete. Yet, the presence of two *soxY* copies belonging to two different phylogenetic clusters and encoded by two different operons of *sox* genes is supported by the complete isolates genomes of *Sulfurovum* sp. NBC37-1 (Nakagawa et al., 2007), *Sulfurimonas denitrificans* (Sievert et al., 2008c) and *Sulfurimonas gotlandica* (Grote et al., 2012).

At first glance, it seems likely that only the conserved version of the SoxY protein is functional, while the other version is subject to random mutations. However, our first insights into metaproteomes from Woody Crack at the Menez Gwen hydrothermal field (unpublished data) and metaproteomes of two actively venting chimney biofilms (P. Pjevac, personal communication) suggest that only the non-canonical SoxY version was

expressed. Confirming metatranscriptomic data of Dahle and colleagues (2013) we find SoxY among the top 10 expressed proteins in the Woody Crack metaproteome. The role of SoxY in the SOX multi-enzyme complex (Wodara et al., 1997; Friedrich et al., 2000; Rother et al., 2001) is the covalent binding of sulfur species anions like thiosulfate or sulfide (Quentmeier and Friedrich, 2001; Quentmeier et al., 2003). As there are no clear sub-clusters within the second variable epsilonproteobacterial SoxY branch, it is unlikely that different variants of the second non-canonical SoxY are adapted to binding of different reduced sulfur species. The high expression of the more variable *soxY* gene might be advantageous in conditions of fluctuating sulfide concentrations in the fluids bypassing the microbial mats of *Epsilonproteobacteria*.

So, what could be the function of the conserved *soxY* copy? Although sulfur storage has never been shown in *Epsilonproteobacteria*, white floc often observed at diffuse hydrothermal venting sites indicates elemental sulfur accumulation in *Epsilonproteobacteria* habitats (Taylor and Wirsen, 1997; Taylor et al., 1999; Meyer et al., 2013). *Arcobacter* related marine and limnic strains have also been shown to form filamentous sulfur (Wirsen et al., 2002; Muyzer et al., 2005; Sievert et al., 2007). This elemental sulfur could be oxidized by vent *Epsilonproteobacteria* in case the supply of dissolved reduced sulfur compounds by diffuse fluid flow is interrupted. Oxidation of a solid phase substrate means no concentration variation. Hypothetically, the conserved SoxY version of *Sulfurimonas* and *Sulfurovum* might be optimized for this emergency scenario and thus help the bacteria survive periods of low fluid flow by oxidation of filamentous sulfur.

Modeling of vent fluid and sea water mixing (Chapter II Fig. 8) showed that within the temperature range likely preferred by the *Sulfurovum* and *Sulfurimonas* species (ca. 10 - 40°C, Inagaki et al., 2003; Inagaki et al., 2004; Takai et al., 2006; Nakagawa et al., 2007; Mino et al., 2014) the H_2S concentrations can vary about 8 fold. Such a decrease can occur within less than a centimeter of a chimney wall according to models of Tivey (2004). Depending on the variations in the strength of advective fluid flow through the chimney walls this gradient is likely to shift spatially. On the one hand, the small spatial scale and temporal fluid flow variations likely prevent the selection of few specific ecotypes of *Sulfurovum* and *Sulfurimonas*. On the other hand, even if the gradient is stable enough to allow for selection and stratification of *Sulfurovum*/*Sulfurimonas* populations, it would happen on a smaller scale than our current sampling resolution.

Our study unveils the microdiversity behind the *Sulfurovum* and *Sulfurimonas* clades by correlating results of 16S rRNA amplicon analysis, full length 16S rRNA sequencing and metagenomics. We propose that their niche in the energy-rich, but instable steep part of the mixing gradient leads to diversification of these clades. Apart from its relevance to hydrothermal environments, this study represents another piece of puzzle on our way to understanding bacterial diversification.

5.1.4 Sulfur and iron oxidizing bacteria on sulfide mineral deposits

While the Chapter II and Chapter IV mainly deal with microbial communities in dynamic mixing gradients of active diffuse venting, chemolithoautotrophic organisms can also be found on inactive hydrothermal deposits not exposed to any reduced compounds input in form of venting fluids (Wirsen et al., 1993; Eberhard et al., 1995; Suzuki et al., 2004; Kato et al., 2010; Sylvan et al., 2012). The third chapter of this thesis addresses yet another sulfur oxidizing and carbon fixing guild: bacteria colonizing inactive sulfide deposits (Fig. 6). These bacteria are not only different from the ones found at actively venting sites or in the background water community, but represent novel (mostly proteobacterial) family level lineages not represented by isolates or sequenced genomes. 16S rRNA gene amplicons based comparison of microbial community structures revealed a clear cut community shift from *Epsilonproteobacteria* dominated active sulfides to *Gammaproteobacteria* dominated inactive sulfides (Chapter III, Fig. 1). We were able to obtain first genomic information from an inactive sulfide chimney community. All investigated metagenomic bins of *Gammaproteobacteria* showed potential for sulfur oxidation and autotrophic carbon fixation. Some of the bins encoded genes potentially involved in iron oxidation (Hedrich et al., 2011; Ilbert and Bonnefoy, 2013; Singer et al., 2013; Barco et al., 2015; Field et al., 2015; Kato et al., 2015).

