Co-existence and niche differentiation of sulfur oxidizing bacteria in marine environments

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Petra Pjevac

Bremen

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1. Gutachter: Prof. Dr. Rudolf Amann
2. Gutachter: Dr. Timothy G. Ferdelman

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‘A *philosopher* is a person who knows less and less about more and more, until he knows nothing about everything.
A *scientist* is a person who knows more and more about less and less, until he knows everything about nothing.’

John M. Ziman

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Summary

Reduced sulfur compounds and sulfur-oxidizing prokaryotes (SOP) are widely distributed in the marine environment. Diverse microbial lineages thrive on the oxidation of reduced sulfur. They co-exist successfully by the adaptive radiation into different physiological and ecological niches. However, the factors determining this differentiation and SOP distribution are largely unknown. Environmental factors, like pH, temperature and salinity, as well as the physiological capabilities of different SOPs for sulfur-oxidation and carbon assimilation likely govern the niche-differentiation. Therefore, as part of multiple collaborative studies, I studied the influence of substrate quality and availability on structuring sulfur-oxidizing microbial communities in different marine habitats.

First, the role of elemental sulfur (S^0), in particular cyclooctasulfur (S_8), as substrate for SOPs in marine benthic habitats was examined (**Chapter II**). We observed a specific association between *Sulfurimonas*/*Sulfurovum*-related *Epsilonproteobacteria* and S^0/S_8 regardless of the habitat. We propose that substrate quality effects SOP diversity and niche differentiation, and the capability to oxidize S_8 probably provides a competitive advantage to the *Sulfurimonas*/*Sulfurovum*-group. Moreover, we investigated the diversity and distribution SOPs along gradients of a sulfide, oxygen and light in a highly sulfidic marine karst lake (Lake Rogoznica, **Chapter III**). The comprehensive analysis of microbial diversity revealed a community shift from phototrophic to chemotrophic sulfur oxidation during holomixis and tight coupling between sulfide and oxygen concentration and the sulfur-oxidizing microbial community in Lake Rogoznica.

In two further studies, we explored different aspects of carbon assimilation in hydrothermally influenced habitats dominated by thiotrophic *Sulfurimonas/Sulfurovum*-related *Epsilonproteobacteria*. We demonstrated the effects of temperature and/or substrate flux on carbon-isotope fractionation during CO_2 assimilation in environmental samples (**Chapter IV**). Furthermore, we showed that these and other hydrothermal vent associated thiotrophs do not incorporate acetate (**Chapter V**), despite their heterotrophic potential. Other microorganisms, not involved in oxidative sulfur cycling at hydrothermal vents, showed high activity and growth after the input of organic substrate.

In summary, this thesis contributes to the general understanding of microbial ecology in sulfur-rich environments by provides novel insights into diversity and niche in sulfur-oxidizing microbial communities.

Zusammenfassung

Reduzierte Schwefelverbindungen und schwefeloxidierende Prokaryoten (SOP) sind weit verbreitet in marinen Habitaten. Phylogenetisch diverse Mikroorganismen können Schwefel oxidieren. Sie koexistieren erfolgreich, da einzelne Gruppen verschiedenen physiologischen und ökologischen Nischen angepasst sind. Die Faktoren, die diese Differenzierung und die Verbreitung von SOP in marinen Habitaten ermöglichen, sind jedoch weitgehend unbekannt. Umweltfaktoren - wie pH-Wert, Temperatur und Salzgehalt - und die physiologischen Unterschiede zwischen verschiedenen SOP in Bezug auf Schwefeloxidation und Kohlenstoffassimilation beeinflussen wahrscheinlich die Nischendifferenzierung. Daher untersuchte ich im Rahmen mehrerer kollaborativere Studien den Einfluss von Schwefel Form und Verfügbarkeit auf die Diversität in Schwefeloxidierenden mikrobiellen Gemeinschaften in verschiedenen marinen Lebensräumen.

Zuerst wurde die Rolle von elementarem Schwefel (S^0), insbesondere Cyclooctaschwefel (S_8), als Substrat für die SOP in bentischen Lebensräumen (**Kapitel II**) untersucht. Wir entdeckten eine spezifische, vom Lebensraum unabhängige Verknüpfung zwischen *Epsilonproteobacterien* aus der *Sulfurimonas*/*Sulfurovum* Gruppe und S^0/S_8. Die Form des Schwefelsubstrats scheint Auswirkungen auf die Diversität und Nischendifferenzierung von SOP zu haben. Die Fähigkeit, S_8 zu oxidieren, stellt für die *Sulfurimonas*/*Sulfurovum* Gruppe einen Vorteil gegenüber anderen SOP da. Darüber hinaus untersuchten wir die Diversität von SOP entlang des Sulfid-, Sauerstoff und Lichtgradienten in einem hoch sulfidischen marinen See (Rogoznica See, **Kapitel III**). Die umfassende Analyse der mikrobiellen Diversität enthüllte den Umschwung von phototorpher zu chemotropher Schwefeloxidation während einer anoxischen Holomixis und die enge Verknüpfung von Sulfid und Sauerstoffkonzentration und der Komposition von Schwefeloxidierenden mikrobiellen Gemeinschaften in dem Rogoznica See.

In zwei weiteren Studien untersuchten wir verschiedene Aspekte der Kohlenstoffassimilation in hydrothermal beeinflussten marinen Lebensräumen. Wir haben gezeigt, dass Temperatur und Substratverfügbarkeit eine Auswirkung auf die Isotopenfraktionierung von Kohlenstoff bei der CO_2-Assimilation durch *Epsilonproteobacterien* der *Sulfurimonas*/*Sulfurovum* Gruppe haben (**Kapitel IV**). Weiterhin zeigten wir, dass diese und andere SOP in hydrothermal beeinflussten Fluiden kein Acetat zu assimilieren scheinen (**Kapitel V**). Stattdessen reagierten andere

Mikroorganismen, die nicht am oxidativen Schwefelkreislauf in Hydrothermalquellen beteiligt sind, mit erhöhter Aktivität und erhöhtem Wachstum auf die Zugabe des organischen Substrats.

Diese Arbeit trägt zum allgemeinen Verständnis der mikrobiellen Ökologie in schwefelreiche marinen Habitaten bei, und bietet neue Einblicke zu Diversität und Nischendifferenzierung in Schwefeloxidierenden mikrobiellen Gemeinschaften.

List of abbreviations

APS	adenosine 5'-phosphosulfate reductase
ATP	adenosine triphosphate
BChl	bacteriochlorophyll
CBB	Calvin-Benson-Bassham cycle
DMSO	dimethyl sulfoxide
DMSP	dimethyl sulfoniopropionate
FISH	fluorescence *in situ* hybridization
GNSB	green non-sulfur bacteria
GSB	green sulfur bacteria
IPL	intact polar lipid
MAR	microautoradiography
NADPH	nicotinamide adenine dinucleotide phosphate
PHA	polyhydroxyalkanoate
PNSB	purple non-sulfur bacteria
PSB	purple sulfur bacteria
rDSR	reverse dissimilatory sulfite reductase system
rRNA	ribosomal RNA
RSS	reduced sulfur species
rTCA	reductive trycarboxylic acid cycle
RubisCO	ribulose 1,5-bisphosphate carboxylase/oxygenase
SI	stable isotope
SIMS	secondary ion mass spectrometry
SIP	stable isotope probing
SOP	sulfur-oxidizing prokaryote
SOX	thiosulfate-oxidizing multi-enzyme system

Chapter I

Introduction

1.1 The sulfur cycle

Sulfur is among the most abundant elements on Earth (McDonough and Sun, 1995). Besides its elemental form - pure or native sulfur (S^0), it can occur is multiple positive and negative stable redox states and therefore reacts with a variety of substances, forming diverse inorganic and organic compounds (Fig. 1). The main sulfur reservoir on Earth is the ocean and its sediments. Large quantities of sulfur are present in the water column in the form of dissolved sulfate (SO_4^{2-}), and on the sea-floor as sulfur-minerals, such as pyrite, gypsum and barite (Sievert *et al.*, 2007a). Sulfide (combined H_2S, HS^- and S^{2-} species) and elemental sulfur (S^0) are likewise abundant; however, their distribution is more limited. Both compounds are common at sites of volcanic and hydrothermal activity (e.g. Ivanov, 1971; Taylor *et al*., 1999), but also occur in coastal and deep-sea sediments (e.g. Zopfi *et al*., 2004; Jansen *et al*., 2009), at cold seep sites (e.g. Omoregie *et al*., 2008; Grünke *et al*., 2011) and in oxygen deficient water columns (e.g. Bura-Nakić *et al*., 2009; Lavik *et al*., 2009). Besides these mentioned, sulfur is present in the ocean in many other inorganic (e.g. thiosulfate - $S_2O_3^{2-}$, sulfite - SO_3^{2-}, polythionates - $S_nO_6^{2-}$) and organic (e.g. dimethyl sulfoxide - DMSO or dimethyl sulfoniopropionate - DMSP) forms (e.g. Andreae *et al*., 1983; Zopfi *et al*., 2004).

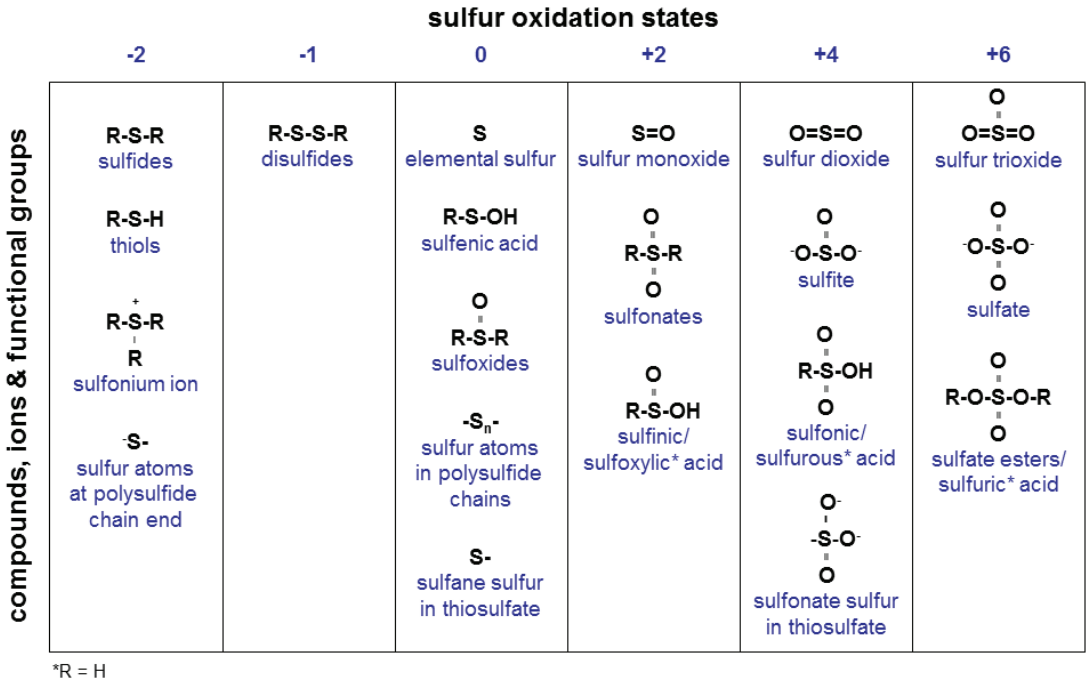

Figure 1: The most common inorganic and organic sulfur compounds, ions and functional groups (represented by simplified covalent binding models) classified according to the oxidation state of their sulfur atom.

Sulfur is an essential constituent of every living organism and all microorganisms assimilate sulfur. It is a component in diverse structural molecules, a carbon carrier and the sixth most abundant element in biomass (Klotz *et al*., 2011). Nevertheless, only a small fraction of the global sulfur pool is bound into biomass (Sievert *et al*., 2007a). The majority of sulfur undergoes biogeochemical cycling through various redox reactions (Fig. 2). The most stable and most abundant sulfur compound in marine ecosystems is sulfate, and sulfate reduction forms the basis of biotic sulfur cycling (Rabus *et al*., 2013). Various anaerobic microorganisms use sulfate (and S^0) as terminal electron acceptor, and an extensive review of their physiology and biochemistry is provided in Rabus *et al*. (2013). In many habitats, microbial sulfate and S^0 reduction is the primary source of electron donors for sulfur-oxidizing prokaryotes (SOPs), which gain energy from the photo- and chemotrophic oxidation of sulfide and other reduced sulfur species (RSS). Some microorganisms even use RSS as both the electron donor and acceptor in the 'inorganic fermentation' process of disproportionation (Bak and Cypionka, 1987; Bak, 1993). Especially the disproportionation of thiosulfate and S^0 are important microbially mediated sulfur transformations in marine systems (Jørgensen, 1990; Canfield and Thamdrup, 1996).

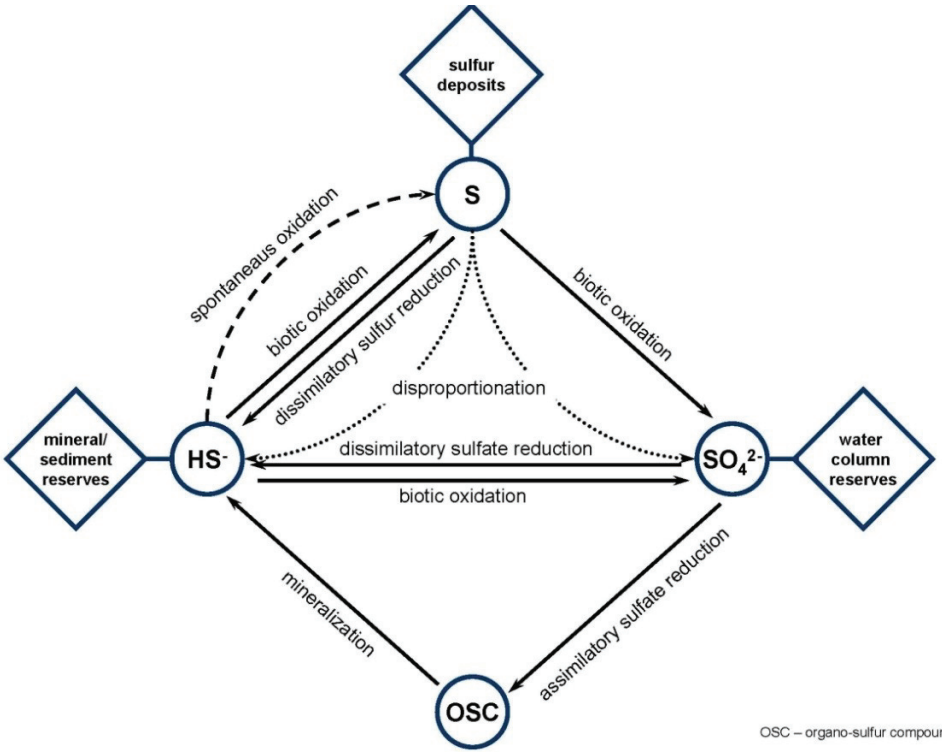

Figure 2: A simplified scheme of the biotic and abiotic sulfur cycle, adapted from Robertson and Kuenen (2006).

Most biotic reactions of the sulfur cycle have abiotic equivalents, but SOPs have evolved to successfully compete with chemical sulfur transformations (Jørgensen and Revsbech, 1983; Zopfi *et al*., 2001). Some of these processes are considered to be the oldest mechanisms of biological energy conservation (Wächtershäuser, 1990; Russell and Hall, 1997; Brocks *et al*., 2005; Wacey *et al*., 2011) and play a key role for the development of microbial biomass and diversity in numerous marine (and terrestrial) habitats.

1.1.1 Sulfur cycling in coastal sediments

The majority of sulfide in marine coastal and near-shore sediments (Fig. 3) originates from microbial sulfate reduction coupled to organic matter degradation (Jørgensen, 1977; 1982). Sulfide concentrations typically increase with sediment depth, reaching micro- to millimolar values in deeper layers, while oxygenated sediment surfaces are sulfide-free (Zopfi *et al*., 2004). A fraction of the sulfide is retained or buried in the sediment as pyrite or other sulfur minerals (Jørgensen, 1982; Chanton *et al*., 1987), while the remaining fraction is reoxidized to sulfate in competing chemical and biotic reactions. With few exceptions, biotic sulfide-oxidation in coastal sediments is dominated by chemotrophic microorganisms. A variety of phylogenetically diverse, but functionally similar SOPs has been detected, enriched and isolated from coastal sediments (e.g. Bowman *et al*., 2003; Musat *et al*., 2006; Webster *et al*., 2010; Lenk *et al*., 2011; 2012).

During sulfide oxidation, different intermediate sulfur species are formed (Zopfi *et al*., 2004). For example, the oxidation of sulfide and pyrite with manganese (IV) oxide leads to the accumulation of S^0, thiosulfate and tetrathionate (Burdige and Nealson, 1986; Schippers and Jørgensen, 2001). The amount of intermediate sulfur species (dissolved or suspended in pore waters) differs across different locations and sediment types, as well as seasonally at single locations. Usually S^0 is the most abundant sulfide oxidation intermediate (Fig. 3), and it accumulates to higher concentrations than other more reactive RSS (e.g. polysulfide, thiosulfate, tetrathionate, sulfite; Zopfi *et al*., 2004).

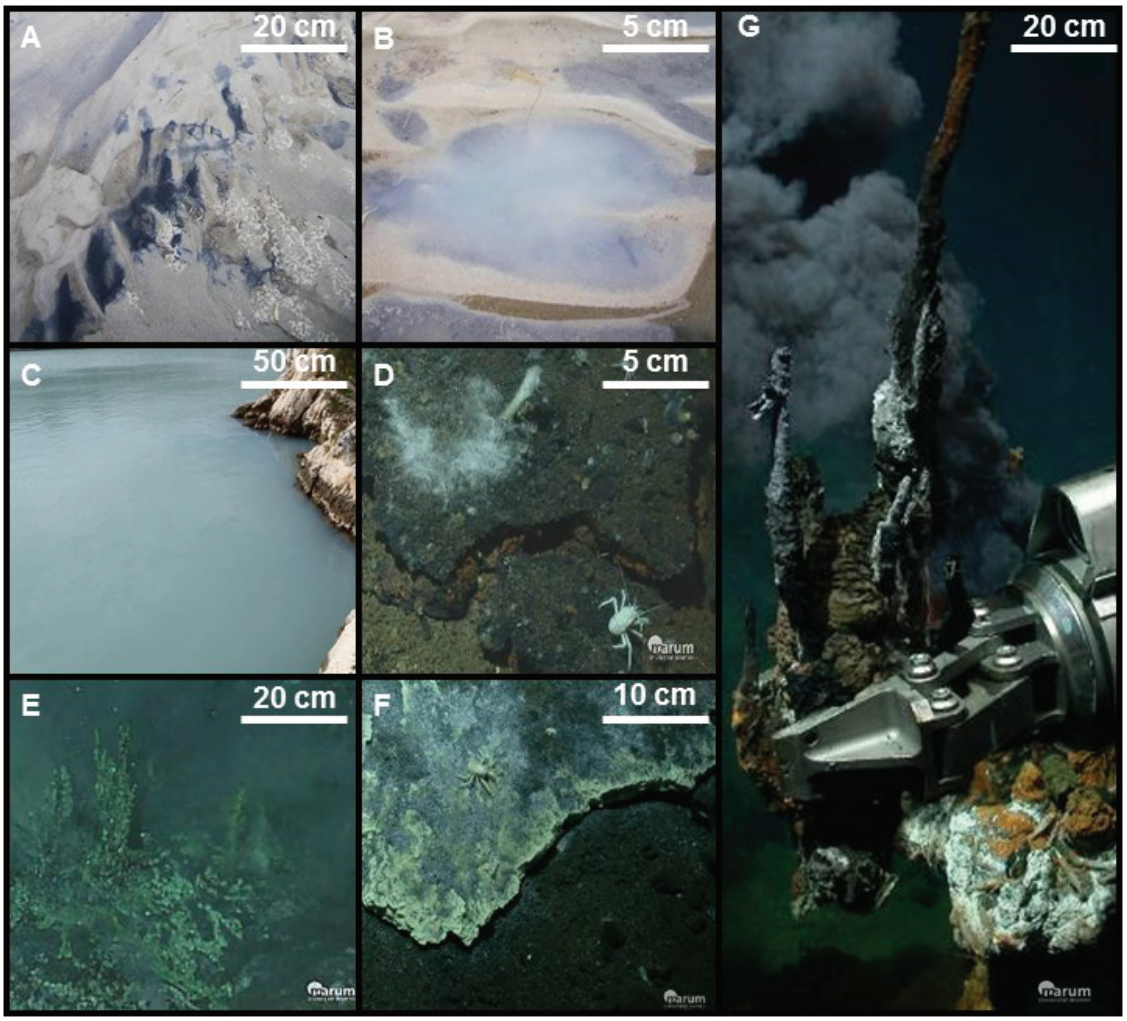

Figure 3: Visible manifestations of the presence of sulfide and elemental sulfur (S^0) in different marine environments. Seepage of sulfide-rich pore water to the sediment surface evidenced by the presence of (**A**) black iron sulfide precipitates and (**B**) white S^0 precipitates at the sediment surface of the Janssand tidal flat (German Wadden Sea). (**C**) A milky turbidity caused by the presence of colloidal S^0 in the water column of Lake Rogoznica (East Adriatic Coast, Croatia). Various displays of sulfur in the Manus Basin deep-sea hydrothermal system (Bismarck Sea, Papua New Guinea): (**D**) a patch of unidentified filamentous sulfide-oxidizing bacteria at a diffuse venting site; (**E**) flows of fresh, liquid and (**F**) older solidified S^0 generated through disproportionation of volcanogenic sulfur dioxide gas; (**G**) a sulfide mineral chimney ('black smoker') actively venting sulfide- and reduced metal-rich hydrothermal fluids.

1.1.2 Sulfur cycling at hydrothermal vent and cold seep sites

Hydrothermal vents are manifestations of geothermal fluid discharge caused by tectonic movement or volcanic activity. At the seafloor, they are most commonly located along mid-ocean ridges, in back-arc spreading centers and at sea mounts (Schrenk *et al*., 2010). The discharging hot fluids are enriched in geofuels (Bach *et al*., 2006) - reduced inorganic and organic compounds, like RSS, hydrogen (H_2) and methane (CH_4). While sulfide (as well as H_2 and CH_4) is mainly produced by high-temperature seawater-rock reactions in the subsurface (Jannasch and Mottl, 1985), other RSS (e.g. thiosulfate, polysulfide, S^0) form during the mixing of vent fluids with oxygenated seawater (Yamamoto and Takai, 2011; Fig. 3). The composition of reduced substrates in hydrothermal fluids can vary significantly between different geological locations (Kelley *et al*., 2002), but RSS are frequently the dominant geofuel and main energy source for vent-associated chemotrophic microorganisms (Amend *et al*., 2011).

Other habitats characterized by the discharge of reduced fluids at the sea-floor are cold seeps, pockmarks and mud volcanoes, commonly located on continental margins. Here, emerging fluids are formed by the compression of sediments during plate subduction at active margins, or compression of massive sediment loads under their own weight at passive margins (Judd, 2003). In comparison to hydrothermal vents, seep fluids are colder, enriched in CH_4 and other hydrocarbons, and exhibit a significantly slower flow rate (Jørgensen and Boetius, 2007). The reduced substrates emerging with fluids at seep sites can be of thermogenic, chemogenic or biogenic origin (Judd, 2003). Much like in coastal sediments, sulfide at cold seep sites is mainly produced by microbial sulfate reduction, in this case coupled to the anaerobic oxidation of methane (Jørgensen and Boetius, 2007). However, the chemosynthetic microbial communities thriving on reduced sulfur at cold seep sites are phylogenetically more similar to those found in deep-sea hydrothermal environments, than to those in shallow and coastal sediments (Omoregie *et al*., 2008; Grünke *et al*., 2011).

1.1.3 Sulfur cycling in the water column

In the water column, the presence of RSS has been reported at and below the chemoclines of permanently (e.g. Black Sea; Jannasch *et al*., 1974; Jørgensen *et al*., 1991) or temporarily (e.g. Baltic Sea; Rheinheimer *et al*., 1989) stratified marine basins, stratified fjords (e.g. Mariager Fjord, Denmark; Zopfi *et al*., 2001) and lakes (e.g. Lago di Cadagno, Tonolla *et al.,* 1999; Lake Rogoznica, Kamyshny *et al*., 2011), as well as in

waters of upwelling regions with episodic sulfide input (e.g. Eastern boundary upwelling systems in Namibia and Peru; Lavik *et al*., 2009; Schunck *et al*., 2013). In these systems, the source of sulfide is most commonly microbial sulfate reduction in underlying anoxic sediments (Brüchert *et al*., 2003; Kamyshny *et al*., 2011) or anoxic layers of the water column (Canfield *et al*., 2010). Unlike in other marine systems discussed here, phototrophic SOPs co-exist with or dominate over chemotrophic SOPs in many sulfidic water columns (Guerrero *et al*., 1985; Stewart *et al*., 2012; Beman and Carolan, 2013).

1.2 Sulfur-oxidizing prokaryotes

The metabolization of sulfur compounds is a widely distributed physiological trade among prokaryotes and various *Bacteria* and *Archaea* gain energy from the oxidation of reduced sulfur compounds (Fig. 4). SOPs are phylogenetically, physiologically and morphologically very diverse (Friedrich *et al*., 2005). This reflects the diversity of environments they inhabit and is likely related to the long evolutionary history of sulfur-dependent microbial metabolisms (Canfield and Raiswell, 1999).

1.2.1 Phototrophic sulfur oxidation

Photosynthesis is considered to be one of the earliest-evolved autotrophic metabolisms (Pierson and Olson, 1989). It is based on the utilization of light energy for the synthesis of organic compounds, transforming electromagnetic energy into chemical energy stored in molecular bonds (Overmann and Garcia-Pichel, 2013). Six prokaryotic lineages harbor photosynthetic members: the *Chloroflexi*, the *Chlorobi*, some *Acidobacteria*, diverse *Proteobacteria*, members of the *Heliobacteraceae* family within the *Firmicutes* and the *Cyanobacteria* (Gest and Favinger, 1983; Woese *et al*., 1985; Stackebrandt *et al*., 1996; Bryant *et al*., 2007). While the *Cyanobacteria* perform oxygenic photosynthesis, all other photosynthetic prokaryotes perform anoxygenic photosynthesis, capturing light energy without the production of oxygen, since electron donors other than water (e.g. sulfide, hydrogen, ferrous iron) are used (Vermass, 2002). Two types of mainly sulfide-oxidizing anoxygenic phototrophs are known: members of the bacterial phylum *Chlorobi* - the ‘green sulfur bacteria’ (GSB); and the ‘purple sulfur bacteria’ (PSB) within the gammaproteobacterial order *Chromatiales* (Stackebrandt *et al*., 1996). Additionally, some *Alphaproteobacteria* and *Betaproteobacteria* referred to as ‘purple non-sulfur bacteria’ (PNSB), some members of the ‘green non-sulfur bacteria’

(GNSB) - the *Chloroflexi*, some *Heliobacteraceae* and even some *Cyanobacteria* are capable of utilizing sulfide and thiosulfate as energy source under certain conditions (Hansen and van Gemerden, 1972; Cohen *et al*., 1975; Hanada and Pierson, 2006; Frigaard and Dahl, 2009).

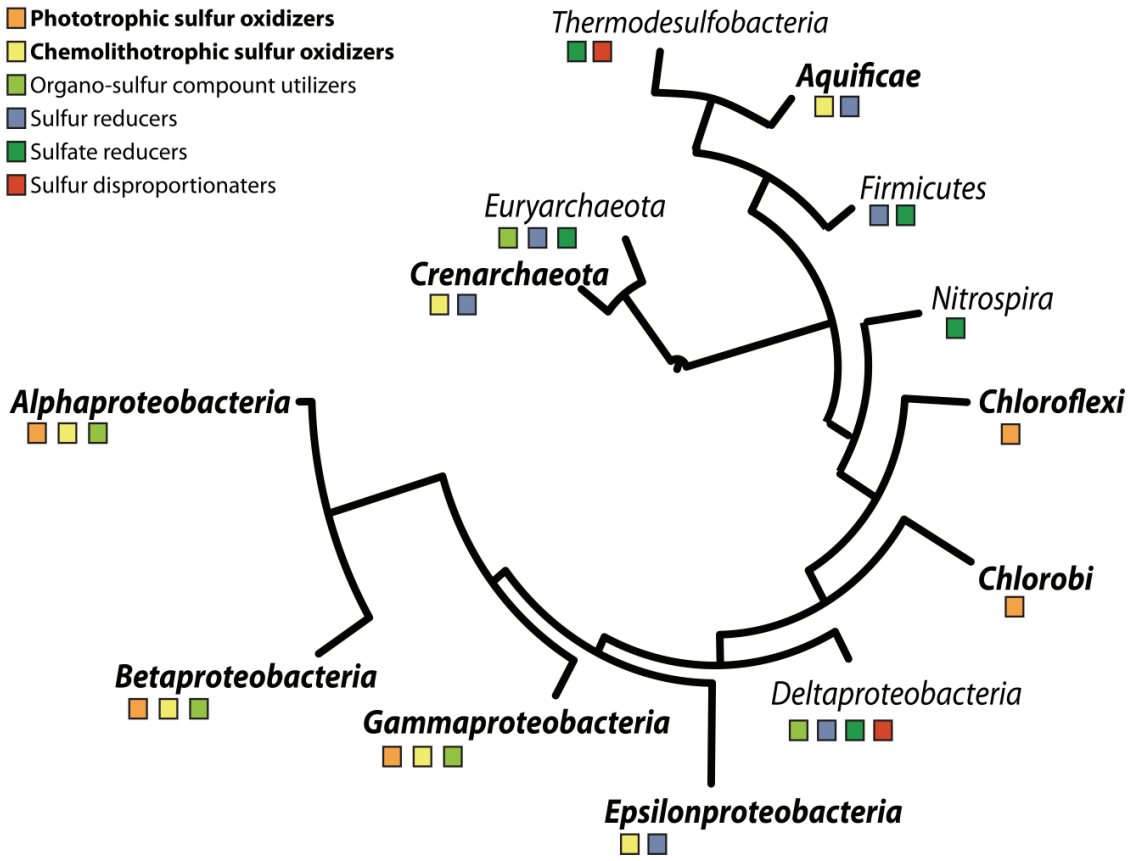

Figure 4: A schematic phylogenetic tree illustrating the diversity of sulfur-metabolizing microorganisms and the distribution of key sulfur metabolisms among major phylogenetic lineages. Lineages with sulfur-oxidizing members are shown in bold. Not all phylogenetic lineages with representatives capable of metabolizing organo-sulfur compounds are shown, as this trait is very common among *Bacteria*. Adapted from Sievert *et al.* (2007a).

Anoxygenic sulfur-oxidizing phototrophs can contribute more than 80% to the total carbon fixation in some habitats (Culver and Brunskill, 1969; Cohen *et al*., 1977), but their impact on global net primary production is low. First of all, the number of suitable habitats for sulfur-oxidizing phototrophs is relatively small. Secondly, carbon fixation driven by phototrophic sulfide oxidation mostly only balances out organic carbon remineralization during sulfate reduction in these habitats (Parkin and Brock, 1981; Overmann *et al*., 1996). Nevertheless, anoxygenic phototrophs play an important role in

the detoxification of sulfide without the consumption of oxygen (Pfennig, 1977). Thus they enable aerobic life (including oxygenic photosynthesis) above the sulfidic zone and facilitate 'real' primary production.

1.2.1.1 *Chlorobi* – the GSB

The isolated, deep-branching phylogenetic lineage of *Chlorobi* exclusively harbors strictly anaerobic, phototrophic bacteria (Stackebrandt *et al*., 1996; Overmann, 2006). Their common name, 'green sulfur bacteria', originates from the typical green or brown coloration of the cells, caused by photosynthetic pigments - bacteriochlorophylls (BChl; predominantly BChl *c*, *d* and *e*) and carotenoids. These pigments are concentrated in special light harvesting complexes called chlorosomes, and in numerous photosynthetic antennae structures (Frigaard and Bryant, 2006; Overmann, 2006).

The *Chlorobi* have very little physiological flexibility. All members are strict photolithoautotrophs, although in the absence of sufficient inorganic carbon some strains can use simple organic molecules (acetate, propionate, and pyruvate) for biomass formation (Bergstein *et al*., 1979; Overmann, 2006). Except for one isolate, all described strains are capable of utilizing sulfide and S^0 during anoxygenic photosynthesis, while some can additionally use polysulfides, thiosulfate, tetrathionate, H_2 and ferrous iron as electron donors (Steinmetz and Fischer, 1982; Brune, 1989; Heising *et al*., 1999; Overmann, 2006; Frigaard and Dahl, 2009). During sulfide oxidation, S^0 is formed and deposited as globules outside of the cells (Overmann, 2006).

As *Chlorobi* require both reduced sulfur compounds and light, they occupy narrow zones in aquatic and sedimentary environments where the opposing sulfide and light gradients overlap. They inhabit chemoclines of stratified water bodies, such as holo- and meromictic lakes, fjords and tropical lagoons (Pfennig, 1968; Trüper and Genovese, 1968; Caumette, 1984; Mas and van Gemerden, 1995). Some species form gas vesicles, which increase buoyancy and minimize cell sedimentation to dark bottom layers (Caldwell and Tiedje, 1975; Overmann, 2006). They may also form thin layers in biofilms and microbial mats at sediment surfaces (e.g. Nicholson *et al*., 1987) and in sulfidic springs and streams (Castenholz *et al*., 1990; Wahlund *et al*., 1991). Regardless of their physiological inflexibility, *Chlorobi* successfully co-exist with competing phototrophs. The molecular architecture and pigment composition of their light harvesting apparatus and their tolerance of high sulfide concentrations allow them to

inhabit the lower end of the sulfide-light gradient and establish populations below layers of *Cyanobacteria* and PSB (e.g. Overmann *et al*., 1992; Mas and van Gemerden, 1995).

1.2.1.2 *Chromatiales* – the PSB

The gammaproteobacterial order *Chromatiales* contains two phylogenetically well-defined families of phototrophic sulfur oxidizers: the *Chromatiaceae* and the *Ectothiorhodospiraceae* (Stackebrandt *et al.*, 1996; Imhoff, 2006a; 2006b). Their common name, 'purple sulfur bacteria', isderived from the color the cells gain from their photosynthetic pigments - BChl *a* and *b*, and diverse carotenoids located in vesicular, tubular or lamellar photosynthetic membranes and antennae structures (Imhoff, 2006a). Members of these two groups are easy to distinguish microscopically: during sulfide oxidation both groups form S^0, and while the *Ectothiorhodospiraceae* deposit S^0 globules extracellularly like GSB, members of the *Chromatiaceae* accumulate S^0 inside the cell (Brune, 1995; Imhoff, 2006a; 2006b; Frigaard and Dahl, 2009). Notably, salt concentrations required for growth differentiate the *Chromatiaceae*, which inhabit both freshwater and marine environments, from the *Ectothiorhodospiraceae*, which are always halophilic (Imhoff *et al*., 1998; 2001). The most halophilic bacterium known so far, *Halorhodospira halophila*, belongs to the *Ectothiorhodospiraceae* (Imhoff *et al*, 1998).

Like the GSB, the PSB are sulfide-oxidizing phototrophs. However, the metabolic versatility of PSB is substantially higher. Aside from H_2, ferrous iron, S^0, thiosulfate, tetrathionate, polysulfides and sulfite (Steudel, 1989; Steudel *et al*., 1990; Widdel *et al*., 1993; Sasikala *et al*., 1993; Ehrenreich and Widdel, 1994), organic compounds can also be used as the electron donor during anoxygenic photosynthesis (Trüper, 1981). Moreover, some PSB can live either chemolithoautotrophically or chemoorganoheterotrophically by respiring oxygen (Gorlenko, 1974; Kämpf and Pfennig, 1980; 1986; Imhoff, 2006a) or can switch to a fermentative metabolism (van Gemerden, 1968; 1974). Also, a variety of simple organic compounds (e.g. acetate, pyruvate, propionate, lactate, fumarate, and malate) can be photoassimilated by some strains (Trüper, 1981; Imhoff, 2006a; 2006b). However, inorganic carbon remains the sole or most important carbon source in most cases (Imhoff, 2006a).

Environmental conditions favoring the development of PSB populations are similar to those described above for GSB, and the two types of sulfur-oxidizing phototrophs often occur together at sulfide, light and oxygen interfaces. However, PSB

prefer lower sulfide concentrations and higher light intensities than GSB and are therefore usually more abundant above GSB layers, both in aquatic and sedimentary environments (Sorokin, 1970; Caldwell and Tiedje, 1975; Guerrero *et al*., 1985). Many PSB are motile and follow the depth fluctuations of chemoclines in stratified aquatic environments (Thar and Kühl, 2001; Tonolla *et al*., 2003). Generally, their metabolic versatility and oxygen tolerance allow the PSB to occupy habitats and niches which are not suitable for GSB. Highly dynamic coastal environments like splash water pools, tidal sediment surfaces, or brine channels in sea-ice are only some examples (Imhoff, 2006a). Specifically, the halophilic and alkaliphilic *Ectothiorhodospiraceae* are uniquely adapted to the niche of sulfur-containing, hypersaline water bodies and soda lakes (Jannasch, 1957; Imhoff and Trüper, 1977; Bryantseva *et al*., 1999; Oren, 2002; Sorokin, 2008).

1.2.2 Chemotrophic sulfur oxidation

At the verge of the 20th century, Sergei Nikolaievich Winogradsky observed and described microorganisms that use the oxidation of inorganic substrates as source of energy for the synthesis of organic molecules. He named them 'inorgoxydants', and his description of their metabolism is essentially equal to the modern-day definition of chemolithotrophy (Kelly, 1990; Kelly and Wood, 2006). The organisms Winogradsky studied were the filamentous *Gammaproteobacteria Beggiatoa,* which grow by oxidizing sulfide to S^0 and sulfate, making sulfur oxidation the first chemolithotrophic process to be discovered.

Winogradsky also coined the term still frequently used to describe non phototrophic SOP, calling them 'colorless sulfur bacteria' due to the lack of photosynthetic pigments in their cells. Today we know that 'colorless sulfur bacteria' do not form a coherent phylogenetic lineage. The capability to chemotrophically oxidize RSS has been ascribed to members of different proteobacterial taxa and the deep-branching thermophilic bacteria of the phylum *Aquificae* (e.g. Reysenbach, 2001; Takai *et al*., 2001; Stöhr *et al*., 2001; Eder and Huber, 2002; Sievert *et al*., 2007a; Fig. 4). Among the *Archaea*, only members of the acido- and hyperthermophilic order *Sulfolobales* are known to thrive on chemotrophic RSS oxidation (Segerer *et al*., 1985; Stetter, 1989; Fig. 4). Although they perform the same energy-generating reactions, the enzymatic machinery and metabolic capabilities of SOPs are very diverse and

similarities between phylogenetically distinct groups are likely a consequence of convergent evolution and horizontal gene transfer (Robertson and Kuenen, 2006).

In the majority of marine systems, proteobacterial sulfur-oxidizers drive the oxidative branch of the sulfur cycle. The *Aquificae* only have a limited distribution, as they generally require thermophilic growth conditions (Burggraf *et al*., 1992; Huber *et al*., 1992; Reysenbach, 2001), and also the *Sulfolobales*-related sulfur-oxidizing *Archaea* are rare in the marine environment (Stetter, 1989).

1.2.2.1 *Alphaproteobacteria*

Several alphaproteobacterial clades can grow by chemotrophic sulfur oxidation. For example, PNSB do not only use sulfide as a photosynthetic electron donor, but can supplement growth with chemotrophic RSS oxidation under aerobic conditions (Siefert and Pfennig, 1979; Yurkov *et al*., 1994). Also, some of the first isolated and most studied facultative sulfur-oxidizers, initially classified as *Thiobacilli*, were recognized to be members of the alphaproteobacterial genera *Paracoccus* and *Starkeya* (Kelly *et al*., 2000; Kelly and Wood, 2000). Furthermore, some *Magnetospirilli* from marine and freshwater sediments can grow by oxidizing reduced sulfur compound (Geelhoed *et al*., 2010).

In marine environments, chemotrophic *Alphaproteobacteria* are intensely involved in the oxidative sulfur cycle. *Roseobacter* clade *Alphaproteobacteria* contribute to sulfur cycling in both pelagic and sedimentary environments (Sorokin, 1995; Sass *et al*., 2010; Lenk *et al*., 2012). Members of the globally abundant SAR11 clade can metabolize organo-sulfur compounds (Gonzales and Moran, 1997; Gonzales *et al*., 1999; Buchan *et al*., 2005; Howard *et al*., 2006; Curson *et al*., 2011). Finally, recent studies showed that chemoautotrophic, sulfur-oxidizing ectosymbionts of abundant marine *Paracatenula* flatworms belong to the *Alphaproteobacteria*, classified as novel *Candidatus* Rigeria clade within the order *Rhodospirillales*, which also includes the PNSB (Gruber-Vodicka *et al*., 2011).

1.2.2.2 *Betaproteobacteria*

Members of the betaproteobacterial genera *Thiobacillus* and *Thiomonas* thrive on the oxidation of RSS (Kelly and Wood, 2006; Robertson and Kuenen, 2006). Like most other chemotrophic SOPs, they inhabit numerous environmental niches at sulfide-oxygen interfaces, such as lake chemoclines, water-sediment interfaces, costal or estuarine sediments and soils (Robertson and Kuenen, 2006). While all thus-far-described

members of the genus *Thiomonas* are facultative chemolithotrophs, which also oxidize complex organic compounds, the genus *Thiobacillus* harbours both facultative and obligate chemolithotrophic sulfur-oxidizers (Wood and Kelly, 1985; 1988; 1999; Robertson and Kuenen, 2006). However, even the obligate sulfur-oxidizing *Betaproteobacteria* are not strict autotrophs, as growth can be supplemented through incorporation of organic carbon, such as acetate and succinate (Kelly and Wood, 2006). Although sulfur-oxidizing *Betaproteobacteria* are present in many sulfidic systems, like other *Betaproteobacteria* they are more common in freshwater than in marine environments (Glöckner *et al*., 1999).

1.2.2.3 *Gammaproteobacteria*

The morphological and physiological versatility among sulfur-oxidizing *Gammaproteobacteria* is higher than within any other sulfur-oxidizing phylogenetic lineage. The gammaproteobacterial order *Chromatiales* does not only include the phototrophic PSB, but also their sister-family, *Halothiobacilliaceae*, which consists exclusively of strictly chemolithotrophic sulfur-oxidizers (Kelly and Wood, 2000). Numerous further chemotrophic sulfur-oxidizing gammaproteobacterial clades are known. Some of the most fascinating and largest bacteria belong to the giant sulfur bacteria, a group including members of the families *Beggiatoaceae*, *Leucotrichaceae* and *Achromatiaceae* within the gammaproteobacterial order *Thiotrichales* (Garrity *et al*., 2005). Some species of giant sulfur bacteria form conspicuous filamentous accumulations and mats at the sea-bed and their vacuolated cells contain various inclusions (Salman *et al*., 2013). The functions and composition of their intracellular inclusions is not yet completely resolved. So far, the storage of S^0, polyhydroxyalkanoates (PHA), polyphosphate and nitrate has been demonstrated (McHatton *et al*., 1996; Schulz *et al.*, 1999; Schulz, 2002; Schulz and Schulz, 2005; Dahl and Prange, 2006; Schwedt *et al*., 2012). Many species are motile and use their storage capabilities to transport nitrate to deeper, more sulfidic sediment layers during sulfide oxidation (Schulz, 2002; Teske and Nelson, 2006).

Other, small and unicellular gammaproteobacterial SOPs are numerous and versatile. Some of the most hostile environments on earth are inhabited by extremophile, sulfur-oxidizing *Gammaproteobacteria*. For example, members of the genus *Acidithiobacillus* grow in acid mine drainage systems at pH values as low as 0.5 (Kelly and Wood, 2000), while representatives of the highly alkaliphilic genera *Thioalkalivibrio,*

Thioalkalimicrobium, Thioalkalispira and *Thioalkalibacter* are abundant in hypo- and hypersaline soda lakes (Sorokin and Kuenen, 2005). Not all sulfur-oxidizing *Gammaproteobacteria* are obligate thiotrophs. The use of alternative inorganic and organic electron donors, as well as a variety of carbon sources has been reported for various representatives (Sorokin, 2003).

Overall, the habitat range of chemotrophic sulfur-oxidizing *Gammaproteobacteria* is vast. They have been detected in diverse sulfidic aquatic environments, preferably at oxic-anoxic interfaces. In the marine environment, they are known for forming sulfide-detoxifying blooms in oxygen minimum zones (e.g. Lavik *et al*., 2009), massive mats overlaying sulfidic deep-sea sediments (Nelson *et al*., 1989; Schulz *et al*., 1999) and the core of sulfur-oxidizing communities in coastal sediments (Jørgensen and Revsbech, 1983; Mussmann *et al*., 2003; Lenk *et al*., 2011). Furthermore, some representatives form endo- and ectosymbiotic associations with marine invertebrates (e.g. clams, mussels, snails, tubeworms, crustaceans; Dubilier *et al*., 2008), while the sulfur-rich sediments and waters surrounding the hosts and their symbionts are often inhabited by closely related, free-living species (e.g. members of the genus *Thiomicrospira* and the SUP05-clade; Ruby *et al*., 1981; Sunamara *et al*., 2004; Naganuma *et al*., 2007).

1.2.2.4 *Epsilonproteobacteria*

The *Epsilonproteobacteria* are classified in two orders, the *Campylobacterales* and the *Nautiliales*, both of which harbor chemotrophic sulfur-metabolizing members. The phylogenetic lineages (genera or environmental sequence groups) within these orders correlate well with common metabolic capabilities and environmental functions of their members (Campbell *et al*., 2006). In marine systems, the predominantly mesophilic, chemolithotrophic *Arcobacter*-, *Sulfurimonas*- and *Sulfurovum*-related *Campylobacterales* are widely distributed as free-living organisms (Taylor *et al*., 1999; Campbell *et al*., 2006; Grote *et al*., 2007; Omoregie *et al*., 2008; Lavik *et al*., 2009; Yamamoto and Takai, 2011) and as sulfur-oxidizing symbionts associated with marine invertebrates (Cary *et al*., 1997; Bright and Giere, 2005; Schmidt *et al*., 2008). The mostly heterotrophic sulfur-oxidizing *Sulfurospirillum* relatives and the moderately thermophilic S^0-reducing members of the order *Nautiliales* show a more limited distribution (Campbell *et al*., 2006).