Earlier 16S rRNA gene based studies already showed a community shift between active and inactive chimneys. The authors hypothesized that the microbes growing on inactive chimneys might utilize the sulfide ores as a source of energy for autotrophic carbon fixation (Suzuki et al., 2004; Kato et al., 2010; Sylvan et al., 2012). Inorganic carbon incorporation was shown by Wirsen and colleagues (1993) by incubating active and inactive chimney biofilms with radioactive tracers. A more recent study by Reeves and colleagues (2014b) has demonstrated that microorganisms on active chimneys mainly fix carbon via reverse TCA cycle, while the CBB cycle is likely the main carbon

fixation on inactive chimneys, once again confirming a major shift in chemolithoautotrophic communities populating the chimneys. Additionally, several bacterial strains capable of growth with poly-metal sulfides as sole electron donor were isolated from hydrothermal deposits (Eberhard et al., 1995; Edwards et al., 2003). The strains isolated by Eberhard and colleagues (1995) were identified as *Thiomicrospira* species (Wirsen et al., 1998), while the strains obtained by Edwards and colleagues (2003) were related to heterotrophic *Alpha-* and *Gammaproteobacteria* clades based on partial 16S rRNA gene phylogeny. However, none of the genomes of these strains was sequenced so far.

We found cytochromes likely involved in iron oxidation on the contigs of sulfur oxidizing *Gammaproteobacteria* as well as on contigs of as yet unclassified bacterial species remotely affiliated to sulfate and iron reducing *Desulfuromonadales* (*Deltaproteobacteria*) (Pfennig and Biebl, 1976; Roden and Lovley, 1993; Coates et al., 1995; Greene et al., 2009; Slobodkina et al., 2012) and iron oxidizing *Leptospirillum ferrooxidans* (*Nitrospirae*), known from acid mine drainage (Schrenk et al., 1998; Edwards et al., 1999; Hippe, 2000). This genomic potential for iron oxidation (Chapter III, Fig. 6) suggests that the microbial communities inhabiting hydrothermal sulfide deposits might be actively leaching sulfide from the ore rather than relying on abiotic oxidation of iron-sulfides by oxygen or ferric iron (Lowson, 1982; Moses et al., 1987; Nicholson et al., 1988; Evangelou et al., 1998; Schippers and Jørgensen, 2001, 2002). Considering the wide distribution of iron-sulfur minerals like pyrite in marine benthic environments, clades of bacteria found on inactive sulfide chimneys might also play a global role in benthic sulfur, iron and carbon cycling. Indeed, abundant sequences in our samples were related to ubiquitous uncultured clades like the *Siboglinidae* symbionts related (SSr) clade recently reported as one of the major carbon fixing microorganisms in coastal sediments (Dyksma et al., 2016) or other *Gammaproteobacteria* found in sediments or mud volcanoes (Chapter III, Fig. S2 - S5) (Pachiadaki et al., 2010; Lenk et al., 2011; Pachiadaki et al., 2011; Pjevac et al., 2014).

The study of microbial communities of inactive sulfides confirms previous hypothesis and insight into these communities, expands the knowledge on involved key bacterial clades and defines their phylogenetic affiliations. Moreover, it provides first insights into genomic information of involved autotrophic bacteria. The dominating autotrophic *Gammaproteobacteria* appear to have sulfur and iron oxidizing potential and are wide spread in marine benthic environments. Inactive hydrothermal chimneys might therefore

offer an opportunity to study microbial clades of global importance for benthic sulfur, iron and carbon cycling in an environment less complex than marine sediments.

5.1.5 The "heterotrophic belt" of hydrothermal vent ecosystems

In the third study included in this thesis (Chapter IV) we investigated the location and identity of heterotrophic microorganisms in deep sea hydrothermal environments.

Free living bacteria dominating the microbial communities immediately adjacent to the diffuse venting sites of Menez Gwen were related to heterotrophic species of *Gamma*- and *Alphaproteobacteria*. These *Gamma*- and *Alphaproteobacteria* were also different from the dominant clades found in the plumes and hydrothermally unaffected oligotrophic water. Their purely heterotrophic metabolic potential was supported by metagenome data and their presence as active cells was confirmed by CARD-FISH. These results draw a picture of a "heterotrophic belt" surrounding the zones of active primary production at diffuse venting orifices (Fig. 6). Sequences of heterotrophic *Alpha*- and *Gammaproteobacteria* genera detected at Menez Gwen were also found in the Manus Basin fluids and some solid surfaces samples (Fig. 8). For example, sequences attributed to the gammaproteobacterial genera *Alcanivorax*, *Acinetobacter*, *Alteromonas* reached abundances of up to 20%, 28%, 22%, respectively. Additionally, in microbial communities of solid surfaces sampled in the Manus Basin we also found elevated read frequencies of *Bacteroidetes* currently represented only by heterotrophic isolates. This indicates that "belts" of heterotrophic microorganisms as well as taxonomic affiliations of its key players might be universal across hydrothermal ecosystems. Supporting this hypothesis, heterotrophic microorganisms had been observed in culture-independent studies of hydrothermal environments before, but were rarely discussed in detail, since most of the studies focused on chemolithoautotrophs (Forget et al., 2010; Meyer et al., 2013; Perner et al., 2013; Sheik et al., 2015).

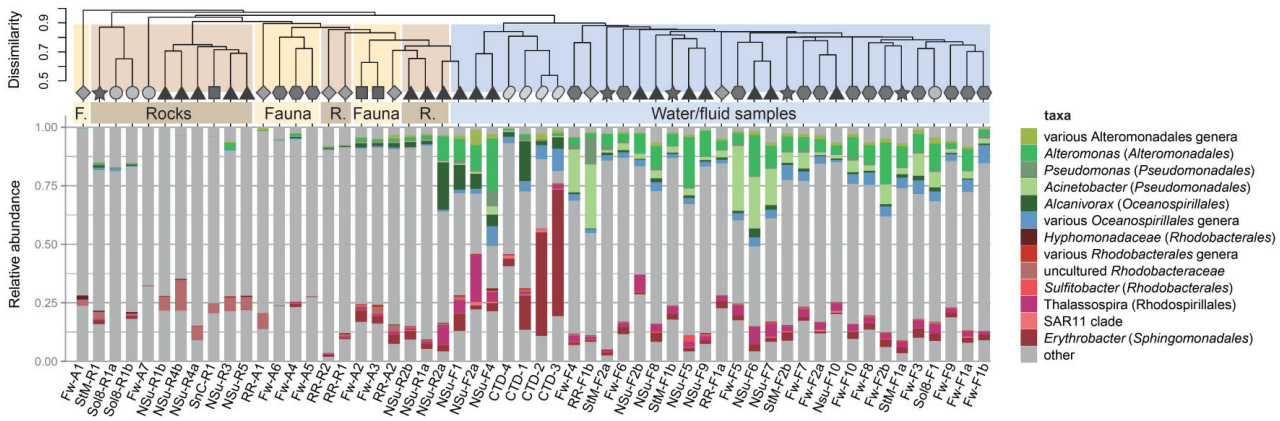

Figure 8: 16S rRNA gene read frequencies of heterotrophic *Alpha-* and *Gammaproteobacteria* in the samples from the Manus Basin hydrothermal fields. Notably, heterotrophic *Proteobacteria* are mainly found in the fluid samples. The heterotrophic niche on solid surfaces is occupied by *Bacteroidetes* (Chapter II, Fig.1 and Chapter III Fig. 1). Clustering and sample ordering as in Chapter II, Fig.1.

Vent fauna and microbial mats represent an obvious source of organic substrates. Some heterotrophic and chemolithoautotrophic vent bacteria were shown to produce exopolysaccharides (Raguenes et al., 1997a; Raguenes et al., 1997b; Stokke et al., 2015). In addition, more recently few geochemical studies provided insights on other possible carbon and energy sources for heterotrophic microorganisms by investigating abundance, origin and identity of organic matter contained in hydrothermal fluids. These studies report methane thiols, short-chain hydrocarbons and volatile fatty acids (Lang et al., 2013; Reeves et al., 2014a; Hawkes et al., 2015; Rossel et al., 2015). In our samples we found evidence for the utilization of different organic substrates by different groups of heterotrophic microorganisms.

The most abundant heterotrophic bacteria in the fluids were related to the *Gammaproteobacteria* isolated by Raguenes and colleagues (1997a,b) (*Vibrio* and *Alteromonas* spp.) and Bertrand and colleagues (2013) (e.g. *Alcanivorax*) (Fig. 8 & Chapter IV, Fig. 4). In the draft genomes of *Gamma*- and *Alphaproteobacteria* obtained from the Menez Gwen metagenomes as well as in the bulk metagenome assemblies of immediate vent vicinity samples we found the metabolic potential for aerobic degradation of alkanes of different lengths. Just as the isolates presented in Bertrand and colleagues (2013) were capable of both, growth on complex sea water medium and growth on dodecane as sole carbon and energy source, our proteobacterial draft genomes also contained transporters and enzymes for degradation of other organic substrates such as sugars, amino acids and oligopeptides, and polysaccharides (xylane, chitin). The substrates for these planktonic heterotrophic bacteria could come from various sources. Sugars and oligosaccharides might derive from “sloppy feeding“ of biofilm attached *Bacteroidetes*. Amino acids and other organic compounds might be excreted by the vent fauna. Finally, alkanes, fatty acids and other organic substrates are probably contained in the hydrothermal fluids as a result of thermogenic transformation of refractory sea water DOM (Hawkes et al., 2015; Rossel et al., 2015).