The *Arcobacter* are physiologically diverse and are not only been common in various marine and terrestrial habitats, but are also known as commensalists and

pathogens in the human and animal intestine, much like related *Epsilonproteobacteria* from the genera *Campylobacter* and *Helicobacter* (Vandamme, 2000; Snelling *et al*., 2006). However, marine *Arcobacter* are most commonly found at sulfur-oxygen transition zones in both pelagic and benthic habitats (Llobet-Brossa *et al*., 1998; Taylor *et al*., 1999; Maugeri *et al*., 2005; Omoregie *et al*., 2008; Lavik *et al*., 2009; Grünke et al., 2011). The extracellular deposition of biogenic elemental sulfur during sulfide-oxidation with oxygen or nitrate is characteristic for marine *Arcobacter* strains (Geveretz *et al*., 2000; Wirsen *et al*., 2002; Sievert *et al*., 2007b). They form sulfur-precipitating mats on sediment surfaces, strongly resembling mats of giant sulfur-oxidizing *Gammaproteobacteria* (e.g. Taylor *et al*., 1999; Wirsen *et al*., 2002).

Sulfurimonas- and *Sulfurovum*-related *Epsilonproteobacteria* often dominate at sulfidic marine cold seep and hydrothermal vent sites (Nakagawa *et al*., 2005; Takai *et al*., 2006; Roalkvam *et al*., 2011; Schauer *et al*., 2011). They are also abundant in oxygen-depleted pelagic waters (Grote *et al*., 2007; Lavik *et al*., 2009) and hypersaline sulfidic brines (Yakimov *et al*., 2007). Less frequently, they were found to occur in sulfidic coastal sediments (Jensen *et al*., 2007; Webster *et al*., 2010). These two genera are phylogenetically well defined and separated, but seem to overlap in ecological function (Campbell *et al*., 2006). *Sulfurimonas*- and *Sulfurovum*-relatives are metabolically versatile. Besides S^0 and thiosulfate, which are used as electron donor by all isolates thus far studied, many strains also oxidize sulfide or H_2 with nitrate or oxygen (Inagaki *et al*., 2003; 2004; Nakagawa *et al*., 2005; Takai *et al*., 2006; Yamamoto *et al*., 2010; Grote *et al*., 2012). Furthermore, these *Epsilonproteobacteria* are recognized for their fast growth and the rapid colonization of diverse surfaces (López-García *et al*., 2003; Campbell *et al*., 2006).

Isolates from all three dominant marine epsilonproteobacterial groups (*Arcobacter*, *Sulfurimonas*, *Sulfurovum*) are microaerophilic and have growth optima at moderate temperatures (25-37°C), although closely related sequences have often been retrieved from cold (~4°C) and strongly hydrothermally influenced (>60°C) environments (Takai *et al*., 2006; Campbell *et al*., 2006; Yamamoto *et al*., 2010; Grünke *et al*., 2011; Schauer *et al*., 2011; Grote *et al*., 2012). They are generally chemolithotrophic, though some strains were experimentally verified to or have the genetic potential to co-assimilate acetate (Sievert *et al*., 2008a; Berg *et al*., 2013; Labrenz *et al*., 2013).

1.2.2.5 *Aquificae*

The *Aquificae* are a deep-branching bacterial phylum, harboring versatile microaerophilic thermo- and hyperthermophilic chemolithotrophs (Burggraf *et al*., 1992; Reysenbach, 2001; Huber and Eder, 2006). Consequently, their distribution is limited to low-oxygen, high-temperature environments, like e.g. hydrothermal vent deposits. Representatives have been isolated from both terrestrial and marine geothermal and volcanic environments (e.g. Kryukov *et al*., 1983; Kawasumi *et al*., 1984; Schima and Suzuki, 1993; Reysenbach *et al*., 2000; Takai *et al*., 2001; Stöhr *et al*., 2001; Eder and Huber, 2002). While their name, 'water maker', refers to the oxidation of molecular hydrogen in the 'Knallgas' reaction performed by many *Aquificae*, most strains also oxidize S^0 and thiosulfate (Huber and Eder, 2006).

1.2.2.6 *Sulfolobales* (sulfur-oxidizing *Archaea*)

Among the *Archaea*, members of the crenarchaeotal order *Sulfolobales* (Stetter, 1989) can oxidize sulfide, S^0, thiosulfate and sulfide minerals. These acidophilic (growth optimum at pH ~2) thermo- and hyperthermophiles are most commonly found in terrestrial solfataras (Brierley and Brierley, 1973; De Rosa *et al*., 1975; Huber and Prangishvili; 2006) and seem to be rare in the marine environment, although some strains have been isolated from marine hydrothermal systems (Segerer *et al*., 1986). Many *Sulfolobales* do not only aerobically oxidize RSS, but can also switch to S^0 reduction with H_2 under anaerobic conditions (Fischer *et al*., 1983; Segerer *et al*., 1985; Kurosawa *et al*., 1998).

1.3 Co-existence and niche differentiation of sulfur-oxidizing microorganisms

The Hutchinsonian niche concept, developed mid last century (Hutchinson, 1957), describes the environment as an n-dimensional hypervolume in which each biotic and abiotic condition or resource represents one dimension. Biological entities (species) are adapted to a certain set or range of environmental conditions and resources, which represents their niche. Species occupying different niches co-exist without competition, while species with overlapping niches are in direct competition. Therefore, co-existing species should exhibit a tendency to differentiate their niches in order to minimize competition (e.g. Hutchinson, 1957; Brand *et al*., 1981; Gorlenko, 1988; Williams, 1988; Grey *et al*, 2004; Polz *et al*., 2006). In light of this theory, SOPs that depend on and

compete for the same resource, namely RSS, are always in competition. Consequently, they should exhibit different preferences concerning substrate quantity/quality and environmental conditions in order to co-exist successfully.

The concept of niche differentiation has often been used to explain the composition and complexity of microbial communities in diverse environments (e.g. Bollmann *et al*., 2002; Rocap *et al*., 2003). However, studies investigating niche differentiation among SOPs are rare. Thus far, sulfide and oxygen concentrations are the best studied niche differentiation factors. For example, Macalady and coworkers (2008) have shown that sulfide to oxygen ratios and advective-diffusive regimes directly correlate with the community composition of sulfur-oxidizing biofilms in the Frasassi cave system (Italy). Filamentous *Epsilonproteobacteria* dominated in high-sulfide/high-flow regimes, while *Thiothrix*-related *Gammaproteobacteria* were more abundant under low sulfide/low flow conditions. Moreover, diffusion-controlled niches were dominantly colonized by *Beggiatoa*-related *Gammaproteobacteria*. Similar observations, linking filamentous *Arcobacter*-related *Epsilonproteobacteria* to high-sulfide/high-flux regions and filamentous *Gammaproteobacteria* to low-sulfide/low-flux regions, were made for sulfur-oxidizing mats in marine hydrothermal (Reysenbach and Cady, 2001) and cold seep environments (Grünke *et al*., 2011). Also, it is known that the layering of PSB and GSB in sulfidic environments is determined by their tolerance towards sulfide and oxygen concentrations and their light requirements (Overmann, 2006; Stomp *et al*., 2007). The large genetic and functional diversity SOPs exhibit concerning mechanisms of reduced sulfur compound oxidation and carbon assimilation probably are of key importance for niche differentiation. The use of different enzymes with different substrate affinities and catalytic properties can facilitate co-occurrence of different SOP in one habitat.

The effect of environmental factors such as temperature and pH on niche differentiation among SOPs is only known for environmental extremes, as only certain SOPs can survive at exceptionally high temperatures (Reysenbach, 2001; Huber and Prangishvili; 2006) and high or low pH (Kelly and Wood, 2000; Sorokin and Kuenen, 2005; Huber and Prangishvili; 2006; Imhoff *et al*., 2006b). The influences of temperature and pH on niche differentiation among mesophilic and neutrophilic SOPs have yet to be determined.

1.4 Exploration of sulfur oxidation and carbon assimilation pathways in sulfur oxidizing prokaryotes

The pronounced phylogenetic, physiological and ecological diversity among SOPs suggest that sulfur-oxidation as metabolic trade has not emerged in a single event during the evolutionary history of microorganisms (Canfield and Raiswell, 1999). This becomes particularly clear when one compares the underlying genetic and enzymatic mechanisms of RSS oxidation employed by different groups of SOPs. Since sulfur compounds have a broad range of oxidation states (Fig. 1), a variety of enzymes that catalyze the oxidation of RSS in energy- (adenosine triphosphate - ATP) and reducing-equivalent- (e.g. nicotinamide adenine dinucleotide phosphate - NADPH) generating reactions have evolved (Ghosh and Dam, 2009). Single enzymes can function together in enzymatic pathways, enabling SOPs to oxidize diverse RSS. As a consequence of functionally convergent evolution, different enzymes and pathways mediate the same RSS oxidation reactions. By mechanisms of horizontal gene transfer some prokaryotes have acquired more than one enzymatic pathway for sulfur oxidation (Ghosh and Dam, 2009; Loy *et al*., 2009). The overall biochemistry, enzymology and genetics of microbial sulfur oxidation are highly complex and multiple comprehensive reviews on these topics are available (Suzuki, 1999; Friedrich *et al*., 2001; 2005; Kletzin *et al*., 2004; Kappler and Dahl, 2006; Frigaard and Dahl, 2009; Ghosh and Dam, 2009; Dahl *et al*., 2013). Best studied are the widely distributed thiosulfate-oxidizing multi-enzyme system (SOX), the reverse dissimilatory sulfite reductase system (rDSR) and the adenosine 5'-phosphosulfate reductase (APS). A summary of key sulfur-oxidation enzymes is provided in Table 1.

SOPs invest the energy and reducing equivalents generated by RSS oxidation in cell growth and proliferation through carbon assimilation processes, thereby forming a link between the biogeochemical cycling of sulfur and carbon. Many SOPs assimilate organic carbon compounds during sulfur-dependent growth, but autotrophic growth (CO_2 fixation) prevails in the majority of sulfur-oxidizing microbial communities as organic carbon sources are a growth-limiting factor in most habitats (e.g. unsedimented hydrothermal systems, anoxic pelagic water columns).

Table 1: Key enzymes for microbial RSS-oxidation and their function.

Enzyme/enzyme system	Encoded by genes	Reaction
sulfide : quinone oxidoreductase	*sqr*	$S^{2-} \rightarrow S^0$
flavocytochrome *c*-sulfide dehydrogenase	*fccAB*	$S^{2-} \rightarrow S^0$
thiosulfate-oxidizing multi-enzyme system *	*soxSRT-VW-XYZABCD-EFGH*	$S^0 \rightarrow SO_4^{2-}$ $SO_3^{2-} \rightarrow SO_4^{2-}$ $S_2O_3^{2-} \rightarrow SO_4^{2-}$
reverse dissimilatory sulfite reductase system	dsrABEFHCMKLJOPNRS	$S^{2-} \rightarrow S^0 \rightarrow SO_3^{2-}$
adenosine 5'-phosphosulfate reductase + ATP sulfurylase	*aprAB* + *sat*	$SO_3^{2-} \rightarrow SO_4^{2-}$
sulfite : acceptor oxidoreductase	unknown	$SO_3^{2-} \rightarrow SO_4^{2-}$
sulfite oxidoreductase	*sorAB*	$SO_3^{2-} \rightarrow SO_4^{2-}$
sulfite dehydrogenase	*soeCBA*	$SO_3^{2-} \rightarrow SO_4^{2-}$
sulfur oxygenase reductase	*sor*	$S^0 \rightarrow S^{2-} + SO_3^{2-} + S_2O_3^{2-}$
thiosulfate : quinone oxidoreductase	*doxDA*	$S_2O_3^{2-} \rightarrow S_4O_6^{2-}$

* In some organisms a 'truncated' version of the SOX multienzyme system, lacking *soxCD* genes, is present (Friedrich *et al.*, 2005).

Sulfur-oxidizing bacteria utilize two different pathways for inorganic carbon assimilation. The sulfur-oxidizing *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria* fix CO_2 via the Calvin-Benson-Bassham (CBB) or reductive pentose phosphate cycle (Fig. 5), which is also used by oxygenic phototrophs and is the most widespread and significant carbon fixation pathway on (modern) Earth (Hügler and Sievert, 2011). Sulfur-oxidizing members of the *Epsilonproteobacteria*, *Aquificae* and *Chlorobi*, on the other hand, fix CO_2 via the reductive tricarboxylic acid (rTCA) cycle (Fig. 5), which is energetically very effective but sensitive to oxygen and therefore operates only in these anaerobic and microaerophilic bacteria (Hügler and Sievert, 2011). In all archaeal sulfur oxidizers thus far studied, CO_2 is assimilated via the recently described 3-hydroxypropionate/4-hydroxybutyrate cycle (Berg *et al*., 2007; Fig. 5).

1.4.1 Cultivation-based approaches

A seemingly straight-forward way to investigate sulfur oxidation and carbon assimilation in different SOPs is the cultivation or enrichment of these microorganisms from environmental samples. Pure cultures allow a comprehensive investigation of an organisms' physiological, biochemical and genetic trades. To date, cultured representatives from all major SOP lineages are available. Detailed studies of sulfur oxidation and carbon assimilation enzymology and biochemistry in some representative species and strains are available (revised in Ghosh and Dam, 2009; Frigaard and Dahl, 2009). A big drawback, however, is that the great majority of microorganisms, including many SOPs, are not readily culturable (Alain and Querellou, 2009; Vartoukian *et al*., 2010). During the last two decades substantial progress in the development of cultivation approaches has been made. These include dilution techniques (e.g. Schut *et al*., 1993; Takai *et al.,* 2000), cell-sorting based techniques (e.g. Zengler *et al*., 2002; Fujitani *et al*., 2013; Dichosa *et al*., 2014) and flow-through systems (e.g. Rappé *et al*., 2002; Webster *et al*., 2011), which facilitated the isolation of representatives from many thus-far uncultured lineages. Regardless of all limitations, cultivation and isolation remain essential and irreplaceable microbiological tools.

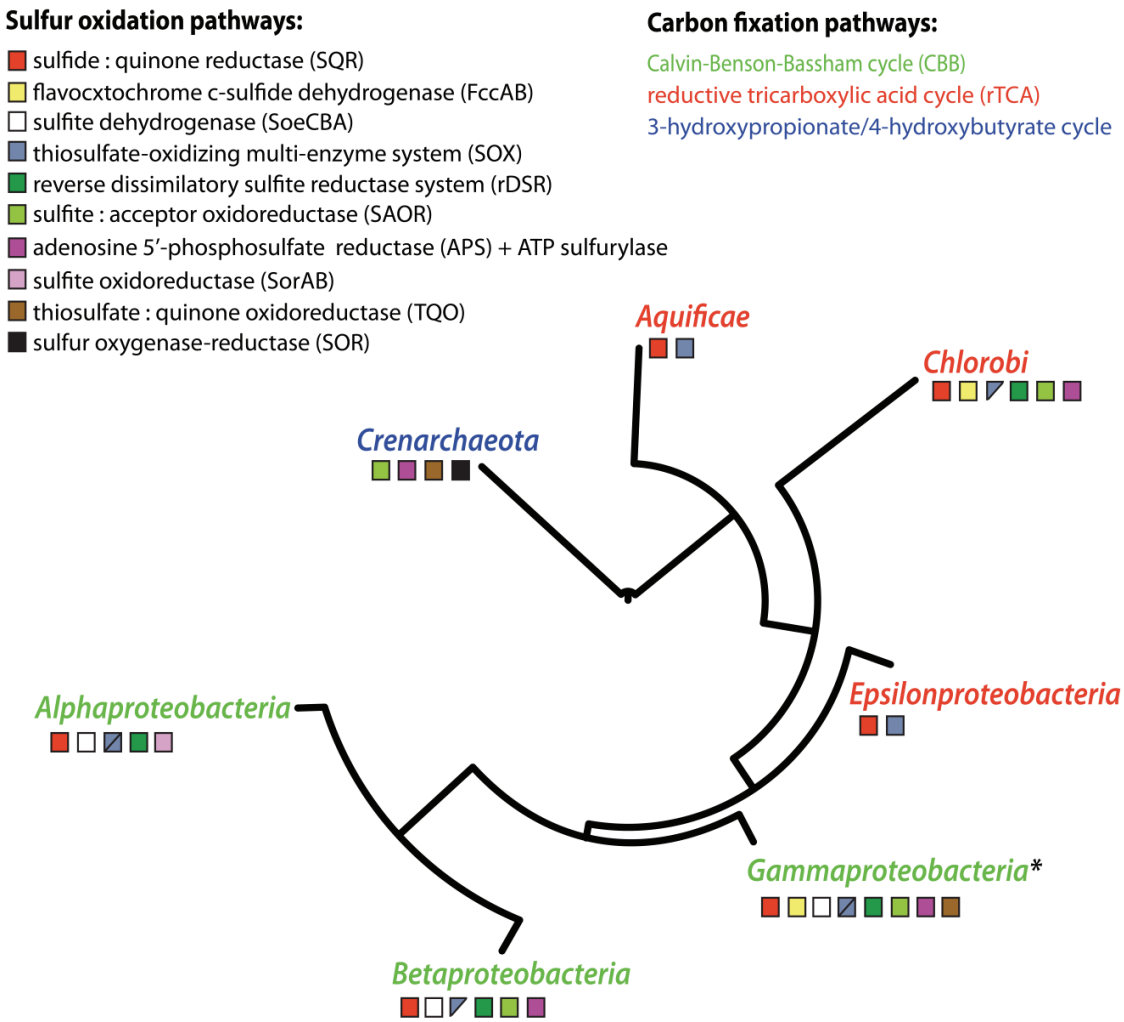

Figure 5: Distribution of key-enzymes and enzymatic pathways involved in sulfur oxidation, and autotrophic carbon assimilation pathways among different phylogenetic groups of SOPs.

1.4.2 Molecular approaches

To circumvent some of the limitations cultivation-based approaches bring along, many so-called molecular tools have been developed. These tools principally do not rely on cultivation or enrichment of the target microorganism, which allows better insights into the *in situ* diversity, composition and function of environmental microbial communities.

The full-cycle rRNA approach is the most widely used cultivation-independent, phylogeny-based tool in microbial ecology (Amann *et al*., 1995). It relies on the identification and *in situ* quantification of microorganisms in environmental samples based on rRNA sequence diversity and identity (Fig. 6). Since the phylogeny of SOPs is well studied, the phylogenetic identity of uncultured microorganisms and its relation to cultured SOPs can be used to infer their environmental function.

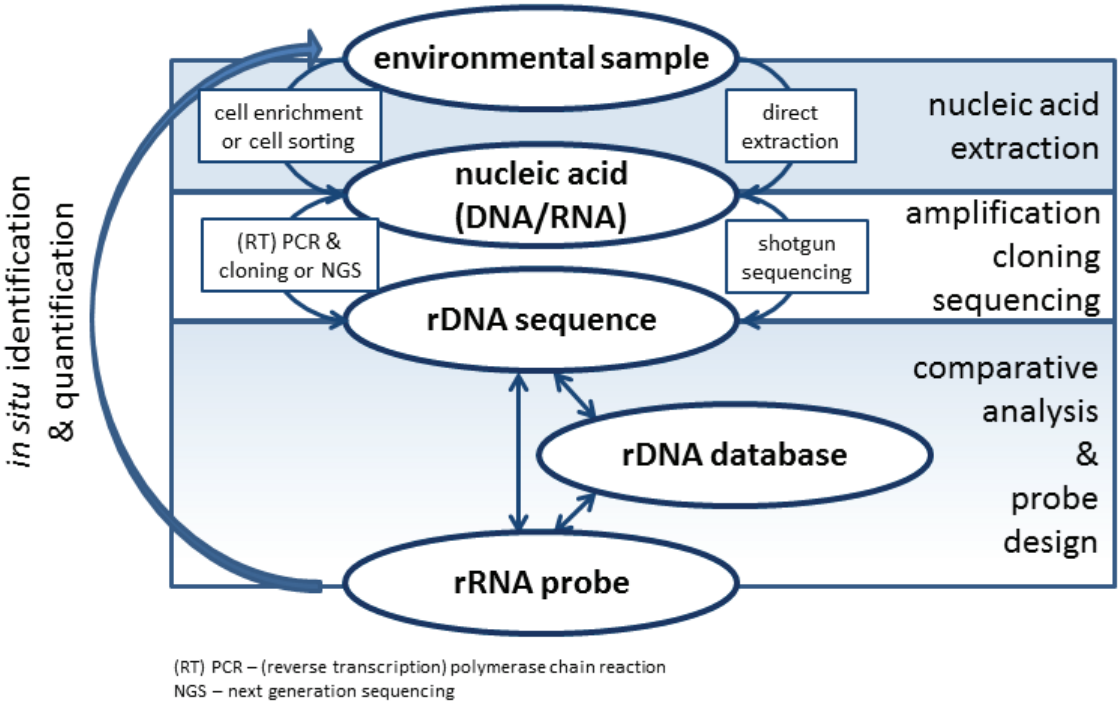

Figure 6: A schematic work-flow of the full-cycle rRNA approach. Adapted from Amann *et al*. (1995).

However, phylogeny does not always necessary reflect physiology. Therefore, the analysis of functional gene diversity and abundance is necessary to directly link an organisms' physiological potential to its phylogenetic identity. Diverse approaches rely on amplification and sequencing or identification and visualization of genes, gene transcripts and enzymes which are unique or essential for certain biochemical pathways

(e.g. Meyer et al., 2007; Meyer and Kuever, 2007; Smith and Osborn, 2008; Moraru *et al*., 2010; Petersen *et al*., 2011; Gros *et al*., 2012). Since the diversity of enzymes involved in microbial sulfur oxidation and carbon fixation is large and certain enzymes and pathways have been identified only recently, currently established functional gene approaches target only a fraction of the environmental variability (Table 2).

Table 2: Functional genes targeted in cultivation-independent studies of microbial sulfur oxidation and carbon fixation.

Gene	Enzyme	Pathway
sulfur oxidation		
aprA / aprB	adenosine 5'-phosphosulfate reductase (APS)	APS reductase / ATP sulfurylase dependent sulfite oxidation
dsrAB	reverse dissimilatory sulfite reductase (DsrAB)	rDSR system
soxB	sulfite dehydrogenase (SoxB)	SOX multienzyme system
carbon fixation		
cbbL / cbbM	ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO)	CBB cycle
aclA / aclB	ATP citrate lyase	rTCA cycle
accC	acetyl-CoA/propionyl-CoA carboxylase	3-hydroxypropionate / 4-hydroxybutyrate cycle

Finally, molecular tools that mostly bypass biases introduced by gene-based approaches are unified under the term 'omics'. These include genomic, transcriptomic, proteomic, metabolomic and lipidomic approaches (Riesenfeld *et al*., 2004; Wenk, 2005; Maran *et al*., 2007). Generally, they rely on the bulk extraction of nucleic acids, proteins, lipids or metabolites from unmodified microbial communities (meta-omics), selected microbial populations or even single cells. Individual and combined 'omic' approaches allow an in-depth analysis on a genetic, compositional and functional level (Morales and Holben, 2011). During the last 25 years the 'omics' toolbox has rapidly developed and expanded and the large amount of data generated has contributed significantly to the advancement of knowledge in microbial ecology. Nevertheless, there are several tradeoffs of these approaches. First of all, the sheer amount of data causes immense computational efforts during analysis. Furthermore, the data volume can complicate the detection of novel and meaningful results. And finally, obtained results are always just a

computational reconstruction and can vary depending on the selected bioinformatic tools (Morales and Holben, 2011).

1.4.3 Stable- and radioisotope approaches

Both sulfur and carbon occur as multiple stable and radioactive isotopes, which can be used to study microbial sulfur oxidation and carbon fixation by isotope labeling approaches. Numerous methods rely on the incorporation of the heavier stable carbon isotope ^{13}C, or the radioactive carbon isotope ^{14}C into constituents of microbial cells during assimilation of organic carbon compounds or CO_2. These include various so-called stable isotope probing (SIP) methods, which examine the composition and diversity of ^{13}C-enriched biomarker molecules (e.g. DNA, RNA, lipids, proteins) after incubation of a microbiological sample with stable isotope (SI)-labeled substrates (e.g. Boschker *et al*., 1998; Radajewski *et al*., 2000; Manefield *et al*., 2002). A drawback of SIP approaches is the long incubation time required to reach sufficient labeling of the desired biomarker pools, which can lead to shifts in the microbial community composition if environmental samples are investigated. Another frequently used tool are radioisotope labeling dependent methods like microautoradiography (MAR; Tabor and Neihof, 1982). MAR can be coupled to 16S rRNA based *in situ* identification of active cells in an approach called MAR-FISH (Lee *et al*., 1999; Nielsen *et al*., 2003; Wagner *et al*., 2006). Less labeling and shorter incubation times are applied, but work with radioactive substances and the photosensitivity of the MAR approach result in a complicated work flow.

A more sensitive SI-approach, requiring less labeling and shorter incubation times, is based on secondary-ion mass spectrometry (SIMS). The technique originates from material science and enables researchers to determine the exact molecular isotopic composition in thin layers of solid materials, including single microbial cells (Wagner, 2009). When coupled to cell identification methods like FISH, SIMS enables the identification of active community members on the single-cell level (Orphan *et al*., 2001; 2002; Musat *et al*., 2008). Moreover, it is important to note that while SIP approaches are only suitable for a small number of essential elements (like carbon, nitrogen, oxygen, hydrogen, and to a certain extent phosphorus and sulfur), SIMS-based methods can theoretically be applied for any element assimilated or stored in the cell (e.g. iron, manganese, silica), as well as for simultaneous detection of multiple element enrichments (Wegner *et al*., 2009). Still, regardless of all methodological improvements

made, stable- and radioisotope labeling techniques always rely on the addition of labeled substrate, and on a well-chosen incubation time. Therefore, experimentally induced biases cannot be entirely excluded.

Labeling can be entirely circumvented by measuring natural isotope abundances and isotope fractionation values specific to a certain reaction or pathway. Isotope fractionation is a result of all processes affecting the isotopic composition of a compound, including the kinetic effects introduced by enzymes catalyzing biotic reactions (Fry, 2008). The isotopic composition or isotope abundance of a compound is usually reported as the thousand-fold relative difference in the heavy to light isotope ratio of a compound in comparison to a standard and denoted as delta (δ) with the unit of per mill (‰)[1].

$$[1] \qquad \delta^{H}I[‰] = \frac{(^{H}I/^{L}I_{sample}) - (^{H}I/^{L}I_{standard})}{(^{H}I/^{L}I_{standard})} \times 1000,$$

if ${}^{H}I$ stands for the heavier and ${}^{L}I$ for the lighter isotope of an element.

Positive δ values mean that a compound is enriched in the heavy isotope in comparison to the standard, while negative δ values indicate heavy isotope depletion in comparison to the standard. In most cases isotope fractionation, denoted as capital delta (Δ), is approximately equal to the absolute difference in the isotopic composition (δ) of the reactant and the product in a chemical reaction[2].

$$[2] \qquad \Delta = |\delta_{product} - \delta_{reactant}|$$

The majority of biological processes result in products depleted in heavy isotopes compared to the reactants. However, the grade of depletion varies depending on the process. For example, different CO_2 fixation pathways result in different ^{13}C-signatures in produced biomass (Fig. 7), which can be used to determine the contribution of individual CO_2 fixation pathways to total primary production and elucidate carbon flow in microbial communities (Biddle *et al*., 2006, Schubotz *et al*., 2011; Olins *et al.*, 2013). However, the interpretation of isotope abundances in an ecological context is rather difficult. First of all, it relies on laboratory-derived knowledge of isotope compositions and fractionation for any given process. Secondly, it is rarely the case that one single reaction affects isotope composition. Lastly, laboratory studies have shown that physical conditions,

such as temperature and substrate abundance can affect isotope fractionation (Jahnke *et al*., 1999; Hayes, 2001; House *et al*., 2003; Bradley *et al.,* 2009), but the extent of their effect in the environment is under-investigated.

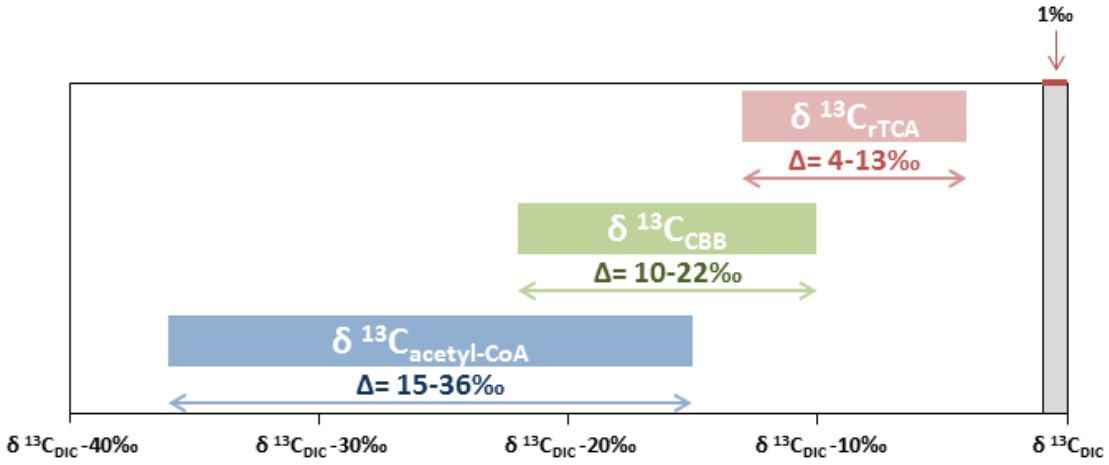

Figure 7: Overall carbon isotope fractionation associated with different pathways of autotrophic carbon fixation. Based on data presented in Hayes (2001).

1.5 Thesis aims and motivation

The goal of this thesis was to obtain a comprehensive picture of microbial sulfur oxidation in marine ecosystems, and to evaluate the impact of particular conditions on the composition and diversity of sulfur-oxidizing microbial communities. Therefore, my collaborators and I investigated the diversity and function of sulfur-oxidizing microbial communities in various sulfidic marine environments. Coastal sediments of the German Wadden Sea, a stratified marine sulfidic lake on the Croatian Adriatic coast and three deep-sea hydrothermal vent systems (Guaymas Basin in the Gulf of Mexico, Menez Gwen at the Mid Atlantic Ridge, and Manus Basin in the Bismarck Sea) were selected as study sites. The studies were designed to appeal to not only microbial ecologists and microbiologist, but also a broader community of marine researches, including biogeochemists and geologists. An interdisciplinary approach, relying on the combined use of cultivation-dependent and -independent microbiological and molecular tools with biogeochemical approaches was selected.

More precisely, the following topics were investigated:

S^0 as substrate for SOPs in marine benthic habitats (**Chapter II** and **VI**) SOPs in marine benthic habitats are very diverse, and different clades dominate in different

environments (Chapter 1.1 and 1.2). Although sulfur cycling in marine environments has been intensely studied, the mechanisms determining the composition of sulfur-oxidizing communities in different habitats are still unclear. A common feature in most benthic marine environments is the accumulation of S^0 as intermediate during biotic and abiotic sulfide oxidation (Zopfi *et al*., 2004). It is, however, unknown how S^0 influences the composition of sulfur-oxidizing communities. We studied the association between S^0 and SOPs in benthic marine habitats using *in situ* colonization experiments, direct sampling of native S^0, enrichment cultures and 16S rRNA gene-based community analysis. In the four studied geochemically and geographically distinct coastal and hydrothermal habitats, different groups of SOP were expected to oxidize S^0.

SOPs in a shallow stratified sea-water lake (**Chapter III**) The chemistry of the highly sulfidic shallow stratified sea-water lake Lake Rogoznica has been intensely studied for the last two decades. Periodic events of total anoxia during holomixis have attracted researchers' attention. However, the microbial diversity and community composition of the lake, with relation to the chemical setting and effects of anoxia on the microorganisms, have been poorly investigated. To determine microbial diversity and microbial community composition in Lake Rogoznica and identify possible microbial key-players, we applied 16S rRNA gene sequencing, *soxB* gene sequencing and CARD-FISH. Alongside with microbiological samplings, we recorded the physico-chemical conditions in Lake Rogoznica, including stratification and holomixis, during one annual cycle.

Thiotrophy and carbon assimilation in hydrothermal environments (**Chapters IV** and **V**) Hydrothermal vents are hot-spots of light-independent primary production in the deep-sea. SOPs are important and abundant in these habitats, as sulfide is one of the main energy sources emerging with hydrothermal fluids. Geochemical and temperature gradients in hydrothermal environments are steep, but the majority of sulfide is oxidized in a narrow mesophilic temperature range. In particular, mesophilic sulfur-oxidizing *Epsilonproteobacteria* are frequently detected in hydrothermal fluids and deposits. Other, mainly gammaproteobacterial SOPs are present, but usually less abundant. The relative importance of these two groups for CO_2 fixation in hydrothermal environments is not yet resolved. We combined the analysis of 16S rRNA gene diversity, IPL diversity and ^{13}C fingerprints of IPLs and TOC to identify the key carbon-assimilating SOPs on sulfide structures from a representative unsedimented hydrothermal system (**Chapter IV**).

Most SOPs inhabiting hydrothermal vents have the genetic potential to assimilate organic carbon compounds and many facultatively heterotrophic SOPs are known. The capability to assimilate organic carbon could be a competitive advantage not only in sedimented hydrothermal systems, rich in organic matter, but also in rock-hosted systems. We used SI-incubations coupled to 16S rRNA gene sequencing, CARD-FISH and nanoSIMS to investigate the heterotrophic potential of SOPs and other microorganisms inhabiting sulfidic, diffuse hydrothermal fluids from two deep-sea hydrothermal fields (**Chapter V**).

Chapter II

Microbial consumption of zero-valence sulfur in marine benthic habitats

Petra Pjevac, Alexey Kamyshny Jr, Stefan Dyksma, Marc Mußmann

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Contributions:

P.P. and M.M. developed concepts and ideas. P.P. collected samples at Janssand and in the Manus Basin, performed experiments, analyzed data, conceived and wrote the manuscript. A.K. performed S^0 measurements in Janssand samples. M.M. collected samples in the Guaymas Basin and established enrichment cultures. S.D. performed FISH experiments on Guaymas Basin samples. A.K., S.D. and M.M. conceived and edited the manuscript.

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Microbial consumption of zero-valence sulfur in marine benthic habitats

Petra Pjevac,[1] Alexey Kamyshny Jr,[2] Stefan Dyksma[1] and Marc Mußmann[1]*

[1]*Max Planck Institute for Marine Microbiology, Bremen, Germany.*

[2]*Department of Geological and Environmental Sciences, The Faculty of Natural Sciences, Ben-Gurion University of the Negev, Beer Sheva, Israel.*

Summary

Zero-valence sulfur (S^0) is a central intermediate in the marine sulfur cycle and forms conspicuous accumulations at sediment surfaces, hydrothermal vents and in oxygen minimum zones. Diverse microorganisms can utilize S^0, but those consuming S^0 in the environment are largely unknown. We identified possible key players in S^0 turnover on native or introduced S^0 in benthic coastal and deep-sea habitats using the 16S ribosomal RNA approach, (*in situ*) growth experiments and activity measurements. In all habitats, the epsilonproteobacterial *Sulfurimonas/Sulfurovum* group accounted for a substantial fraction of the microbial community. Deltaproteobacterial *Desulfobulbaceae* and *Desulfuromonadales* were also frequently detected, indicating S^0 disproportionation and S^0 respiration under anoxic conditions. Sulfate production from S^0 particles colonized *in situ* with *Sulfurimonas/Sulfurovum* suggested that this group oxidized S^0. We also show that the type strain *Sulfurimonas denitrificans* is able to access cyclooctasulfur (S_8), a metabolic feature not yet demonstrated for sulfur oxidizers. The ability to oxidize S^0, in particular S_8, likely facilitates niche partitioning among sulfur oxidizers in habitats with intense microbial sulfur cycling such as sulfidic sediment surfaces. Our results underscore the previously overlooked but central role of *Sulfurimonas*/*Sulfurovum* group for conversion of free S^0 at the seafloor surface.

Received 6 June, 2013; accepted 20 January, 2014. *For correspondence. E-mail mmussman@mpi-bremen.de; Tel. (+49) 421 2028936; Fax (+49) 421 2028580.

Introduction

Zero-valence sulfur (S^0) is a central intermediate in the biotic or abiotic oxidation of sulfides (Troelsen and Jørgensen, 1982; Schippers and Jørgensen, 2002; Wirsen *et al*., 2002; Holmkvist *et al*., 2011a). Notably, S^0 is an important intermediate in microbial sulfide oxidation, which accounts for a major fraction of oceanic dark CO_2 fixation (Middelburg, 2011). This process often leads to conspicuous accumulations of S^0, regularly emerging in pelagic oxygen minimum zones (OMZs), at hydrothermal vents and at sediment surfaces (Taylor *et al*., 1999; Wirsen *et al*., 2002; Jansen *et al*., 2009; Lavik *et al*., 2009). In marine sediments, S^0 typically ranges between 0.1 and 10 μmol cm^{-3} but also reaches up to 41 μmol cm^{-3} (Troelsen and Jørgensen, 1982; Panutrakul *et al*., 2001; Zopfi *et al*., 2004). Besides, historic and recent volcanic activities cause widespread and massive accumulations of crystalline S^0 at marine and terrestrial surfaces (Ivanov, 1971). In the oceans, S^0 occurs as cyclooctasulfur (S_8), polymeric sulfur (S_n) or polysulfides (S_n^{2-}) (Holmkvist *et al*., 2011b; Lichtschlag *et al*., 2013).

In the absence of free sulfide, the solubility and reactivity of S^0 is relatively low. Nevertheless, many cultured, phylogenetically diverse microorganisms conserve energy by using S^0 as electron donor. Members of the *Chlorobi*, *Chloroflexi*, *Aquificales*, *Proteobacteria* and many thermophilic *Archaea* oxidize S^0 either phototrophically or chemotrophically (Kletzin *et al*., 2004; Overmann, 2006; Robertson and Kuenen, 2006; Sievert *et al*., 2007). Furthermore, S^0 is respired with diverse organic compounds and hydrogen by members of the *Deltaproteobacteria*, *Epsilonproteobacteria*, *Gammaproteobacteria*, *Aquificales*, *Thermotogae*, *Firmicutes* and also by some thermophilic *Archaea* (Janssen and Morgan, 1992; Kletzin *et al*., 2004; Campbell *et al*., 2006; Rabus *et al*., 2006; Sievert *et al*., 2007; Schwedt *et al*., 2011). Various sulfate-reducing *Deltaproteobacteria* grow on S^0, while others are inhibited by it (Rabus *et al*., 2006). Moreover, S^0 is disproportionated to sulfate and sulfide by members of the *Desulfobulbaceae* (Bak, 1993; Thamdrup *et al*., 1993; Finster, 2008), by *Dissulfuribacter thermophilus* (Slobodkin *et al*., 2012) and by *Thermodesulfobacteria* (Slobodkin *et al*., 2011).

While cultivation-independent 16S ribosomal RNA (rRNA) approaches indicated a large diversity and abun-

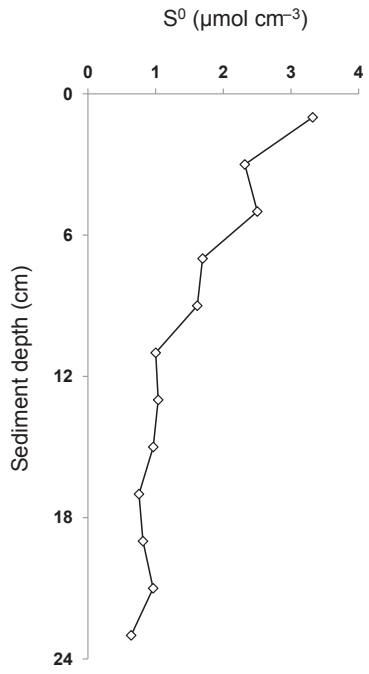

Fig. 1. Amounts of S^0 (µmol cm^{-3}) in the upper 25 cm of Janssand tidal flat sediment (May 2011).

dance of sulfur-cycling microorganisms in the environment, their high metabolic versatility hampers the accurate identification of those actually thriving on S^0. Edwards and colleagues (2003) introduced solid S^0 for *in situ* colonization, but the S^0-associated microbes were not phylogenetically identified in detail. Several studies suggested an important role of uncultured *Alphaproteobacteria* and *Gammaproteobacteria* in sulfur oxidation at marine sediment surfaces (Ravenschlag *et al*., 1999; Bowman *et al*., 2003; Musat *et al*., 2006; Lenk *et al*., 2011; 2012), whereas *Epsilonproteobacteria* were rarely detected in these habitats (Llobet-Brossa *et al*., 1998; Jensen *et al*., 2007; Webster *et al*., 2010; Lenk *et al*., 2011). In contrast, *Epsilonproteobacteria* dominate in sulfidic hydrothermal and cold-seep habitats (Nakagawa *et al*., 2005; Campbell *et al*., 2006; Sievert *et al*., 2007; Omoregie *et al*., 2008; Yamamoto and Takai, 2011), and in OMZs of the oceanic water column (Grote *et al*., 2007; Lavik *et al*., 2009; Bruckner *et al*., 2012). In anoxic habitats, potentially S^0-disproportionating or S^0-respiring *Deltaproteobacteria* account for a significant fraction of the microbial community (Purdy *et al*., 1997; Ravenschlag *et al*., 1999; Dhillon *et al*., 2003; Mußmann *et al*., 2005).

In this study, we identified potential key players of S^0 consumption in three contrasting, marine benthic habitats that are naturally rich in S^0 (Supporting Information Fig. S1). First, we performed *in situ* S^0-colonization experiments in surface sediments of a sulfidic tidal sand flat of the German Wadden Sea (site 'Janssand'), which typically shows intense sulfur cycling leading to conspicuous accumulations of S^0 during low tide (Jansen *et al*., 2009; Kamyshny and Ferdelman, 2010). Since *Gammaproteobacteria* and *Alphaproteobacteria* dominate the sulfur-oxidizing community in these sediments (Musat *et al*., 2006; Lenk *et al*., 2011; 2012), we hypothesized that mainly these organisms also colonize and oxidize S^0. Furthermore, we expected *Deltaproteobacteria* as the abundant S^0 consumers performing either S^0 respiration or disproportionation under anoxic conditions. For comparison, we studied the microbial diversity in volcanogenic S^0 in a deep-sea back-arc spreading centre (Manus Basin, Papua New Guinea) and in a S^0-precipitating microbial mat covering deep-sea sediments (Guaymas Basin, Mexico). Here, we predicted *Gammaproteobacteria* and *Epsilonproteobacteria* as dominant S^0-colonizers and oxidizers. To identify microorganisms, we performed 16S rRNA gene sequencing and catalysed reporter deposition-fluorescence *in situ* hybridization (CARD-FISH) on all S^0 samples. The activity of S^0-colonizing microorganisms was tested in enrichment cultures and sulfate production experiments.

Results

S^0 amounts in Janssand tidal sediment

Biogeochemical sulfur cycling in the Janssand tidal flat has been studied before (Jansen *et al*., 2009; Kamyshny and Ferdelman, 2010), but amounts of S^0 in the sediment were still unknown. Therefore, we determined S^0 over a vertical sediment profile in May 2011. Amounts of S^0 ranged from 3.3 µmol cm^{-3} at the surface to 0.6 µmol cm^{-3} in 18–25 cm depth (Fig. 1).

16S rRNA diversity in S^0-, glass- and pyrite-colonizing biofilms from Janssand tidal sediment

Since S^0 cannot be separated from the sediment without distorting the microbial community, we introduced S^0 particles for *in situ* colonization. Therefore, nylon bags containing several S^0 particles of 4–6 mm in diameter were deposited in two tidal flats sediments in the German Wadden Sea. These were incubated *in situ* for 4 weeks or 12 months. To study the influence of redox conditions on S^0 colonization, experiments were performed in the upper 15 cm of the sediment, spanning layers with different oxygen regimes. Based on previous oxygen measurements at these sites (de Beer *et al*., 2005; Røy *et al*., 2008), hereafter we refer to three different sediment

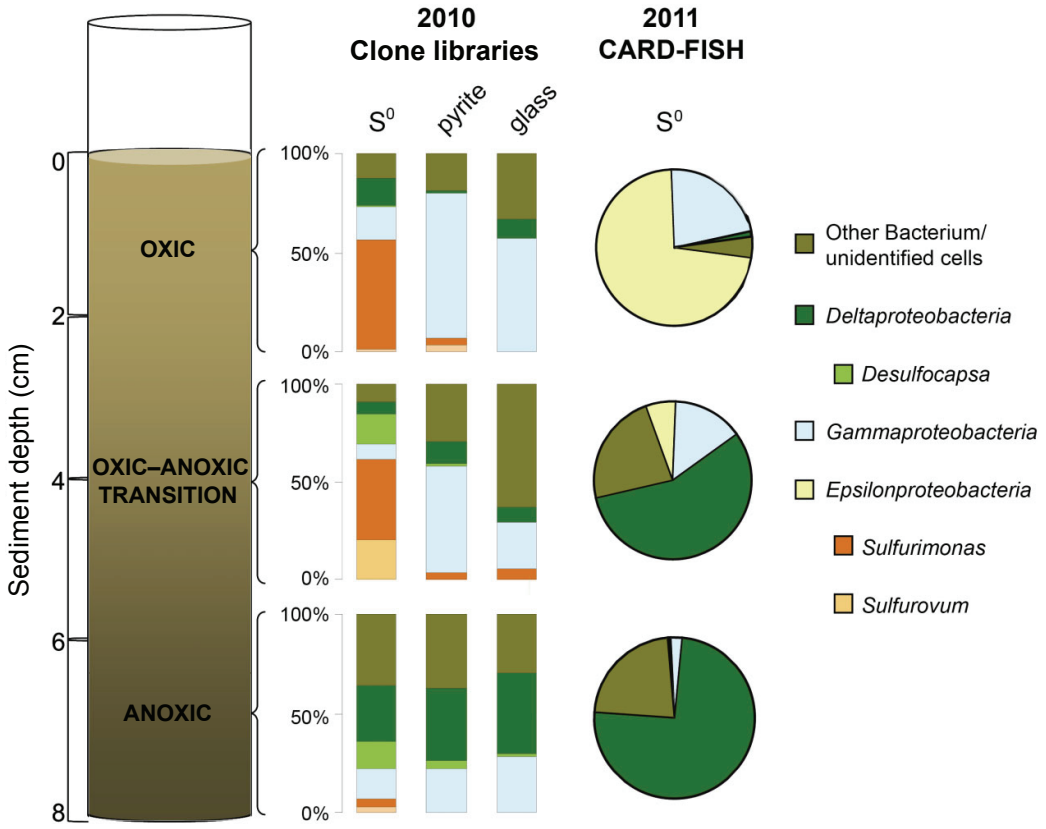

Fig. 2. Microbial community composition in biofilms grown on S^0, pyrite and glass particles recovered from three sediment layers (Janssand tidal flat). For the 2010 experiment, the frequency of deltaproteobacterial, epsilonproteobacterial and gammaproteobacterial sequences in 16S rRNA gene libraries was determined ($n = 632$). For the 2011 experiment, the relative cell abundance in % of total cell counts (DAPI) was determined by CARD-FISH in S^0-grown biofilms. For the 2010 experiment, semiquantitative data on relative cell abundances are given in Supporting Information Table S1.

layers as 'oxic', 'oxic-anoxic transition' or 'anoxic' layer. To distinguish preferential colonization and consumption of S^0 by specific phylogenetic bacterial groups from unspecific colonization, we also introduced pyrite and/or glass particles as controls. Furthermore, we ensured that our S^0 source stimulated growth of physiologically and phylogenetically diverse sulfur-oxidizing strains such as *Thiobacillus denitrificans*, *Allochromatium vinosum*, *Thiomicrospira crunogena*, *Sulfurimonas autotrophica* and *Sulfurimonas denitrificans*. Here, it is important to point out that the used S^0 was not purified and thus contained several S^0 species such as polymeric or polysulfidic sulfer S, which is essential to support S^0-dependent growth of e.g. *A. vinosum* (Franz *et al.*, 2007).