The heterotrophs on the surfaces of vent fauna sampled for the Manus Basin study were mostly members of *Bacteroidetes* just like those on chimneys and rock surfaces (Chapter II, Fig. 1). *Bacteroidetes* are known polysaccharide degraders in surface waters and are often observed in high abundance after an algal bloom (Cottrell and Kirchman, 2000; Pinhassi et al., 2004; Bauer et al., 2006; Gomez-Pereira et al., 2012). A detailed study of a single hydrothermal biofilm revealed heterotrophic *Bacteroidetes* feeding on the cellulose- or chitin-like sheath of chemolithoautotrophic *Sulfurovum* sp. (Stokke et al.,

2015). Direct attachment to the biofilms grants *Bacteroidetes* spp. an almost exclusive access to this energy-rich substrate. We observed *Bacteroidetes* sequences not only in all samples from *Sulfurovum* dominated surfaces (Chapter II, Fig. 1), but also in samples of inactive sulfide chimneys dominated by chemolithoautotrophic *Gammaproteobacteria* (Chapter III, Fig. 1). Notably, *Bacteroidetes* OTUs on inactive chimneys were completely different from the OTUs on active chimneys. Degradation of polysaccharides after phytoplankton blooms is currently suggested to be a substrate controlled process, where different members of the microbial community specialize on different substrates (Gomez-Pereira et al., 2012; Teeling et al., 2012). If the same is true for the hydrothermal *Bacteroidetes* lineages, it would imply that *Gammaproteobacteria* on inactive sulfide chimneys produce different types of polysaccharides than *Epsilonproteobacteria* on active chimneys.

Taken together, this study complements the current knowledge on possible organic substrates, heterotrophic isolates and single vent case studies by providing a first systematic evaluation of the distribution, relative abundance and identity of heterotrophic bacteria in hydrothermal environments.

5.2 Bioinformatic methods for environmental sequence analysis

Apart from high- resolution descriptions of microbial diversity at hydrothermal vent systems, this thesis also provides interesting applications of recently developed methods of sequence analysis. Numerous bioinformatics tools and approaches were tested in the process of data analysis. In the following paragraphs the methods we found to be most efficient and reliable for our data are introduced and discussed.

5.2.1 Advantages of novel OTU clustering methods

As one of the first studies, the investigation of sulfur oxidizing bacterial communities of the Manus Basin relies completely on percentage-identity-independent generation of operation taxonomic units (OTUs). OTU clustering based on percentage identity was used for over 15 years in molecular ecology research of microbial communities (Seguritan and Rohwer, 2001; Schloss and Handelsman, 2005; Sun et al., 2009; Caporaso et al., 2010; Edgar, 2010; Fu et al., 2012). Apart from arbitrary chosen thresholds, which do not apply equally to all variable regions (Yu and Morrison, 2004;

Youssef et al., 2009) of the SSU rRNA gene and to all clades of organisms (Sogin et al., 2006; Schloss and Westcott, 2011; Koeppel and Wu, 2013; Yarza et al., 2014), its weakness was the amount of calculation for pairwise alignments exponentially increasing with the number of analyzed reads. In addition, OTUs of short read amplicons have been shown to be paraphyletic (Koeppel and Wu, 2013). The limitation by computational resources was at first relaxed by more efficient, “greedy” clustering algorithms like CD-HIT or USEARCH (Li and Godzik, 2006; Edgar, 2010; Fu et al., 2012). However, their sequence clustering depends on the input order of sequences (Mahe et al., 2014). Two novel methods of grouping sequences into meaningful units, both independent of arbitrary thresholds and computationally efficient, were published recently: Minimum entropy decomposition (MED, Eren et al., 2015) and SWARM (Mahe et al., 2014, 2015).