In the first colonization experiment in the Janssand tidal flat (October 2010), S^0, glass and pyrite particles were incubated *in situ* for 4 weeks in all three sediment layers, and the 16S rRNA gene diversity in particle-grown biofilms was analysed. We recovered 632 bacterial 16S rRNA gene sequences, thereof 53% from S^0-grown biofilms and 47% from control surfaces (26% from pyrite- and 21% glass-grown biofilms). Sequences related to *Epsilonproteobacteria* made up 56% of all clone sequences in S^0-grown biofilms from the oxic layer and 62% of sequences from S^0-grown biofilms in the transition layer. In contrast, < 1% of sequences from S^0 biofilms grown in the anoxic layer were related to *Epsilonproteobacteria* (Fig. 2). Only few (< 5%) epsilonproteobacterial sequences were obtained from glass- or pyrite-grown biofilms, suggesting that *Epsilonproteobacteria* preferentially colonized S^0 particles. Phylogenetic reconstruction of 61 operational taxonomic units (OTUs) [97% sequence identity (SI) cut-off] revealed that most

OTUs displayed 87–96% SI to S^0-oxidizing strains of the genus *Sulfurimonas* (Supporting Information Fig. S2). Fewer OTUs grouped with the S^0-oxidizing strain *Sulfurovum lithotrophicum* (89–96% SI). All recovered OTUs were more closely related (97–99% SI) to sequences of uncultured representatives from sediments, hydrothermal vents and pelagic OMZs (Supporting Information Fig. S2).

S^0 particles from the oxic-anoxic transition and anoxic sediment layers appeared to be preferentially colonized by *Deltaproteobacteria* (Fig. 2). Here, of all deltaproteobacterial sequences 74% from the oxic-anoxic transition and 29% from the anoxic sediment affiliated with *Desulfocapsa/Desulfobulbus*-related (90–97% SI) sequences that were retrieved from a S^0-disproportionating enrichment culture established from the same site (provided by Kai Finster, Supporting Information Fig. S3). Only few *Desulfobulbaceae*-related sequences were retrieved from control particles (Fig. 2). Similarly, sequences related to metal- and S^0-respiring *Desulfuromonadales* were more diverse and more frequently found on S^0 than on control particles (Supporting Information Fig. S3). In contrast, no specific S^0 colonization was observed for sequences of sulfate-reducing *Desulfobacteraceae*, which were detected in similar relative frequencies on S^0 and on control particles.

Gammaproteobacterial 16S rRNA gene sequences were recovered from all biofilms, but their relative clone frequencies (Fig. 2) and their phylogenetic affiliation did not indicate the presence of sulfur oxidizers or other populations that preferentially colonized S^0. In fact, these sequences grouped with non-sulfur-oxidizing members of pelagic groups such as *Alteromonadales* and *Oceanospirillales*, and with uncultured *Gammaproteobacteria* from Janssand sediments retrieved in an earlier study by Lenk and colleagues (2011) (data not shown).

CARD-FISH of particle-grown biofilms from tidal flat sediments

After identifying candidate key players in S^0 consumption by 16S rRNA gene sequencing, we performed CARD-FISH on biofilms to confirm these results in the 2010 experiment and in two additional colonization experiments. In the 2010 experiment, all tested S^0 and control particles were colonized by microorganisms [4′,6-diamidino-2-phenylindole (DAPI) stain, Supporting Information Fig. S4]. However, S^0 particles were more densely colonized than control particles, indicating a preferential colonization of S^0 over rather inert surfaces. In agreement with the 16S rRNA diversity analysis, *Epsilonproteobacteria* clearly dominated the S^0 biofilms grown in the uppermost sediment layers in two independent, 4-week colonization experiments in October 2010 (Supporting Information Table S1, Fig. S4) and in October 2011 (Fig. 2). Here, they accounted for up to 72% of all cells. Furthermore, they were abundant in biofilms from the transition layer, while almost no epsilonproteobacterial cells were detected on control particles and on S^0-grown biofilms from the anoxic layer (Supporting Information Table S1). To confirm the importance of *Epsilonproteobacteria* for S^0 colonization and consumption in oxic tidal sediments, we additionally incubated S^0 and glass particles in oxic sediments of the Königshafen tidal flat at the Island of Sylt, located roughly 150 km north of the Janssand flat. Similarly, S^0-grown biofilms were dominated by *Epsilonproteobacteria* (56% of DAPI), whereas they were almost absent on glass particles (Supporting Information Table S2).

We also examined the abundance of *Epsilonproteobacteria* by CARD-FISH in three surface sediment samples (0–1 cm depth) in the vicinity of colonization experiments (Janssand October 2010, 2011; Königshafen October 2011) and the sediment core used for S^0 measurements (Janssand, May 2011). In all samples potential epsilonproteobacterial cells were detected, but these accounted for less than 0.5% ($< 10^7$ cells ml^{-1}) of all cells and were not clearly distinguishable from background (Supporting Information Table S2).

In accordance with the 16S rRNA gene diversity, deltaproteobacterial cells were almost absent on S^0-grown biofilms from oxic sediment layers (Janssand 2010, 2011), while they dominated in S^0-grown biofilms from anoxic and transition layers (56% and 75% of all cells, Fig. 2). *Gammaproteobacteria* were detected by FISH in all biofilms, but no preferential colonization of S^0 was apparent (Supporting Information Table S1). This observation was in line with the random association of gammaproteobacterial 16S rRNA gene sequences with S^0 and control particles.

Community composition of S^0-colonizing microorganisms in two deep-sea systems

To compare S^0-consuming microorganisms from tidal sediments to those in other benthic S^0-rich habitats, we sampled naturally occurring S^0 at two deep-sea sites. First, we studied the microbial community on volcanogenic S^0 in the Manus Basin off the coast of Papua New Guinea (Supporting Information Fig. S1). Second, we sampled a S^0-precipitating microbial mat that covered sulfidic hydrothermal sediments in the Guaymas Basin/Gulf of California, Mexico (Supporting Information Fig. S1). It is important to note that in both settings, low temperatures ($< 4°C$ Manus Basin, 3–8°C Guaymas Basin) prevailed, which most likely supported psychrophilic to mesophilic microbial communities.

In volcanogenic S^0 from the Manus Basin, *Epsilonproteobacteria* accounted for 11–21% of all cells, while

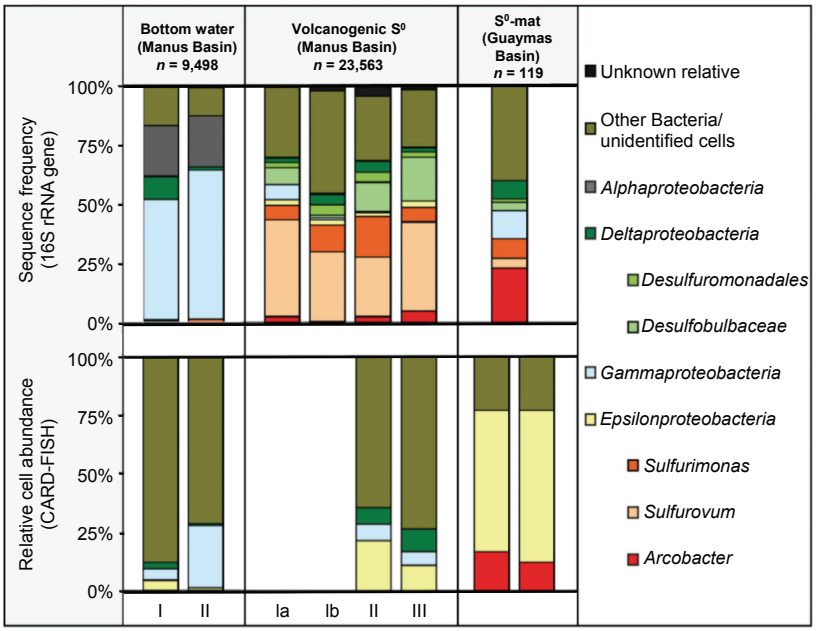

Fig. 3. Microbial community composition in bottom water (Manus Basin), in a volcanogenic S^0 outcrop (subsamples Ia and Ib), in two S^0 boulders (II and III) (Manus Basin) and in a S^0-precipitating mat (Guaymas Basin). Upper panel: 16S rRNA gene diversity, determined by pyrotags (Manus Basin, 33 058 sequences) or by a 16S rRNA gene library (Guaymas Basin, 119 sequences). Lower panel: relative abundance of deltaproteobacterial, epsilonproteobacterial and gammaproteobacterial cells in % of total cell counts (DAPI).

Deltaproteobacteria made up 7–10% (Fig. 3). The majority of microorganisms remained unidentified. Analysis of 16S rRNA pyrotag sequences confirmed the importance of *Epsilonproteobacteria*. From in total 23 563 sequences, a large fraction (42%) grouped with the genera *Sulfurimonas* and *Sulfurovum*. Sequences related to other *Epsilonproteobacteria* such as *Arcobacter* were clearly minor. Deltaproteobacterial sequences accounted for on average 16%, and were related to *Desulfuromonadales* and to the S^0-disproportionating and S^0-respiring genera *Desulfurivibrio*, *Desulfobulbus* and *Desulfocapsa* within the family *Desulfobulbaceae* (Fig. 3). The microbial community in two bottom-sea water samples (< 4°C) was clearly different from the S^0-associated one, as *Epsilonproteobacteria* accounted only for < 1% of both pyrotags and relative cell abundances (Fig. 3) pointing to a preferential colonization of S^0 by members of the *Sulfurimonas/Sulfurovum* group.

The filamentous S^0-precipitating mat from the Guaymas Basin was also dominated by *Epsilonproteobacteria*. They accounted for > 60% of total cells and for up to 85% of all detected *Bacteria* (Fig. 3). *Arcobacter*-related microorganisms constituted up to 16% of all cells. To identify the remaining epsilonproteobacterial cells, a 16S rRNA gene library was established. Here, 35% of all sequences affiliated with epsilonproteobacterial genera, namely *Sulfurimonas*, *Sulfurovum* and *Arcobacter* (Fig. 3). An *in silico* analysis revealed that all recovered *Arcobacter*-related sequences were indeed targeted by the *Arcobacter*-specific probe Arc94. As no other epsilonproteobacterial sequences were recovered, we presume that cells only targeted by the general *Epsilonproteobacteria* probes most likely affiliated with *Sulfurimonas* and *Sulfurovum*.

Moreover, few sequences were related to *Desulfuromonadales* and to *Desulfobulbaceae* (Supporting Information Fig. S3). The *Desulfobulbaceae* relatives were affiliated with sequences retrieved from a S^0-disproportionating enrichment culture, established from Guaymas Basin surface sediments during our sampling campaign in 2009 (kindly provided by Kai Finster, Supporting Information Fig. S3).

To confirm the role of the detected *Epsilonproteobacteria* in S^0 consumption in the Guaymas Basin, we set up enrichment cultures that favoured microbial S^0 oxidation with oxygen (treatment A) or with nitrate (treatment B) as electron acceptors. After 5 months of incubation at 4°C and two transfers, both enrichment cultures were dominated by

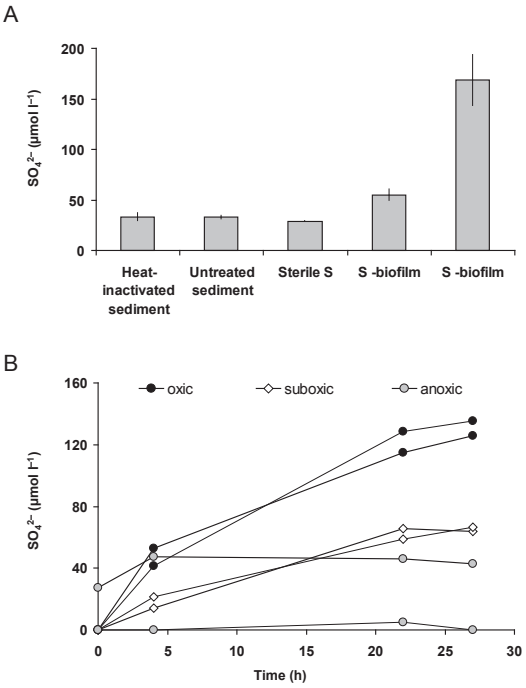

Fig. 4. Sulfate (SO_4^{2-}) production by S^0-grown biofilms from the Janssand tidal flat. Sulfate concentrations were calculated from IC measurements based on a Na_2SO_4 standard curve.
A. 2010 experiment: S^0-grown biofilms from the oxic sediment layer and controls after 72 h. This experiment was performed in triplicates.
B. 2011 experiment: S^0-grown biofilms from all layers. This experiment was performed in duplicates. Values were corrected for background sulfate.

Epsilonproteobacteria and *Gammaproteobacteria* as revealed by CARD-FISH (Supporting Information Fig. S5). Most epsilonproteobacterial 16S rRNA gene sequences from both treatments were related to *S. denitrificans* and to *Sulfurimonas paralvinellae* (92–98% SI) (Supporting Information Fig. S2), while the majority of gammaproteobacterial sequences from the oxic enrichment (treatment A) grouped with the sulfur-oxidizing strain *T. crunogena* (not shown).

To test for S^0 respiration of the *Deltaproteobacteria*, we set up an anaerobic enrichment culture from the S^0-precipitating mat with 2 mmol l^{-1} acetate as electron donor and S^0 as electron acceptor. This enrichment culture produced large amounts of sulfide indicated by iron sulfide precipitation and strong sulfide odour, and was dominated by close relatives of the S^0-respiring strain *Desulfuromonas acetooxidans* (95–98% SI, Supporting Information Fig. S3).

Sulfate production from S^0-grown biofilms and enrichments

To test the hypothesis that the detected *Sulfurimonas/Sulfurovum*-related *Epsilonproteobacteria* grew lithoautotrophically by oxidizing S^0, we measured sulfate production by *in situ* S^0-grown biofilms from tidal flats and in the enrichment cultures. S^0 particles from the oxic sediment layer in the Janssand tidal flat (October 2010) were removed from the sediment, rinsed and transferred to sulfate-free artificial sea water (ASW) for incubation. The S^0-grown biofilms formed 50–150 µmol l^{-1} sulfate during 72 h from approximately 2–3 g S^0 particles, which was twofold to sixfold higher than sulfate concentrations measured in control incubations (sterile S^0, untreated sediment and heat-inactivated sediment) (Fig. 4). Furthermore, S^0-grown biofilms from the oxic sediment layer from Königshafen (October 2011) produced ~ 60 µmol l^{-1} sulfate (in 72 h from 1 g S^0 particles), which was twice as much as in background controls (Supporting Information Table S3). Important to note is that a fraction (~ 20 µmol l^{-1}) of sulfate in the experiments including controls was most likely background sulfate originating from impurities in otherwise sulfate-free ASW.

In a time-series experiment, we measured sulfate formation by S^0-grown biofilms from different sediment layers (Janssand, October 2011). Again, sulfate was produced by S^0-grown biofilms from the oxic sediment layer, while little to no sulfate formation was observed by S^0-grown biofilms from the transition or the anoxic sediment layers (Fig. 4).

We could also confirm S^0-oxidation activity in the oxygen- and nitrate-respiring enrichment cultures established from a S^0-precipitating mat. During 30 days of incubation the nitrate-respiring treatments formed more sulfate (up to 4.5 mmol l^{-1}) than the oxygen-respiring treatments (up to 1.2 mmol l^{-1}) (Supporting Information Fig. S6), which may reflect the adaptation to the generally microoxic conditions at Guaymas Basin surface sediments (Gundersen *et al.*, 1992).

Sulfate production from S_8 by S. denitrificans

The prevalence of the *Sulfurimonas/Sulfurovum* group in S^0 samples and the high activity in the S^0 enrichment cultures led us to hypothesize that these microorganisms are able to activate and oxidize S_8, the main constituent of S^0 provided in our experiments. As no pure cultures could be retrieved from our environmental studies, we tested this hypothesis with the type strain *S. denitrificans* that is closely related to sequences from our study. In sulfate-free mineral medium with pure S_8 as the sole electron donor, cultures of *S. denitrificans* formed 4 mmol l^{-1} sulfate already during the first day (Fig. 5). For comparison, the photoautotroph *A. vinosum* was incubated with

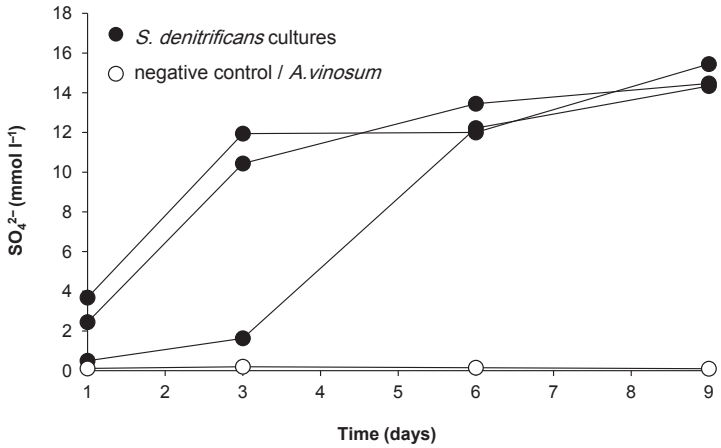

Fig. 5. Sulfate (SO_4^{2-}) production from S_8 as sole electron donor by active cultures of *S. denitrificans* (black circles). As controls, we used cell-free medium and active cultures of *A. vinosum* (both white circles). In controls, no sulfate was formed. Experiments were performed in triplicates.

S_8 under photoautotophic conditions. This strain oxidizes free S^0 only in its polymeric or polysulfidic form (Franz *et al.*, 2007). In line with this earlier study, *A. vinosum* cultures did not produce sulfate over a period of 9 days (Fig. 5). These results indicated substantial differences between both strains in their ability to oxidize S^0 species.

Discussion

We studied the microorganisms associated with solid S^0 and its consumption in contrasting marine benthic habitats. Although these strongly differed in their environmental settings, similar phylogenetic groups were associated with native and introduced S^0. The detection of mainly three functional groups indicated that S^0 is metabolized in a reductive and oxidative fashion (Fig. 6). In fact, S^0 occurred in oxic and anoxic sediment layers in high quantities, similar to other sulfidic tidal sediments (Troelsen and Jørgensen, 1982; Panutrakul *et al.*, 2001). In deep-sea habitats, S^0 formed conspicuous structures that were directly sampled and used for molecular analyses. The identification of the microbial communities

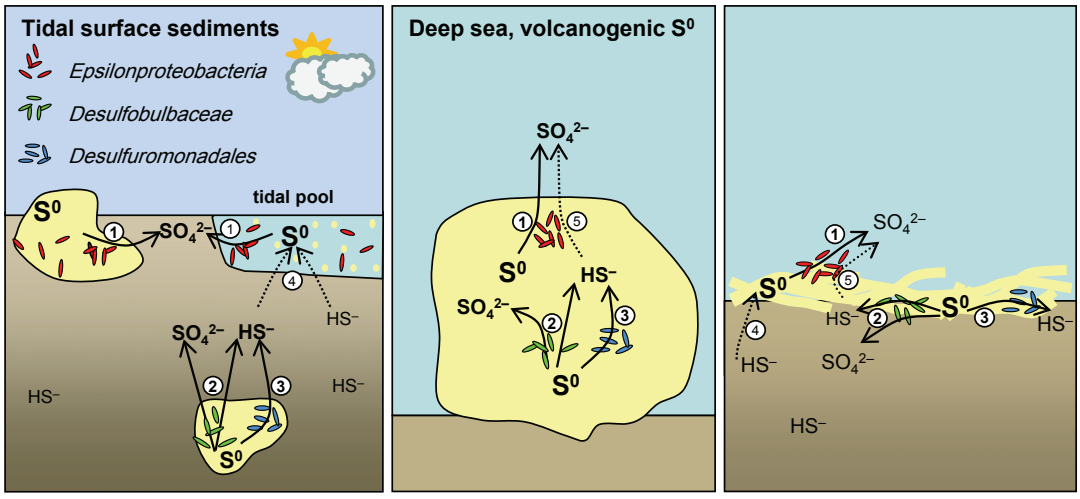

Fig. 6. Model of microbial S^0 consumption in the three studied benthic habitats. Three distinct functional groups catalyse turnover of S^0. (1) S^0 oxidation by *Epsilonproteobacteria*, (2) S^0 disproportionation by *Desulfobulbaceae* and (3) S^0 respiration by *Desulfuromonadales*. In tidal sediments, sulfide is oxidized chemically (4) and by uncultured *Gammaproteobacteria* and *Alphaproteobacteria* (Lenk *et al.*, 2011; 2012). In S^0-precipitating mats, *Epsilonproteobacteria* may also oxidize ambient sulfide to S^0 (4) or directly to sulfate (5).

associated with S^0 *in situ* combined with activity measurements provides an important extension to previous, mostly laboratory-based experiments on microbial S^0 consumption.

Deltaproteobacteria *colonize and consume* S^0 *under anoxic conditions*

In line with our initial hypothesis, deltaproteobacterial *Desulfobulbaceae* and *Desulfuromonadales* were associated with S^0 in all three habitats, while other *Deltaproteobacteria* appeared to be rare. To date, the environmental function of *Desulfuromonadales* is still largely unresolved because these organisms may also reduce Fe(III) and Mn(IV) in marine surface sediments (Roden and Lovley, 1993; Mußmann *et al*., 2005; Lovley, 2006; Rabus *et al*., 2006). Likewise *Desulfocapsa* and *Desulfobulbus* relatives have been proposed as important S^0-disproportionating bacteria in various habitats (Canfield and Thamdrup, 1996; Tonolla *et al*., 2000; Mußmann *et al*., 2005; Finster, 2008; Hügler *et al*., 2011), but have not been found directly associated with environmental S^0. The preferential colonization of S^0 particles in transition and anoxic tidal sediments compared with control particles and their enrichment in anoxic cultures from Guaymas sediments suggest that S^0-disproportionating *Desulfobulbaceae* and S^0-respiring *Desulfuromonadales* are possible key players in anoxic S^0 consumption at the sea floor (Fig. 6).

Epsilonproteobacteria *are important* S^0 *colonizers in deep-sea and tidal habitats*

Consistent with the well-documented prevalence of *Epsilonproteobacteria* in sulfidic cold-seep and hydrothermal systems, we found high frequencies of epsilonproteobacterial cells and sequences related to the *Sulfurimonas/Sulfurovum* group in volcanogenic S^0, in S^0-precipitating mats and in S^0-oxidizing enrichments cultures. However, we did not expect that the *Sulfurimonas*/*Sulfurovum* group also constituted a major fraction of microbial communities associated with S^0 in tidal sediments because *Epsilonproteobacteria* are usually hardly detectable in these sediments (this study; Lenk *et al*., 2011; S. Dyksma, unpublished) and are clearly outnumbered by sulfur-oxidizing *Alphaproteobacteria* and *Gammaproteobacteria* (Musat *et al*., 2006; Lenk *et al*., 2011; 2012). Consistent with this observation, *Epsilonproteobacteria* also accounted for high relative abundances of up to 22% in S^0-rich tidal pools emerging during low tide (Supporting Information Fig. S4; Lenk, 2011). While *Epsilonproteobacteria* are uniquely suited to rapidly colonize surfaces (López-García *et al*., 2003; Campbell *et al*., 2006), their lower relative abundance in glass- and pyrite-grown biofilms and particularly in the corresponding sediments (Lenk *et al*., 2011; this study) indicated a strong preference for S^0, although the *Sulfurimonas/Sulfurovum* group also uses sulfur compounds besides S^0 (Inagaki *et al*., 2003; 2004; Nakagawa *et al*., 2005; Takai *et al*., 2006; Yamamoto *et al*., 2010; Grote *et al*., 2012; Labrenz *et al*., 2013). Sulfate production by S^0-grown biofilms and enrichment cultures, both dominated by *Sulfurimonas/Sulfurovum* sequences, supported that these bacteria indeed thrived on S^0 oxidation using oxygen or nitrate as electron acceptors. Our comprehensive molecular analysis of S^0-associated microbial communities in two tidal surface sediments strongly suggest a major role for the *Sulfurimonas*/*Sulfurovum* group in environmental S^0 oxidation in tidal sediments, but most likely also in deep-sea habitats (Fig. 6).

Utilization of S^0 *by sulfur-oxidizing bacteria*

The prevalence of *Epsilonproteobacteria* and the minor role of other sulfur-oxidizing populations on S^0-grown biofilms suggest principal physiological differences in the S^0 metabolism between distinct groups of sulfur-oxidizing bacteria. Following up this idea, we show that *S. denitrificans* is able to grow with S_8, commonly the main constituent of commercially available S^0. This metabolic feature has not yet been demonstrated for any other sulfur-oxidizing bacterium. In fact, in our control experiments the sulfur-oxidizing gammaproteobacterium *A. vinosum* did not grow on pure S_8 just as in previous experiments by Franz and colleagues (2007). Our environmental data are consistent with this observation and indicate that the ability to access S^0, in particular S_8, may differ among sulfur-oxidizing bacteria and influences their environmental distribution. Further experiments need to test how widespread this phenomenon is. Notably, abiotic and biogenic S_8 is a main constituent of S^0 in natural aquatic systems including marine surface sediments and filamentous S^0-precipitating mats (Engel *et al*., 2003; Macalady *et al*., 2006; Holmkvist *et al*., 2011b; Lichtschlag *et al*., 2013), and may offer a yet unrecognized niche for specialized sulfur oxidizers. Therefore, we hypothesize that similar to *A. vinosum*, the sulfur-oxidizing *Alphaproteobacteria* and *Gammaproteobacteria* dominating sediment biofilms do not use free S^0/S_8, but may rather thrive on other sulfur compounds such as sulfide, which is easily available in large amounts in these sediments (de Beer *et al*., 2005; Jansen *et al*., 2009). In this way, the limited usability of S_8 as electron source may facilitate niche partitioning simply by their diverging substrate preferences among sulfur oxidizers. This contrasts previous studies that ascribed niche separation of *Gammaproteobacteria* and *Epsilonproteobacteria* to variations in sulfide and oxygen concentrations/ratios

(Macalady *et al.*, 2008; Jones *et al.*, 2010; Grünke *et al.*, 2011).

Conclusion and outlook

In this study, we identified three distinct phylogenetic and functional groups that likely consume S^0 in marine benthic habitats. Although the phylogenetic diversity of S^0-consuming microorganisms in culture collections is high, the consistent detection of relatively few phylotypes such as the *Desulfobulbaceae*, *Desulfuromonadales* and *Sulfurimonas*/*Sulfurovum*-related *Epsilonproteobacteria* suggests that these groups probably harbour the key players in microbial S^0 consumption. By accessing S^0, in particular S_8, more efficiently the *Sulfurimonas*/*Sulfurovum* group may be able to coexist with other sulfur-oxidizing bacteria in sulfur-rich environments or even outcompete them in systems, where free S^0 prevails over other sulfur species, e.g. in the Manus Basin. Moreover, our study provides first insights, how substrate quality may influence diversity of sulfur-oxidizing bacteria in benthic ecosystems. In this regard, it is intriguing that *A. vinosum* did grow in the presence of S_8 only when in co-culture with *S. denitrificans* (P. Pjevac, unpubl. data). With the recent methodological progress in discrimination of environmental sulfur compounds, especially of S^0 species (Kamyshny and Ferdelman, 2010), future studies will reveal the role of different reduced sulfur compounds in sustaining coexistence of physiologically similar sulfur-metabolizing bacteria. The relative contribution of the different functional groups to S^0 turnover remains to be elucidated. Our study enhances the understanding of microbes and processes in the benthic marine sulfur cycle, primarily of the largely unexplored sulfur oxidation, which is a major light-independent CO_2 sink in the oceans.

Experimental procedures

Sampling sites in tidal flats of the German Wadden Sea

We studied the colonization and consumption of S^0 in surface sediments of two sulfidic, sandy tidal flats in the German Wadden Sea. The Janssand tidal flat is located in the back barrier of the East Frisian Island Spiekeroog (53.7°N, 7.6°E). The Königshafen tidal flat is located at the North Frisian Island Sylt (55.0°N, 8.4°E) (Supporting Information Fig. S7). The geochemistry of both flats has been described elsewhere (Janssand: Neira and Rackemann, 1996; Røy *et al.*, 2008; Königshafen: Kristensen, 2000; de Beer *et al.*, 2005). In both tidal flats, sampling and experiments were conducted in the upper 25 cm of the sediment, spanning the oxic, transition and anoxic sediment layers.

Colonization experiment in tidal flats of the German Wadden Sea

We performed in total three independent S^0-colonization experiments in the two tidal flats. In the Janssand flat, colonization experiments were performed for 4 weeks each in 2010 and 2011 (October to November). In the Königshafen tidal flat (Island of Sylt), a similar colonization experiment was performed with an extended incubation time of 12 months (starting October 2010). Colonization devices with S^0, glass and pyrite particles were assembled in the laboratory and deposited in three different sediment depths representing different oxygen regimes (Røy *et al.*, 2008; Jansen *et al.*, 2009): the oxic (0–1 cm), the oxic–anoxic transition (2–5 cm) and the anoxic (~ 15 cm) sediment layer in the Janssand. In the Könighafen tidal flat, S^0 particles were deposited only in the uppermost sediment layer. S^0 particles were generated by melting powderous S^0 (Sulfur Ph Eur, Fluka, Buchs, Switzerland) in a sterile glass Petri dish at ~ 120°C. After S^0 solidified, the 1–2 mm thick S^0 plates were crushed into irregular-shaped particles with a diameter of 4–6 mm. Microscopy glass slides (Carl Roth, Karlsruhe, Germany) and pyrite crystals (Krantz Rheinisches Mineralien-Kontor GmbH KG, Bonn, Germany) were crushed into particles of 3–8 mm in diameter. Approximately 2–5 g of S^0, glass or pyrite particles were filled into nylon bags with a mesh size of 150 μm (Biologie-Bedarf Thorns, Göttingen, Germany), which were closed by cable ties. Before deposition, the bags were washed for 2 h in 1 M HCl and methanol. These bags were cable tied to 40 cm-long iron poles, which were deposited vertically in predrilled sediment holes. These holes were then refilled with sediment. After incubation for a period of 4 weeks or 12 months, the colonized particles were retrieved and processed for molecular analyses and sulfate production experiments. Further details on sampling locations, incubation conditions and experiments are given in Supporting Information Table S4.

Growth tests on S^0 used for colonization experiments were performed with the following strains: *T. denitrificans* (DSM-12457), *T. crunogena* (DSM-12353), *A. vinosum* strain 180^T (DSM-180), *S. autotrophica* (DSM-16264) and *S. dentrificans* (DSM-1251).

Sampling of tidal sediments for CARD-FISH

At the Janssand tidal flat, we sampled 0.5 cm^{-3} of the oxic surface sediment layer (0–1 cm depth) in the direct vicinity of colonization experiments (October 2010 and 2011) and from the 25 cm-deep core sampled for S^0 measurements (May 2011). Moreover, oxic surface sediment was sampled and fixed for CARD-FISH close to the colonization experiment in the Königshafen tidal flat (Sylt, October 2011).

Sampling sites in the deep sea at the Manus Basin, Papua New Guinea

S^0 samples from the hydrothermally active area North Su (3.8°S, 152.1°E) and adjutant inactive areas of the Manus Basin back-arc spreading centre, Bismarck Sea, Papua New Guinea (Supporting Information Fig. S7), were collected during the R/V SONNE cruise SO-216 between 14 June and 23 July 2011. North Su is a conical shaped volcano rising between 1700 m and 1150 m water depth, hosting numerous black smoker, white smoker and diffuse vent sites on its flank regions. In both hydrothermally active and inactive regions of the Manus Basin, volcanogenic S^0 flows, sills, outcrops and

boulders (Supporting Information Fig. S1) were frequently observed on and between the volcanic talus (Bach *et al.*, 2011). The geology and geochemistry of the Manus Basin and North Su have been described previously (Tivey *et al.*, 2006; Reeves *et al.*, 2011).

Sampling in the North Su area/Manus Basin

We sampled volcanogenic S^0 boulders and outcrops of unknown age (Supporting Information Fig. S1) that were surrounded by ~ 4°C-cold, oxic sea water. No recent hydrothermal activity was indicated, and the sampled volcanogenic S^0 was located in 210–590 m distance from active diffuse hydrothermal vent sites (Supporting Information Fig. S8). Two fist-sized S^0 boulders were collected with help of the remotely operated vehicle Quest (MARUM, Bremen, Germany), while two larger pieces from a massive S^0 outcrop were broken off with a TV grab and divided into two subsamples based on different coloration. Furthermore, bottom sea water was collected in 180–610 m distance from the sampled S^0 using a Niskin 24-bottle rosette (10 l) mounted on a Sea-Bird 911 plus CTD (Sea-Bird-Electronics, Bellevue, WA, USA; Supporting Information Fig. S8). Another bottom sea water sample was collected 7.3 km aside of the North Su system as external reference. All samples were processed for CARD-FISH and 16S rRNA gene diversity analysis. More details on sampling locations and types of sample collection are given in Supporting Information Table S4.

Sampling in the Guaymas Basin, Gulf of California, Mexico

During the R/V Atlantis cruise AT15–56 (22 November–5 December 2009), a sediment core was taken in 2010 m water depth in the vicinity of a *Beggiatoa* mat covering hydrothermal sediment in the Guaymas Basin, Gulf of California (Supporting Information Fig. S7). The push core was retrieved at site Cathedral Hill (27°0069 N, 111°2427 W) during dive 4565 with the submarine DSV Alvin. The sediment was covered with white, fluffy sulfur mats (Supporting Information Fig. S1). *In situ* temperature for the sampling position ranged from 3.3°C at 2 cm above surface to 14.4°C at 8 cm sediment depth. During ascend in the water column, the sediment surface was resuspended because of outgassing of methane and sulfide. The sediment core was transferred to 4°C, and after 1 day the fluffy white mat settled and re-established at the surface. Microscopic inspection revealed filamentous precipitates typical for sulfur mats generated by the sulfide-oxidizing *Arcobacter sulfidicus* that are commonly observed at the Guaymas Basin and other hydrothermal sites (Taylor *et al.*, 1999). This mat was sampled for molecular analyses. Furthermore, enrichment cultures supporting S^0 oxidation with oxygen (A), nitrate (B) and S^0 respiration with acetate (C) were inoculated with mat material onboard. Details are given in the supporting information. Within 5 months, these enrichments were transferred twice on sulfate-free ASW, and the final enrichment cultures were sampled for CARD-FISH and 16S rRNA gene libraries. S^0 oxidation was monitored by measuring sulfate production in the oxygen- (A) and nitrate-respiring (B) enrichments. Sulfide formation in S^0-respiring enrichment was evident by formation of iron-sulfide precipitates and intense sulfide odour.

Sample fixation and CARD-FISH

All samples from volcanogenic S^0, S^0-precipitating mat, sediment and *in situ* colonized particles were fixed in a formaldehyde solution [2% final in sterile ASW or in phosphate-buffered saline (PBS)] at 4°C overnight, washed twice in PBS (1×) and transferred to PBS : ethanol (1:1 or 2:3) for storage at −20°C. Cells were detached from colonized particles and sediment by ultrasonication (Ishii *et al.*, 2004) with the sonication probe Sonoplus HD70 (Bandelin, Berlin, Germany) and filtered on polycarbonate (GTTP) membranes (0.22 μm pore size, Millipore, Darmstadt, Germany). Sea water and enrichment samples were mixed with formaldehyde stock solution (37%) to a final concentration of 2% and were fixed at 4°C overnight. After fixation, samples were filtered on polycarbonate membranes and stored at −20°C. Samples of the S^0-precipitating mats from the Guaymas Basin were fixed in 50 % ethanol and stored at −20°C. CARD-FISH was performed on agarose-embedded filter sections according to Pernthaler and colleagues (2002) and Ishii and colleagues (2004). The applied probes and hybridization conditions are listed in Supporting Information Table S5. Filters were counterstained with DAPI and mounted in a 4:1 mixture of Citifluor (Citiflour, Leicester, UK) and VectaShield (Vector Labs, Burlingame, CA, USA). Cell were identified and counted using an epifluorescence microscope (AxioSkop 2 mot plus, Carl Zeiss, Göttingen, Germany), and images were taken with the AxioCam MRm camera (Carl Zeiss). Image processing was performed with the AXIO VISION 4.7. software (Carl Zeiss).

Sample processing and DNA extraction for 16S rRNA gene analysis

Colonized particles, volcanogenic S^0, S^0-precipitating mat and sediment samples designated for DNA extraction were kept at −20°C. Bottom sea water samples and samples of S^0-enrichment cultures were filtered through cellulose acetate or polyethersulfone membrane filters (0.22 μm pore size, Millipore) and stored at −20°C. DNA was extracted using the Power Soil DNA Kit or the Ultra Clean Soil DNA Kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturers' protocols. Approximately 0.25–1 g of colonized particles, volcanogenic S^0 or S^0-precipitating mat were used for DNA extraction. Membrane filters from bottom sea water or enrichment cultures were sliced prior to extraction. The extracted DNA was stored at −20°C.

16S rRNA gene libraries from particle biofilms (Janssand), S^0-precipitating mat and enrichments (Guaymas Basin)

Bacterial 16S rRNA gene libraries were constructed from S^0-, glass- and pyrite-grown biofilms retrieved from the Janssand tidal flat in 2010. In addition, gene libraries were generated from the S^0-precipitating mat and S^0-enrichment cultures from the Guaymas Basin. From extracted DNA, triplicate polymer-

ase chain reactions (PCRs) with the primer pair GM3F/GM4R (Muyzer *et al*., 1995) were performed, and pooled and gel-purified PCR products were cloned using the TOPO-TA cloning kit (Invitrogen, Karlsruhe, Germany), according to the manufacturer's instructions. Vector inserts were Sanger-sequenced using a Sequencer 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) or by GATC Biotech (Konstanz, Germany). All sequences were manually quality checked and nearly full-length sequences were generated with the SEQUENCHER Version 4.6.1 software package (GeneCodes Corporation, Ann Arbor, MI, USA). Details are given in the supporting information.

16S rRNA gene amplicon 454-pyrotag sequencing of volcanogenic S^0 and deep-sea bottom water (Manus Basin)

The bacterial diversity in volcanogenic S^0 and bottom sea waters from the Manus Basin was analysed by 454-pyrotag sequencing of partial 16S rRNA gene amplicons. Fragments were amplified using modified, bacteria-specific primers GM3F and 907RM (Muyzer *et al*., 1995) in 10 replicate reactions with the proof-reading Phusion High Fidelity Polymerase (New England BioLabs, Ipswich, MA, USA). PCR products were gel purified and sequenced using the Roche's 454 FLX Titanium technology (Roche/454 Life Sciences, Branford, CT, USA). A total of 36 469 sequence reads were analysed with the NGS pipeline (SILVAngs) of the SILVA rRNA gene database project (Quast *et al*., 2013). Details are given in the supporting information. Analysis statistics are given in Supporting Information Table S6.

Phylogenetic analysis

Phylogenetic analysis was performed with the ARB software package (Ludwig *et al*., 2004). Sequences were aligned with the SINA (SILVA Incremental) aligner (Pruesse *et al*., 2012), and phylogenetic trees were reconstructed based on the SILVA 16S rRNA SSU reference database, release 111. Only nearly full-length sequences were used for phylogenetic reconstructions with the maximum-likelihood method ARB RAxML, applying 50% nucleotide conservation base frequency filters to exclude highly variable positions. Partial 16S rRNA gene sequences were subsequently added using maximum-parsimony criteria. No changes to the general tree topology were allowed during this step. Sequences were grouped in OTUs based on a 97% SI cut-off. All nucleotide sequences of this study have been deposited in GenBank or the ENA Sequence Read Archive under the following accession numbers: Janssand: KF623748-KF623752, KF623971-KF624600; Manus Basin: sample accession numbers ERS34975-ERS34980; Guaymas Basin: KF623953-KF623970.

S^0 measurements in tidal sediments

In May 2011, a sediment core was sampled in the Janssand tidal flat to determine the amount of S^0 in a vertical profile of the tidal sediments. The sediment core was sectioned in 2 cm horizons and fixed in zinc acetate (4.4%) a few hours after sampling in the home laboratory. Total extractable S^0 was determined by high-performance liquid chromatography after chloroform extraction, as described in Kamyshny and Ferdelman, 2010. Between 18 and 25 g (wet-weight) zinc acetate fixed sediment were used for extraction.

Sulfate production in S^0-grown biofilms (Janssand, Königshafen) and in S^0-enrichment cultures (Guaymas Basin)

First, S^0 particles that were colonized under oxic conditions in the Janssand tidal flat in 2010 were tested for sulfate production. As controls, we used: (i) heat (microwave)-inactivated surface sediment, (ii) untreated surface sediment and (iii) sterile S^0 powder. Of each sample, 2–3 g were transferred to 50 ml sulfate-free ASW and incubated for 72 h in the dark at room temperature. For the Janssand tidal flat colonization experiment from 2011, we incubated approximately 1 g each of colonized S^0 particles retrieved from oxic, transition and anoxic sediment layers for 27 h in 20 ml ASW at 14°C. Heat-inactivated S^0 particles served as control. These incubations were subsampled (1 ml ASW) at 0 h, 4 h, 22 h and 27 h. Aditionally, we incubated approximately 1 g of S^0 particles colonized under oxic conditions in the Königshafen tidal flat in 50 ml sulfate-free ASW for 72 h at room temperature in the dark. Untreated surface sediment and sterile S^0 powder served as controls. Besides, sulfate production was monitored for 30 days in S^0-oxidizing enrichment cultures (treatments A and B) from the S^0-precipitationg mat (Guaymas Basin) to confirm S^0-oxidizing activity.

For sulfate measurements, all supernatants were sterile filtered through 0.22 μm membrane filters (Milipore) and diluted 10- or 100-fold with MilliQ water. Sulfate concentrations were measured by suppressed ion chromatography on a Metrohm 761 compact IC (Metrohm AG, Herisau, Switzerland). For each run standards prepared from Na_2SO_4 in a range from 5 to 400 μmol l^{-1} and quality control samples prepared from calibrated sea water (International Association for the Physical Sciences of the Ocean) were analysed.

Growth experiments on pure S_8

The ability of *S. denitrificans* to oxidize pure S_8 was tested by measuring sulfate production in pure cultures. Sulfate-free medium (medium DSMZ 113 with sulfate salts replaced by chloride salts) containing S_8 as sole electron donor was inoculated with active cells of *S. denitrificans*. Pure S_8 was generated by dissolving S^0 powder in CS_2 and subsequent precipitation by evaporation of CS_2 (Steudel and Eckert, 2003; A. Kamyshny, unpublished). S_8 prepared in this manner is stable for several weeks. Precipitates of S_8 were ground, resuspended in water and immediately added to the culture flasks. For comparison and as control for residual polymeric or polysulfidic S^0 active cells of the photoautotroph *A. vinosum* 180T was added to medium (DSMZ 28 with sulfate salts replaced by chloride salts) and incubated under photoautotophic conditions. This strain is known to oxidize free S^0 only when in its polymeric or polysulfidic form (Franz *et al*., 2007). To test for abiotic sulfate production, a cell-free control was set up. Triplicate experiments were performed at

room temperature. Supernatants were sampled daily and sulfate concentration was determined.

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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. (A) S^0 precipitations in a sulfidic pool during low tide at the Janssand tidal flat (German Wadden Sea), (B) volcanogenic S^0 boulder and (C) S^0 outcrop at the North Su Volcano rising in the back-arc spreading centre Manus Basin (Bismarck Sea, Papua New Guinea), and (D) S^0-precipitating microbial mat covering hydrothermal sediments in the Guaymas Basin (Gulf of California, Mexico).

Fig. S2. Phylogenetic reconstruction of *Epsilonproteobacteria*-related 16S rRNA gene sequences using maximum likelihood (RAxML). Sequences were retrieved from particle-colonization experiments (Janssand 2010), from the S^0-precipitating mat and from oxygen- and nitrate-respiring S^0-enrichment cultures (Guaymas Basin). Representative OTUs (97% SI cut-off) were selected for presentation, *n* = number of sequences per OTU. Scale bar indicates 10% estimated sequence changes.