MED, on the one hand, offers high resolution of sequence clusters down to the single nucleotide level and is at the same time an efficient method to distinguish random noise originating from PCR and sequencing errors (Huse et al., 2007; Quince et al., 2009; Kunin et al., 2010; Degnan and Ochman, 2012) from meaningful patterns (Eren et al., 2015). Such high resolution is important for clinical studies or environments with prevalence of few closely related strains (Eren et al., 2011; McLellan et al., 2013; Eren et al., 2014). SWARM, on the other hand, is valuable in revealing continuous clusters of sequences independent of percentage identity thresholds. For example, with the (standard) setting of “*d*=1” sequence reads are linked to each other as long as they differ not more than by one position in their alignment (Mahe et al., 2014, 2015) (Fig. 9). Clustering of sequences continues until there is a “gap” of more than one position difference to the next similar sequence. If the sample is sequenced deep enough, such “gap” should represent a natural boundary between two distinct units (Mahe et al., 2014, 2015).

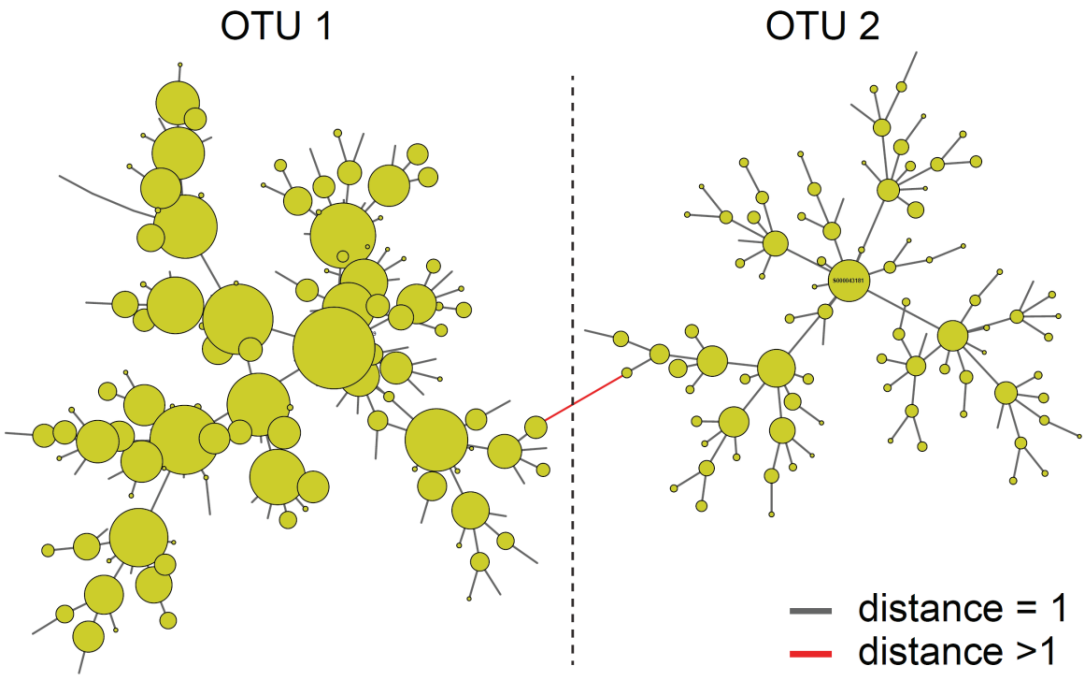

Figure 9: Example of two SWARM-generated OTUs. Nodes represent sequence reads grouped into one node by MED. The circle size is proportional to the number of included sequences. Edges represent distance between reads based on pairwise alignment. Note, the length of the edges caries no value. Black edges represent a difference of one base, red edges - difference larger than one base pair. All represented sequences were classified as *Sulfurovum*. The graph was generated based on SWARM output in Cytoscape.

The logic of SWARM can be considered analogous to the "sequence-discrete" populations introduced by Caro-Quintero and Konstantinidis (2012) while investigating existence of bacterial species by analysis of metagenomes. Caro-Quintero and Konstantinidis (2012) recruited metagenome sequence reads to assembled contigs, stepwise decreasing the minimum percentage identity required for a read to be recruited. What they observed was a rapid decrease in recruited reads from 100% to 95% identity followed by a gap from about 95% to 80% sequence identity, where almost no reads were mapping. We propose that this "genomic discontinuity" (Caro-Quintero and Konstantinidis, 2012) could be seen as analogous to the sequence differences separating the individual SWARMs. An analysis of 16S rRNA genes with SWARM parallel to the analysis of sequence-discrete populations in the metagenome of exactly same sample, ideally derived from exactly same DNA, could help relate to each other the patterns observed in both.