Fig. S3. Phylogenetic reconstruction of *Deltaproteobacteria*-related 16S rRNA gene sequences using maximum likelihood (RAxML). Sequences were retrieved from particle-colonization experiments (Janssand 2010), from the S^0-precipitating mat and from S^0-respiring enrichment culture (Guaymas Basin). Sequences from S^0-disproportionating cultures from Janssand tidal sediment and from Guaymas Basin sediments were provided by Kai Finster. Representative OTUs (97% SI cut-off) were selected for presentation, *n* = number of sequences per OTU. Scale bar indicates 10% estimated sequence changes.

Fig. S4. (A) Epifluorescence images of DAPI-stained cells (in blue) in (a) S^0-, (b) pyrite- and (c) glass-grown biofilms from the oxic sediment layer in Janssand tidal sediment (October 2010). Scale bar refers to 5 μm. (B) Epifluorescence images of (a) *Epsilonproteobacteria* (probe Epsy549) in a S^0 biofilm from the oxic sediment layer, Janssand 2010, (b) *Epsilonproteobacteria* (Epsy914) accounting for up to 22% of DAPI in a S^0-rich tidal pool (Janssand, May 2011), (c) *Epsilonproteobacteria* (probe mix Epsy549/Epsy914) in a volcanogenic S^0 boulder (Manus Basin) and (d) *Epsilonproteobacteria* (probe mix Epsy549/Epsy914) and *Arcobacter* (inset, Arc94) in a S^0-precipitating mat (Guaymas Basin). In green, CARD-FISH signal (Alexa 488); in blue, DAPI stain. Scale bar refers to 10 μm.

Fig. S5. Epifluorescence images of *Epsilonproteobacteria* (probe Epsy549) in (A) oxygen- and (B) nitrate-respiring S^0-enrichment cultures from the S^0-precipitating microbial mat (Guaymas Basin). In green, CARD-FISH signal (Alexa 488); in blue, DAPI stain.

Fig. S6. Sulfate production in (●) oxygen- and (◆) nitrate-respiring S^0-enrichment cultures from the S^0-precipitating microbial mat (Guaymas Basin).

Fig. S7. Bathymetric map (Ocean Data View, ODV) of sampling locations in (A) tidal flats of the German Wadden Sea; (B) the Manus Basin back-arc spreading centre, Bismarck Sea, Papua New Guinea; (C) the Guaymas Basin, Gulf of California, Mexico. Map was constructed with help of Ocean Data View (Schlitzer, R., Ocean Data View, http://odv.awi.de, 2012.)

Fig. S8. Detailed bathymetric map of the North Su sampling area (Manus Basin, Papua New Guinea). Volcanogenic S^0 (yellow stars) and bottom water sample I (blue circle), active venting sites (gray circles). Scale bar indicates distance in meters. Figure adapted from Bach and colleagues (2011).

Table S1. Semiquantitative relative abundance of *Epsilonproteobacteria*, *Gammaproteobacteria* and *Deltaproteobacteria* in biofilms grown on introduced S^0, pyrite and glass particles in the Janssand tidal flat colonization experiment in October 2010, as determined by CARD-FISH. Legend: ++ abundant, + present, – absent.

Table S2. Total cell counts (TCC, DAPI) and relative abundance (%) of selected populations determined by CARD-FISH in Janssand (JS, 2010; 2011) and Königshafen (KH, 2011) tidal sediments.

Table S3. Sulfate (SO_4^{2-}) production by S^0-grown biofilms incubated in Königshafen tidal sediments (October 2011). Sulfate concentrations were calculated from IC measurements based on a Na_2SO_4 standard curve.

Table S4. Details of sampling sites and dates, incubation periods and deposition/retrieval method.

Table S5. Horseradish peroxidase-labelled oligonucleotide probes used for CARD-FISH.

Table S6. Statistics of the 454-pyrotag data obtained from the NGS pipeline (SILVAngs) of the SILVA rRNA gene database project (Quast *et al.*, 2013).

Table S7. Sediment porosity, density and total extractable S^0 (μmol g^{-1}) used to calculate total S^0 concentration in different sediment layers (Janssand, May 2011).

Appendix S1. Supplementary methods.

Supporting Information

Supplementary Methods

S^0-enrichment cultures from a filamentous S^0-precipitating mat, Guaymas Basin

Enrichment cultures were started onboard by inoculating 100 ml of filtered bottom sea water (in 250 ml flasks) supplied with approximately 5-10 g of methanol-washed powdered sulfur (Sulfur Ph Eur, Fluka, Buchs, Switzerland) with top sediment from the above described push core, including material from the S^0-precipitating mat. Treatment A was kept oxic by sealing the bottle with air-permeable parafilm. In treatments B and C bottom sea water was purged with N_2, supplied with 8 mmol l^{-1} sodium nitrate (B) or 2 mmol l^{-1} sodium acetate (C) and sealed with an air-tight butyl rubber septum. All flasks were incubated at 4°C and were cooled during transport to the home laboratory. After 6 weeks subsamples of S^0 from these enrichments were transferred to 50 ml of carbonate-buffered, sulfate-free, artificial sea water medium (ASW), which sterilized powderous S^0. The enrichment cultures were incubated at 4°C either (A) oxically, (B) anoxically with 2-8 mM nitrate as electron-acceptor or (C) with 2 mmol l^{-1} sodium acetate and S^0 as the sole electron acceptor. Sulfate production indicated growth of S^0-oxidizing microorganisms (Fig. S6), while sulfide was formed in acetate/S^0 enrichments. After a second transfer and a total incubation period of approximately 5 months enrichments were sampled for CARD-FISH and 16S rRNA gene libraries

16S rRNA gene libraries

Primers GM3F and GM4R (Muyzer *et al*., 1995) were selected for 16S rRNA gene amplification, since they cover a large diversity (Klindworth et al., 2012) within our groups of interest (79.0% in the *Epsilonproteobacteria* and 74.1% in the *Gammaproteobacteria*, 72.9% *Alphaproteobacteria*, 79.7% *Deltaproteobacteria*). PCR was performed in a volume of 20 µl, containing 1x *Taq* PCR buffer, 2 µl of 3 mg ml^{-1} BSA, 320 µM of each dNTP, 2 µM of each primer, 0.04 U of *Taq* polymerase (Eppendorf, Hamburg, Germany) and 1 µl of DNA template. Triplicate PCR amplicons were pooled to reduce bias (Polz and Cavanaugh, 1998). PCR was performed as follows: initial denaturation at 94°C for 5 min, 30 cycles of 30 sec denaturation at 94°C, 90 sec annealing at 48°C and 120 sec elongation at 72°C, followed by 10 min of final elongation at 72°C. Finally, the replicates were pooled and purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany).

16S rRNA gene tags – amplification, library construction and sequencing

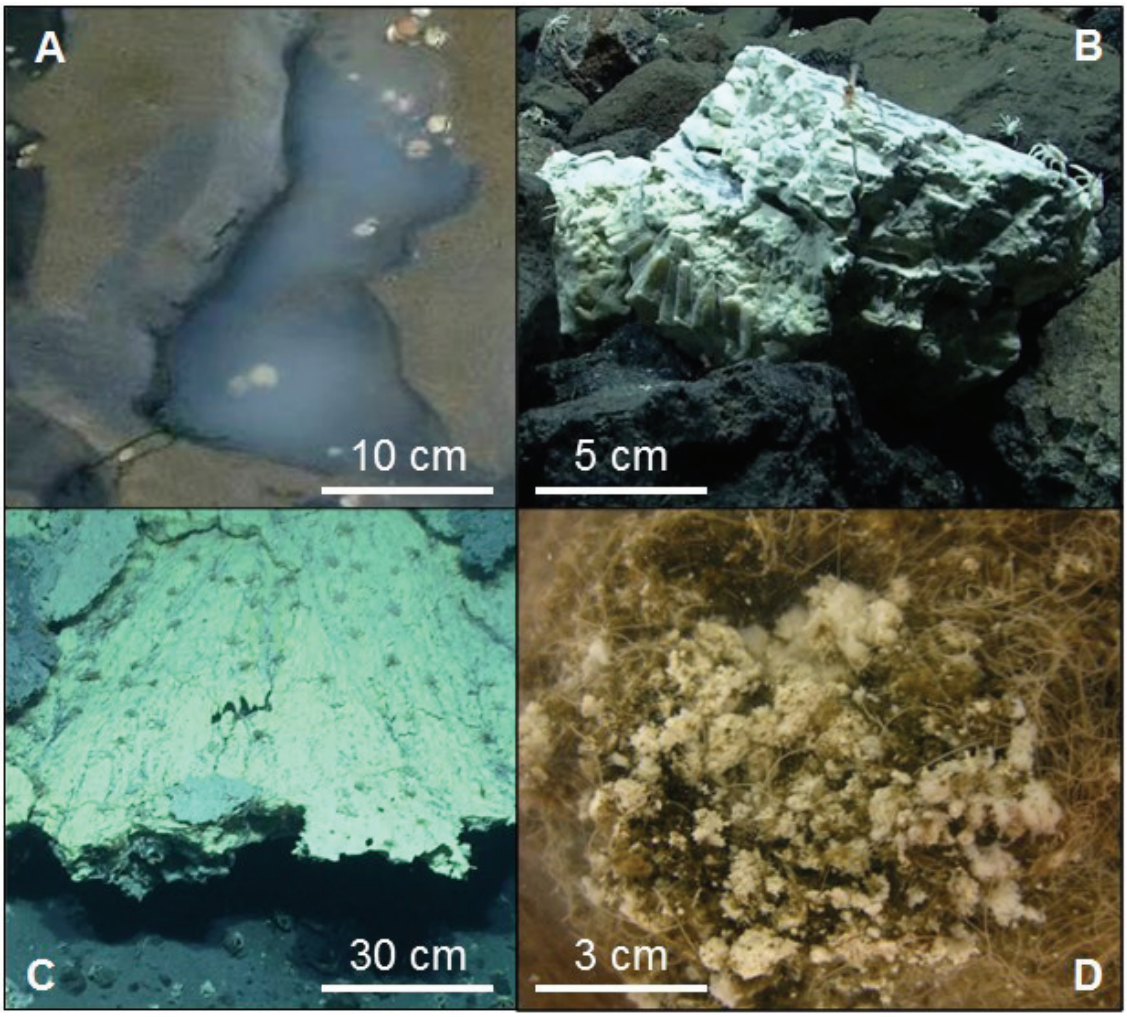
For 454-pyrotag sequencing of 16S rRNA gene amplicons, bacterial primers GM3F and 907RM (Muyzer *et al*., 1995) were modified to introduce a sample-specific barcode at the 5' end, enabling the sample distinction after sequencing. Both primers were extended with a Sfi I restriction site at the 5' end, for ligation of the 454 adapters. Ten parallel PCR reactions for each sample were performed using the Phusion High Fidelity Polymerase Kit (Finnzymes, New England BioLabs Inc). Briefly, 1x Phusion HF Buffer, 250 µM of each deoxynucleoside triphosphate, 0.5 µM of each primer, 0.4 U of Phusion DNA polymerase were mixed with 1 µl of DNA template in a 20 µl PCR reaction. PCR was carried out as follows: 3 min initial denaturation at 98°C, 25 to 30 cycles of denaturation at 98°C for 10 sec, followed by 30 sec of annealing at 52°C and 30 sec elongation at 72°C, finalized by 10 min of final elongation at 72°C. All replicate reactions were pooled and DNA was precipitated by addition of ethanol and sodium-acetate (ph 5.2). After 60 minutes incubation on ice, samples were centrifuged for 20 min at 15000 rpm. Supernatant was discarded, and the DNA pellet was re-suspended in 20 µl of TE buffer. Afterwards, the re-suspended DNA was gel-purified using SYBR Green I Nucleic Acid Gel Stain (Invitrogen) and the MinElute Gel Extraction Kit (Qiagen) according to the manufacturers' protocol. DNA concentration was determined fluorometrically at 260 nm, using the Qubit 2.0 Fluorometer and the Qubit dsDNA HS Assay KIT (Invitrogen), Amplicons were pooled and pyrosequenced at the Max Planck-Genome Centre (Cologne, Germany).

16S rRNA 454-pyrotag analysis

After adapter and quality clipping, sequence reads retrieved from 454-pyrosequencing were aligned against the SILVA SSU SEED alignment using the SILVA Incremental Aligner - SINA (Pruesse *et al*., 2012) and quality checked (Quast *et al*., 2013), excluding all reads containing >2% ambiguities or homopolymers, reads shorter than 200 base pairs and reads with an alignment identity <50% hereafter. Remaining reads were de-replicated, clustered (using Cd-hit; see Li and Godzik, 2006) and finally assigned to a taxonomic path based on a local nucleotide BLAST (Camecho *et al*., 2009) search against the SILVA 16S rRNA SSU reference database, release 111.

Supplementary Figures and Tables

Figure S1: A) S^0 precipitates in a sulfidic pool during low tide at the Janssand tidal flat (German Wadden Sea), **B)** Volcanogenic S^0-boulder and **C)** S^0-outcrop at the North Su Volcano rising in the back-arc spreading center Manus Basin (Bismarck Sea, Papua New Guinea), and **D)** S^0-precipitating microbial mat covering hydrothermal sediments in the Guaymas Basin (Gulf of California, Mexico).



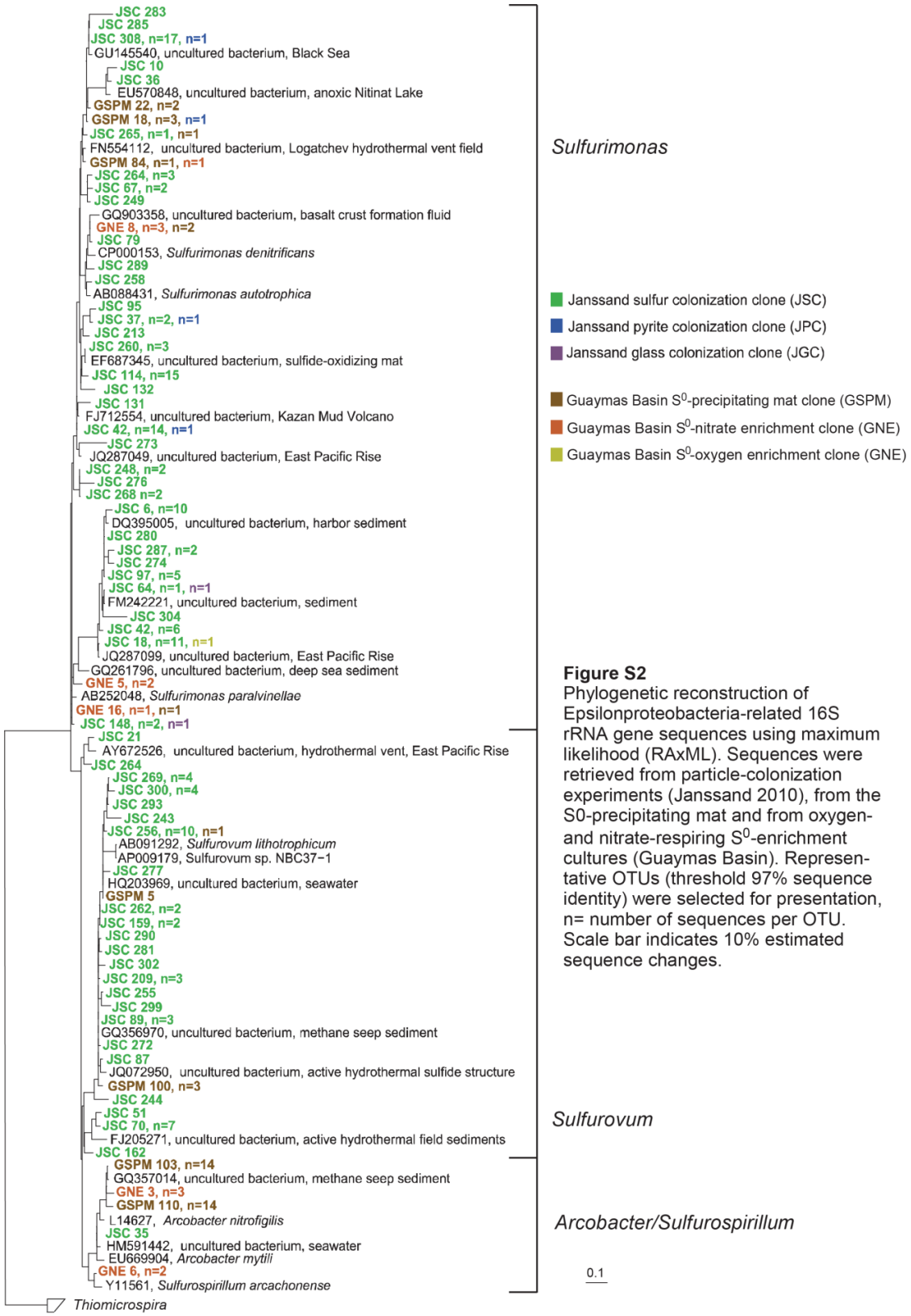


Figure S2
Phylogenetic reconstruction of Epsilonproteobacteria-related 16S rRNA gene sequences using maximum likelihood (RAxML). Sequences were retrieved from particle-colonization experiments (Janssand 2010), from the S0-precipitating mat and from oxygen- and nitrate-respiring S^0-enrichment cultures (Guaymas Basin). Representative OTUs (threshold 97% sequence identity) were selected for presentation, n= number of sequences per OTU. Scale bar indicates 10% estimated sequence changes.

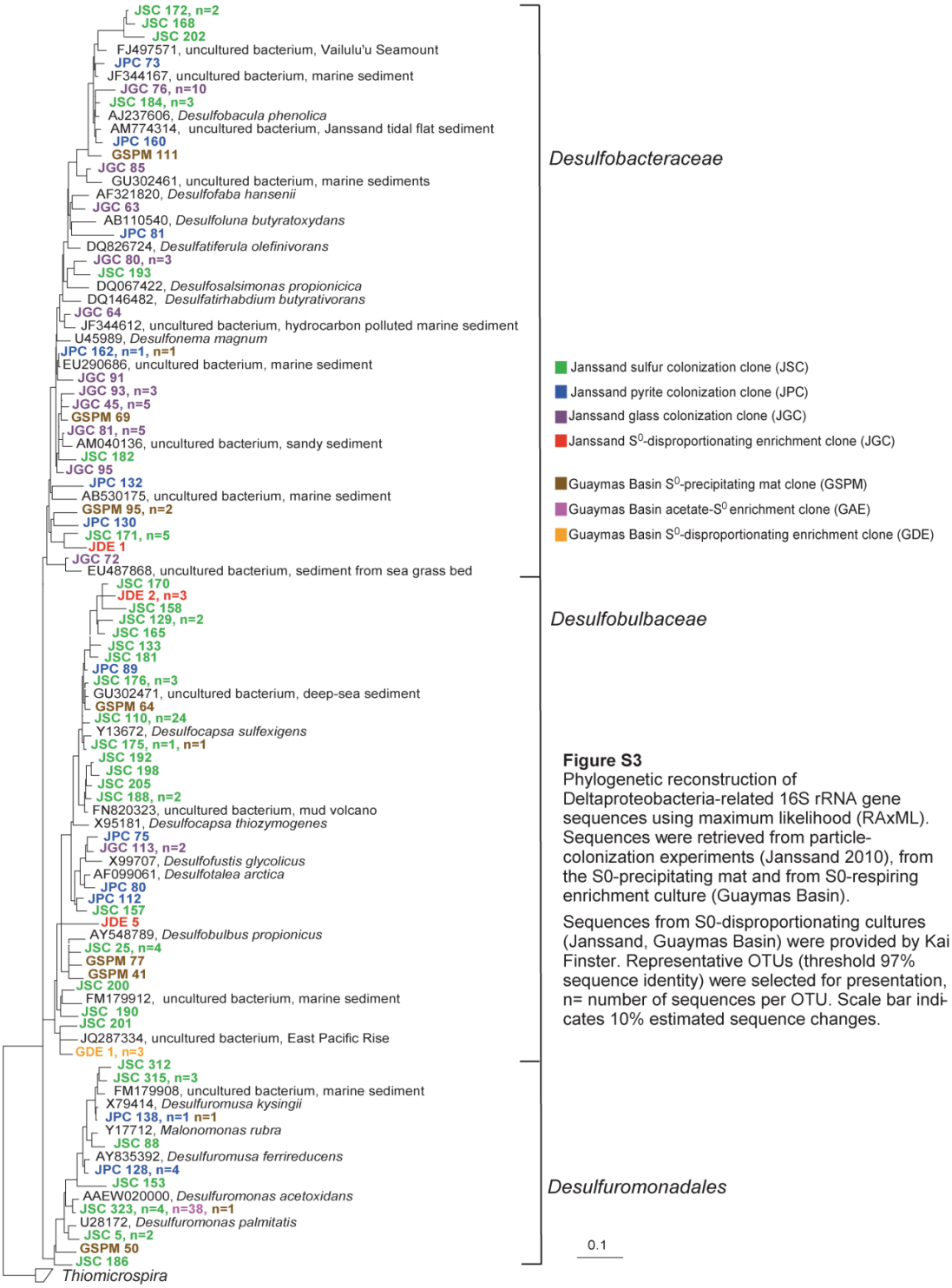


Figure S3
Phylogenetic reconstruction of Deltaproteobacteria-related 16S rRNA gene sequences using maximum likelihood (RAxML). Sequences were retrieved from particle-colonization experiments (Janssand 2010), from the S0-precipitating mat and from S0-respiring enrichment culture (Guaymas Basin).

Sequences from S0-disproportionating cultures (Janssand, Guaymas Basin) were provided by Kai Finster. Representative OTUs (threshold 97% sequence identity) were selected for presentation, n= number of sequences per OTU. Scale bar indicates 10% estimated sequence changes.

Figure S4:
A) Epifluorescence images of DAPI-stained cells (in blue) in a) S^0-, b) pyrite- and c) glass-grown biofilms from the oxic sediment layer in the Janssand tidal flat (October 2010). Scale bar refers to 5 µm.
B) Epifluorescence images of a) *Epsilonproteobacteria* (probe Epsy549) in a S^0-biofilm from the oxic sediment layer (Janssand, October 2010), b) *Epsilonprotoebacteria* (probe Epsy914) accounting for up to 22% of DAPI in a S^0-rich tidal pool (Janssand, May 2011), c) *Epsilonproteobacteria* (probe mix Epsy549/914) in a volcanogenic S^0-boulder (Manus Basin), and d) *Epsilonprotoebacteria* (probe mix Epsy549/914) and Arcobacter (inset, probe Arc94) in a S^0-precipitating mat (Guaymas Basin). In green: CARD-FISH signal (Alexa 488); in blue: DAPI-stain. Scale bar refers to 10 µm.