The analysis of microbial communities in the fourth chapter is focused rather on genus level classification of the OTUs, than on individual OTUs. Such classification-based approach was sufficient for addressing the questions posed, since the shift from chemolithoautotrophic to heterotrophic microbial community was well detectable at the genus level. In the Manus Basin studies (Chapter II, III), we used a combination of MED with a subsequent application of SWARM. For the finest generated units, the MED nodes, we could not identify any correlation between environmental parameters or sample types and relative abundances of individual MED nodes. Therefore, we interpret the MED nodes as neutral in regards to the tested parameters. It cannot be excluded though, that the existence of so many different "nodes" within one clade is explained by other, unmeasured parameters. We clustered the representative sequences of the nodes generated by MED (sequence with the highest copy number within a node) with SWARM applying the maximum distance required for linkage of two nodes set to one difference ("*d*=1"). The fact that we obtained roughly an order of magnitude less SWARM OTUs than MED nodes shows that MED did resolve the sequence space down to a level of single nucleotide difference. At the same time the presence of multiple discrete OTUs shows that there is discontinuity in the 16S rRNA sequences of the microdiverse *Sulfurovum* and *Sulfurimonas* genera. The OTUs created by SWARM did show significant correlation with respect to sample category (plume, fluid, fauna surface, rock/chimney surface). However, the correlation of community structure to measured geochemical parameters of the fluids was only significant on the level of genera, which might again contribute to the fact that resolution of sampling did not correspond to the scale of differentiation between individual OTUs.

5.2.2 Metagenome assembly and analysis techniques

Throughout this doctoral thesis I employed an "organism-centric approach" (McMahon, 2015) to metagenome analysis, including assembly, binning and targeted re-assembly of specific population genomes. Such approaches appeared already early in the history of metagenomics and were usually applied in low complexity environments (Tyson et al., 2004; Martín et al., 2006; Woyke et al., 2006; Walsh et al., 2009; Meyerdierks et al., 2010). For more complex microbial communities, however, a rather gene-centric whole metagenome analysis was largely in use until recently (Venter et al., 2004; DeLong et al., 2006; Rusch et al., 2007; Wegley et al., 2007; Yooseph et al., 2007; Brazelton and

Baross, 2009; Gilbert et al., 2010; Xie et al., 2011; Perner et al., 2014). For highly complex environments such as soil, metagenome assembly remains a challenge (Howe et al., 2014). A major limit of all metagenome approaches is difficulty in assignment of a function to a specific organism or population (Tyson and Hugenholtz, 2004; Warnecke and Hugenholtz, 2007).

In the past few years, a variety of binning software has emerged, such as e.g. MetaWatt (Strous et al., 2012), GroupM (Imelfort et al., 2014), CONCOCT (Alneberg et al., 2014), MetaBAT (Kang et al., 2015), MaxBin (Wu et al., 2014). Differential coverage between different samples is central to most of these tools, although some also employ a combination of binning criteria and include taxonomic classification based on reference databases. Yet, the possibility for the generation of chimeric bins remains (Luo et al., 2012; Sharon and Banfield, 2013) and thorough evaluation of binning results is crucial.

In our studies, we chose MetaWatt for metagenomic binning, first of all because of the wide range of criteria it combines to assign sequences to the bins, its computational speed and comprehensive visualization capabilities. Its modularity enables the user to switch particular binning criteria on and off or to give them different weights. For example, in the Manus Basin fluids co-occurrence of several *Sulfurovum*, *Sulfurimonas* and SUP05 species posed a considerable challenge to binning. While the contig bins of *Epsilonproteobacteria* could be well separated based on different coverage profiles across the five fluid metagenomes and divergent tetranucleotide sequences, the SUP05 bins were much harder to bin, due to their synchronized abundance pattern across the metagenomes (Chapter II, Figure S5). As a result, the bins of *Epsilonproteobacteria* are more complete and less redundant than the SUP05 bins. For the inactive chimney metagenome the differential coverage could not be applied, since the majority of the assembled sequences were exclusive to this sample and received no coverage in the fluid genomes.

Another important factor influencing binning is the contig length. Length of assembled contigs usually increases with lower data complexity (Albertsen et al., 2013; Howe et al., 2014). Following this logic, we collected the raw sequence reads mapping to the binned contigs as well as their sometimes unmapped read pairs and assembled them de-novo using a more sophisticated, but computationally expensive assembler (SPAdes) than for the bulk metagenome assembly (IDBA-UD). The results were longer contigs and improved binning. This process can be repeated several times with increasing stringency of mapping and binning criteria narrowing down the focus of re-assembly and leading to

a further improvement of the bins. The number of iteration remains dependent on the nature of the dataset, the questions asked by the researcher and the “cost-benefit” ratio of additional re-assembly rounds in terms of quality increase of the bin.

In conclusion, we have found an effective and relatively fast way of obtaining information from environmental DNA. First, analyzing the 16S rRNA gene amplicon data helps to understand the patterns of interaction between environmental factors and microbial community structure, identifying OTUs reacting to the change of environmental parameters and selecting representative samples for further metagenomic analysis. Instead of analyzing whole metagenomes, binning techniques and targeted re-assembly can be used to narrow the approach down to populations of interest, identified by the 16S rRNA genes analysis. Ideally, 16S rRNA information from the bins can be related to the amplicon based OTUs. Unfortunately, when several closely related genomes appear in the metagenome, 16S rRNA seems to end up on separate short contigs, probably due to regions conserved among all these genomes “confusing” the assembler. 16S rRNA genes are difficult to bin as they might 1) have higher coverage than the rest of the genome due to often multiple copies of the gene, and 2) have different tetranucleotide frequency and GC content due to high conservation of the 16S rRNA gene in microbial genomes. A high quality assignment of metagenomic 16S rRNA sequences to metagenome bins enabling to connect the amplicon based screening approach with insights from metagenomes remains a challenge for the future.

5.3 Conclusions & Outlook

Deep sea hydrothermal vents are unique habitats hosting extensive ecosystems sustained by the energy of Earth's geosphere. Physico-chemical conditions at hydrothermal vents were suggested to resemble the conditions of the early Earth and, therefore, offer valuable insights into the origin of life and the course of evolution. However, hydrothermal vent habitats are also interesting from the ecological perspective. Located in the deep sea, these oases of life receive only extremely low supply of organic matter from the photic water column. At the same time the mixing of emitted hot fluids rich in reduced compounds and oxygenated oligotrophic sea water generate a large variety of microenvironments in immediate vicinity of the vents. These settings make hydrothermal fields an exciting system to study microbial niche partitioning and adaptations to contrasting environments. The three studies presented here address these topics based on an unprecedented number of samples, high spatial resolution and supported by comprehensive geochemical data.

First, we were able to identify multiple lines of differentiation between various sulfur oxidizing autotrophic bacteria populating hydrothermal vents. These comprise the dissolved or precipitated type of sulfur compounds and their concentrations, as well as differences in lifestyle and adaptations to environmental stress. While *Sulfurovum* and *Sulfurimonas* related Epsilonproteobacteria were mainly found attached to surfaces in turbulent high sulfide zones, SUP05-clade *Gammaproteobacteria* dominated more calm and homogeneous low-sulfide areas as planktonic organisms. Yet other clades of *Gammaproteobacteria* specialize on the oxidation of sulfide minerals constituting the hydrothermal chimney structures. The additional ability to oxidize iron might be an important distinctive feature allowing them to access this niche by actively dissolving the minerals. These examples show that niche differentiation of microorganisms does always depend on more than one parameter. It is based on a combination of different features. In order to further understand the interplay of environmental factors and assign weights to the different dimensions of niche partitioning, experimental testing in a defined environment is unavoidable. Too many parameters are co-varying in natural gradients preventing the full elucidation of niche differentiation mechanisms. Recent developments in *in situ* incubation technologies, enrichment and isolation of further vent bacteria, as well as construction of artificial gradients will certainly be useful in the future for systematic testing of different conditions.

Second, we were able to generate a hypothesis explaining the high diversity within *Sulfurovum* and *Sulfurimonas Epsilonproteobacteria*. With respect to the measured geochemical parameters, *Sulfurimonas* and *Sulfurovum* seem to behave as entities, showing a clear preference in their position in the mixing gradient. Yet, our results suggest that the diversification between, e.g., different *Sulfurovum* OTUs might reflect the wide range of variation of environmental parameters, especially sulfide concentrations, in the steep part of the mixing gradient. The overall niche inhabited by *Sulfurovum* at hydrothermal vents could be described as a typical “intermediate disturbance” habitat. It provides high amounts of energy and spatially and temporally fast changing conditions at the same time, favoring diversification and preventing directed selection. However, it was also previously hypothesized that *Epsilonproteobacteria* in general are impaired in their DNA repair mechanisms and therefore prone to random and rapid diversification. A comparison of vent *Epsilonproteobacteria* to *Epsilonproteobacteria* populations in other habitats with less fluctuating conditions would be a straight forward way to test this hypothesis. If our hypothesis holds true, it should be possible to enrich different *Sulfurovum* OTUs from a diverse environmental sample under different discrete incubation conditions. Analogies to other prokaryotic organisms inhabiting intermediate disturbance niches in other habitats should also be investigated, in order to assess how wide spread this mode of diversification is in the microbial world.

Our metagenomic studies of microbial communities inhabiting inactive poly-metal sulfide chimneys have identified a possibly globally important guild of marine iron- and sulfur-oxidizing *Gammaproteobacteria*. Representatives of these barely described family level clades are found in many marine benthic habitats. Their genomic potential for iron oxidation revealed here, allows us to speculate that active pyrite oxidation might be another important energy source for carbon-dioxide fixation at hydrothermal vents, in marine sediments and ocean crust. However, first, activity studies of these microbial communities are needed in order to confirm their pyrite dissolving activity.

This study does not only cover chemolithoautotrophic processes in hydrothermal ecosystems, but also addresses carbon remineralization and microbial heterotrophy. We systematically located and identified the heterotrophic members of microbial communities at hydrothermal vents which have too long been out of focus in studies addressing hydrothermal systems. Again, a separation into surface attached *Bacteroidetes*, probably associated to chemolithoautotrophic microbial mats, and planktonic *Gamma-* and *Alphaproteobacteria* was apparent. Whereas the carbon source

for the *Bacteroidetes* most probably lies in the extracellular polysaccharides produced by the microbial mats, the carbon source(s) of the *Gamma*- and *Alphaproteobacteria* enriched in the vicinities of venting remains to be clarified. Alkane degrading potential and relatedness to known alkane degrading species could mean that these bacteria utilize organic carbon contained in the hydrothermal fluids. Recent studies suggested that refractory dissolved organic matter (DOM) becomes bioavailable through hydrothermal transformation (Hawkes et al., 2015; Rossel et al., 2015). Measurements and identification of organic matter content in hydrothermal fluids parallel to biological sampling could improve our understanding of heterotrophic bacterial niches in hydrothermal environments. Incubations of viable samples of hydrothermal microbial communities with heat-transformed refractory DOM could lead to an enrichment of the main bacterial clades utilizing it. As soon as abundant organic substances resulting from such transformation of DOM are identified, they could also be used in tracer experiments to show their uptake by certain hydrothermal microbial clades.

Finally, apart from the results of hypothesis testing and novel insights, the collected data itself offer valuable detailed information. Fast changing patterns of microbial community composition reflected in our samples illustrate how important precise sampling is in such dynamic environments. Our data will be used to guide future studies targeting specific microbial groups at hydrothermal vents, testing hypothesis phrased or refined in this study.

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Acknowledgements

Prof. Dr. Rudi Amann – Thank you for giving me the opportunity to do my PhD in the Molecular Ecology department. It was a pleasant experience. Thank you for reviewing my publications and thesis, for inspiration, discussions, valuable comments and support.

Prof. Dr. Wolfgang Bach – thank you for agreeing to review my thesis and for the help in understanding geochemistry and thermodynamics of the deep ocean.

Prof Dr. Michael Friedrich, David Probandt and Tina Enders for their participation as members of my examination board ("Prüfungsausschuss").

Anke – for the four years of supervision, all the patience and effort, for always having time for explanations and discussions. Thank you for the trust, for giving me space to develop ideas and concepts and for supporting their realization.

Petra – for really fun scientific discussions, spontaneous brainstorms, good advice and sharing your experience. It is a great pleasure working with you. Another big thanks for reading and commenting on large parts of this thesis.

The people of the **Molecol and the MPI** for making these four years a very pleasant and exciting experience. This PhD in this environment was science how I imagined it to be.

Special thanks goes to **Nicole** and **Kathrin** for all the help in the lab. Without your backup I would still be doing DNA extractions, PCRs and FISH on all these filters and rocks… Another special thanks goes to **Harald, Michi, Liz, Juliane, Chris, Pelin** for introducing me to the world of scripts, pipelines clusters, reads, bins, and mappings.Thanks to **Hanno** for making things computable within sane time frames.
Emil and **Kathrin,** thanks for the healthy madness in the office!

All the **fellow crazy scientists and hobby-artists, -DJs, -platypuses and just party people** - for all the nights out in Bremen and beyond, cultural and "not so cultural". If there is something these four years were not, then for sure they were not boring.

Tooze, NuKing Moose and all the jammers – life would be sad without music. And the tension of a PhD would be unbearable without some rock'n'roll noise. So lets rock again!

Clara – all the big words are too big to fit here ;) So I use the opportunity for the often forgotten simple things: Thank you for the mornings, the evenings, the weekends! For color in my life!

My Parents and family – for unlimited, invaluable support I could always count on. You made me what I am and you make possible what I do. Вы научили меня думать. Большое вам спасибо за это!

Appendix A: Contributions to other studies

Ubiquitous *Gammaproteobacteria* dominate dark carbon fixation in coastal sediments

Stefan Dyksma, Kerstin Bischof, Bernhard M Fuchs, Katy Hoffmann, Dimitri Meier, Anke Meyerdierks, Petra Pjevac, David Probandt, Michael Richter, Ramunas Stepanauskas and Marc Mußmann

In press, *The ISME Journal*

doi:10.1038/ismej.2015.257

Rapid succession of uncultured marine bacterial and archaeal populations in a denitrifying continuous culture.

Beate Kraft, Halina E. Tegetmeyer, Dimitri Meier, Jeanine S. Geelhoed and Marc Strous

In *Environmental Microbiology*, (2014) 16(10), 3275–3286

doi: 10.1111/1462-2920.12552

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(Vorname, Name, Anschrift, Matr.-Nr.)

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