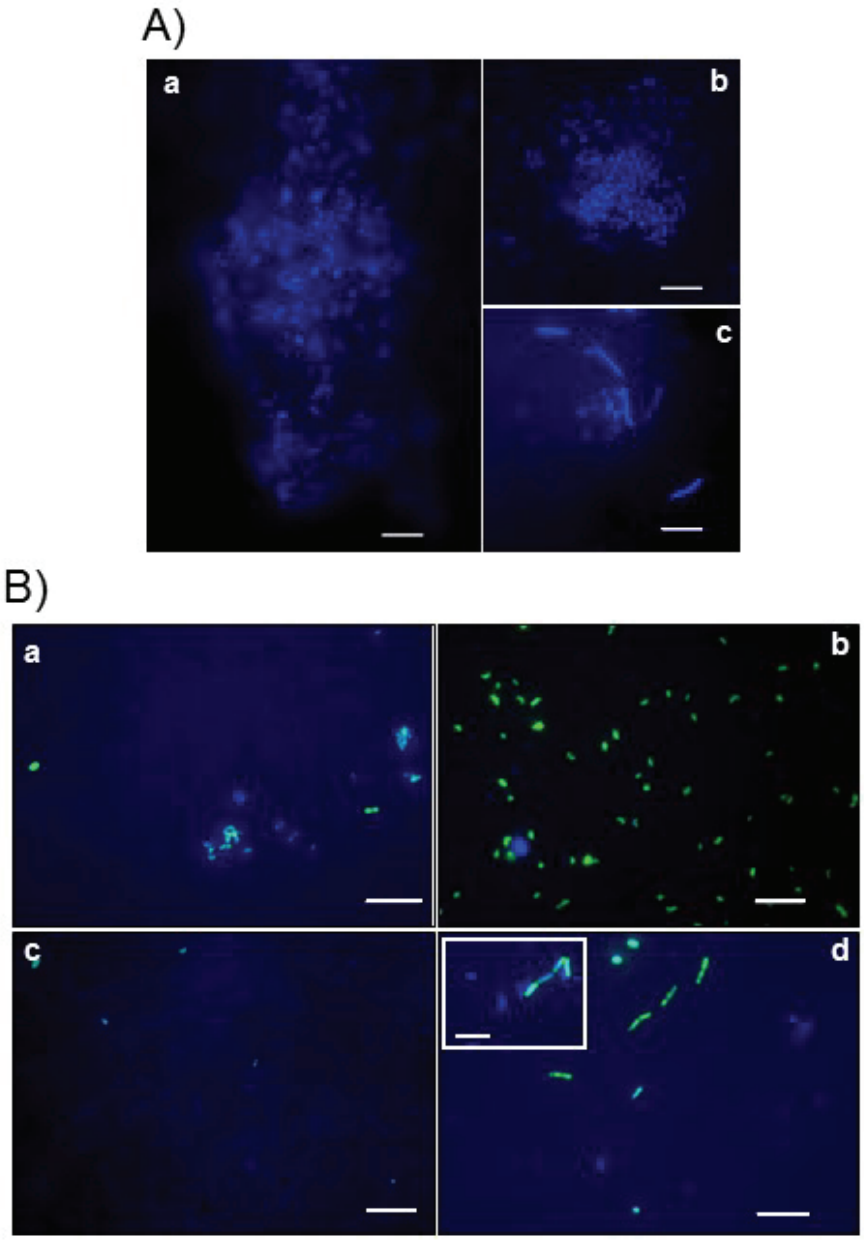


Figure S5: Epislourescence images of *Epsilonprotoeobacteria* (probe Epsy549) in **A)** oxygen- and **B)** nitrate-respiring S^0-enrichment cultures from the S^0-precipitating microbial mat (Guaymas Basin). In green: CARD-FISH signal (Alexa 488); in blue: DAPI-stain.

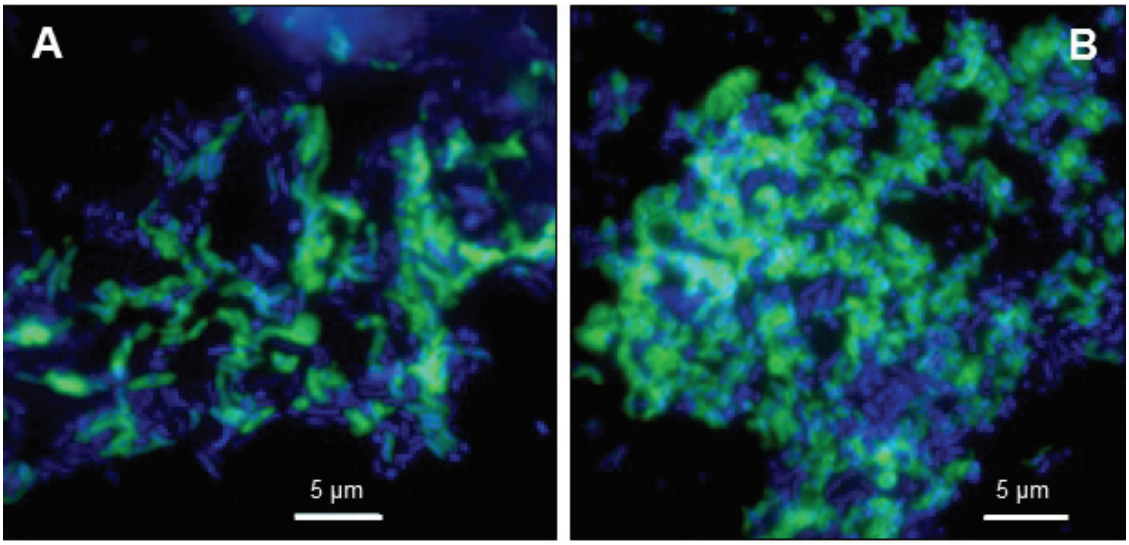


Figure S6: Sulfate production in oxygen- (○) and nitrate- (♦) respiring S^0-enrichment cultures from the S^0-precipitating microbial mat (Guaymas Basin).

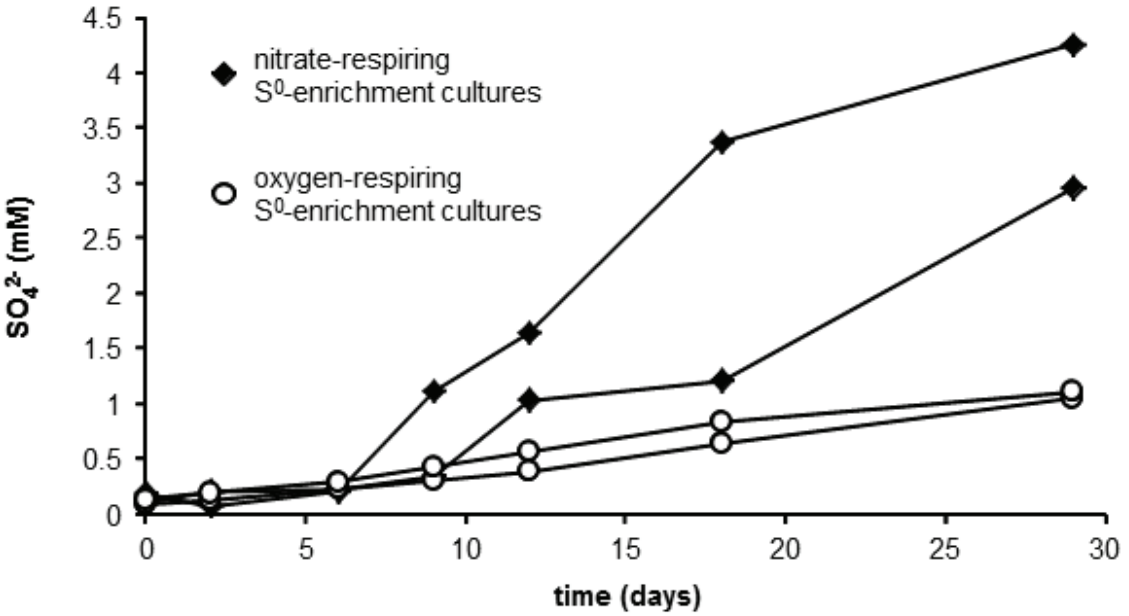


Figure S7: Bathymetric map (Ocean Data View, ODV) of sampling locations at **A**) Janssand and Königshafen tidal flats of the German Wadden Sea; **B**) the Manus Basin back-arc spreading center, Bismarck Sea, Papua New Guinea; **C**) the Guaymas Basin, Gulf of California, Mexico. Map was constructed using Ocean Data View (Schlitzer, R. Ocean Data View, htto://odv.awi.de, 2012).

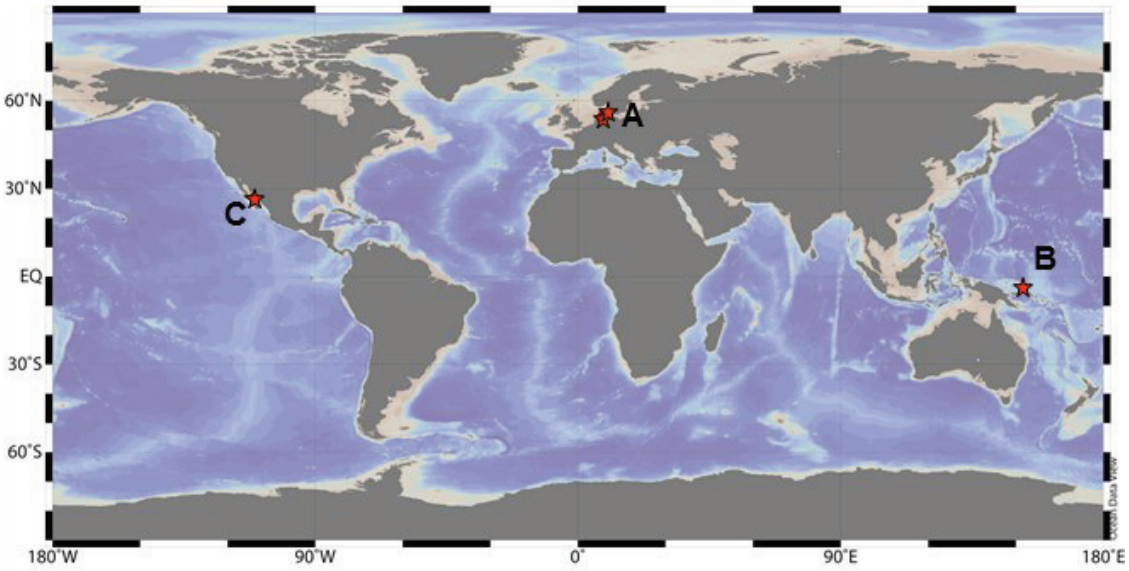


Figure S8: Detailed bathymetric map of the North Su sampling area (Manus Basin, Papua New Guinea). Volcanogenic S^0 (yellow stars) and bottom water sample I (blue star), active venting sites (gray circles). Scale bar indicates distances in meters. Figure adapted from Bach *et al*. (2011)

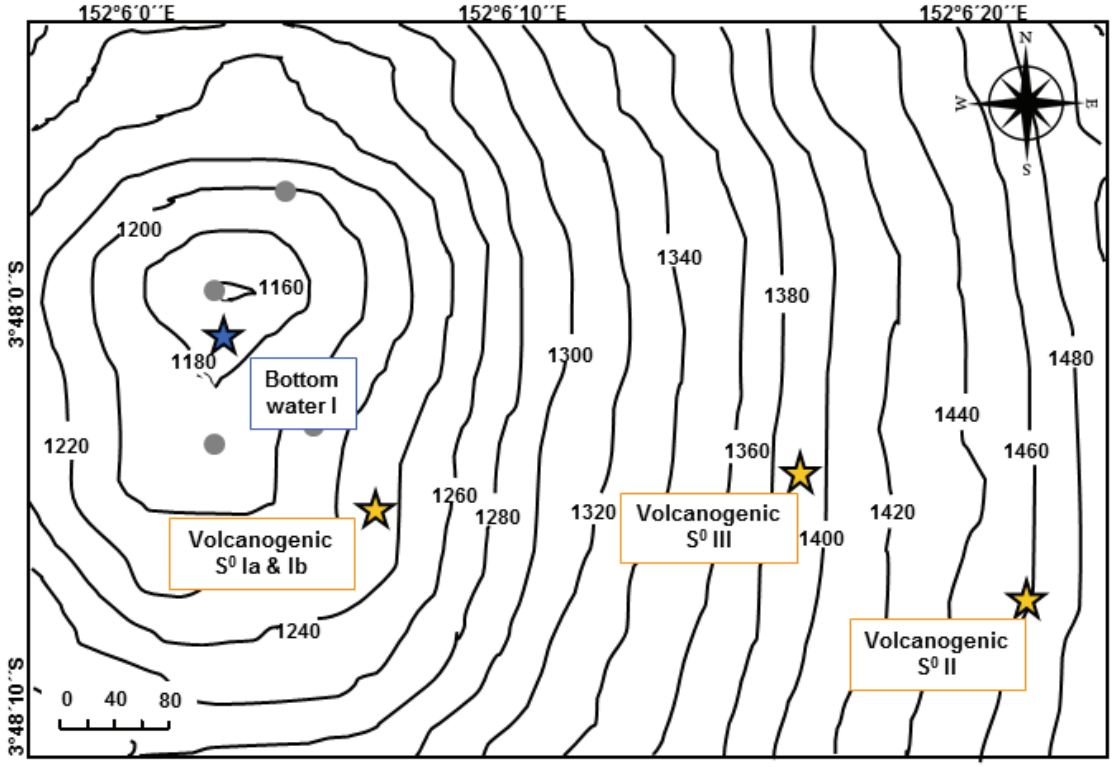


Table S1: Semi-quantitative relative abundance of *Epsilonproteobacteria*, *Gammaproteobacteria* and *Deltaproteobacteria* in biofilms grown on introduced S^0-, pyrite- and glass-particles in the Janssand tidal flat colonization experiment in October 2010, as determined by CARD-FISH. Legend: ++ abundant, + present, – absent.

Sample	*Epsilonproteobacteria*	*Gammaproteobacteria*	*Deltaproteobacteria*
oxic layer			
S^0-grown biofilm	++	+	-
glass-grown biofilm	-	++	-
pyrite-grown biofilm	-	+	+
oxic-anoxic transition layer			
S^0-grown biofilm	+	+	+
glass-grown biofilm	-	+	+
pyrite-grown biofilm	+	-	+
anoxic layer			
S^0-grown biofilm	-	+	+
glass-grown biofilm	-	+	+
pyrite-grown biofilm	-	+	+

Table S2: Total cell counts (TCC, DAPI) and relative abundance (%) of selected populations determined by CARD-FISH in Janssand (JS, 2010; 2011) and Königshafen (KH, 2011) tidal sediments.

Sample	TCC [cells ml^{-1}]	% of TCC			
		EUBI-III	NON388	EPSY549	EPSY914
JS surface sediment (Nov 2010)	4.0 x 10^8	86%	<0.5%	-	<0.5%
JS surface sediment (May 2011)	4.2 x 10^8	92%	<0.5%	-	<0.5%
JS surface sediment (Nov 2011)	3.7 x 10^8	79%	<0.5%	-	<0.5%
KH S^0-grown biofilm (Oct 2011)	-	89%	<0.5%	56%	
KH glass-grown biofilm (Oct 2011)	-	87%	<0.5%	<0.5%	
KH surface sediment (Oct 2011)	5.3 x 10^8	84%	<0.5%	<0.5%	

Table S3: Sulfate (SO_4^{2-}) production by S^0-grown biofilms incubated in Königshafen tidal sediments (October 2011). Sulfate concentrations were calculated from IC measurements based on a Na_2SO_4 standard curve.

Sample	Sulfate [µmol l^{-1}]
S^0-grown biofilm	63.1 ± 96.3
surface sediment	-*
heat-inactivated surface sediment	23.8 ± 5.8

* below detection limit (0.2 µmol l^{-1})

Table S4: Details of sampling sites and dates, incubation periods and deposition/retrieval method.

Sample origin and type	Location	Incubation period and/or sampling time	Sample description	Deposition and retrieval method
Janssand colonization experiments	53.7383 N, 7.6858 E	4 weeks (Oct-Nov 2010; Oct-Nov 2011)	S^0, glass and pyrite particles colonized in the sediment T(sediment) = 8-12°C oxic = 0-1 cm oxic-anoxic transition = 2-5 cm anoxic = ~15 cm	manually
	53.7358 N, 7.6988 E			
Janssand Sediment	53.7 N, 7.6 E	Nov 2010; May 2011; Nov 2011	oxic surface sediment (0-1 cm depth) T(sediment) = unknown	
Königshafen colonization experiments	55.0346 N 8.4236 E	12 months (Oct 2010 - Oct 2011)	S^0 and glass particles colonized in the oxic surface sediment (0-1 cm depth) T(sediment) = unknown	
Königshafen sediment		Oct 2011	oxic surface sediment (0-1 cm depth) T(sediment) = unknown	

Table S4 (continued): Details of sampling sites and dates, incubation periods and deposition/retrieval method.

Sample origin and type	Location	Sampling time	Sample description	Deposition and retrieval method
Manus Basin bottom water	3.8580 S, 152.1332 E	June 2011	Bottom water sample Depth = -1650 m T(water) = ~4°C	Niskin Bottle
	3.4812 S, 152.0606 E		Bottom water sample Depth = -1166 m T(water) = ~4°C	
North Su volcanogenic S^0	3.8015 S, 152.1019 E	June 2011	S^0-outcrop Depth = -1230 m T(surrounding water) = ~4°C	TV Guided GRAB
	3.8019 S, 152.1062 E		S^0-boulder Depth = -1480 m T(surrounding water) = ~4°C	ROV Quest
	3.8001 S, 152.1049 E		S^0-boulder Depth = -1404 m T(surrounding water) = ~4°C	
Guaymas Basin S^0-precipitating mat	27.0069 N, 111.2427 E	Dec 2009	S^0-precipitating filamentous microbial mat Depth = -2010 m T(surrounding water) = ~4°C	DSV Alvin

Table S5: HRP-labeled oligonucleotide probes used for CARD-FISH.

Probe name	Target group	Probe sequence (5'→3')	% FA**	Reference
EUBI-III	Most *Bacteria*	GCTGCCTCCCGTAGGAGT GCAGCCACCCGTAGGTGT GCTGCCACCCGTAGGTGT	35	Amann *et al*., 1990 Daims *et al*., 1999
NON388	negative control	ACTCCTACGGGAGGCAGC	35	Wallner *et al*., 1993
Epsy549	some *Epsilonproteobacteria*	CAGTGATTCCGAGTAACG	35	Lin *et al*., 2006
Epsy914	some *Epsilonproteobacteria*	GGTCCCCGTCTATTCCTT	35	Loy *et al*., 2003
Arc94	*Arcobacter*	TGCGCCACTTAGCTGACA	20	Snaidr *et al*., 1997
Gam42a* (+ competitor)	*Gammaproteobacteria*	GCCTTCCCACATCGTTT (GCCTTCCCACTTCGTTT)	35	Manz *et al*., 1992
Delta495a (+ competitor)	most *Deltaproteobacteria* most *Gemmatimonadetes*	AGTTAGCCGGTGCTTCCT (AGTTAGCCGGTGCTTCTT)	35	Loy *et al*., 2002 Lücker *et al*., 2007
Delta495b (+ competitor)	some *Deltaproteobacteria*	AGTTAGCCGGCGCTTCCT (AGTTAGCCGGCGCTTCKT)	35	
Delta495c (+ competitor)	some *Deltaproteobacteria*	AATTAGCCGGTGCTTCCT (AATTAGCCGGTGCTTCTT)	35	

* targets 23S rRNA
** formamide concentration in hybridization buffer (v/v). All hybridizations were performed at 46°C.

Table S6: Statistics of the 454-pyrotag data obtained from the NGS pipeline (SILVAngs) of the SILVA rRNA gene database project (Quast *et al*., 2013).

Sample	Sequences (before filtering)	Average length	Sequences (after filtering)	OTUs*	Clustered**	Replicates***
Bottom water 1	7479	452.2	7189	1691	4146	1352
Bottom water 2	2413	458.9	2306	574	1158	574
Volcanogenic S^0-outcrop 1	870	424.2	818	206	434	178
Volcanogenic S^0-outcrop 1	10781	431.1	10212	1127	5870	3215
Volcanogenic S^0-boulder 1	7724	420.2	6076	1071	3937	1068
Volcanogenic S^0-boulder 2	7184	418.6	6457	626	4291	1540

* number of unique reads in sample
** number of reads in sample assigned to clusters with 98% identity
*** reads identical (100%) to another read within the same sample

Table S7: Sediment porosity, density and total extractable S^0 (µmol g^{-1}) used to calculate total S^0 concentration in different sediment layers (Janssand, May 2011).

Depth [cm]	Porosity	Wet sediment density [g cm^{-3}]	Total S^0 (measured) [µmol g^{-1}]	Total S^0 (calculated) [µmol cm^{-3}]
0-2	0.30	1.81	1.83	3.32
2-4	0.29	1.65	1.41	2.32
4-6	0.36	1.90	1.32	2.50
6-8	0.37	1.97	0.86	1.69
8-10	0.35	1.76	0.92	1.62
10-12	0.31	1.63	0.61	1.00
12-14	0.35	1.81	0.57	1.04
14-16	0.30	1.51	0.64	0.97
16-18	0.35	1.84	0.41	0.75
18-20	0.39	2.00	0.41	0.81
20-22	0.33	1.85	0.52	0.96
22-24	0.27	1.64	0.39	0.64

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Chapter III

Community shift from phototrophic to chemotrophic sulfide oxidation during holomictic conditions in a shallow stratified sea-water lake

Petra Pjevac, Marino Korlević, Elvira Bura-Nakić, Irena Ciglenečki, Rudolf Amann and Sandi Orlić

Manuscript in preparation

Contributions:

P.P. and S.O. developed concepts and ideas. P.P. conceived and wrote the manuscript, performed CARD-FISH experiments, construction and sequencing of soxB gene clone libraries, and analysis of all in the manuscript presented data. M.K. performed DNA isolation, constructed 16S rRNA gene clone libraries and CARD-FISH experiments. E.B.-N. measured and calculated POC, DOC and RSS concentrations and hydrographical data. S.O collected samples, performed CARD-FISH experiments. M.K., E.B.-N, I.C., R.A. and S.O. conceived and edited the manuscript.

Community shift from phototrophic to chemotrophic sulfide oxidation during holomictic conditions in a shallow stratified sea-water lake

Petra Pjevac[1*], Marino Korlević[2], Elvira Bura-Nakić[2], Irena Ciglenečki[2], Rudolf Amann[1] and Sandi Orlić[2]

[1]Max Planck Institute for Marine Microbiology, Celsiusstraße 1, 28359 Bremen, Germany
[2]Ruđer Bošković Institute, Bijenička cesta 54, 10000 Zagreb, Croatia

*corresponding author

Running title: Chemotrophic SOPs dominate a stratified lake during holomixis

Keywords: stratified lake, holomixis, anoxia, *Prosthecochloris*, *Halochromatium*, SUP05 clade *Gammaproteobacteria*

Abstract

Lake Rogoznica is a shallow stratified sea-water lake cut into the karst relief of the eastern Adriatic coast. Most of the year, lake waters are divide in an oxic epilimnion and a sulfidic hypolimnon. Although the lakes water column has been offten investigated during the last 20 years, little is known about the microbial communities in Lake Rogoznica. We investigated microbial diversity and microbially mediated sulfur cycling in Lake Rogoznica with culture-independent microbiological methods (16S rRNA gene sequencing, functional gene sequencing and CARD-FISH) and compared results to recorded sulfide and oxygen concentrations and hydrological parameters (pH, temperature, salinity). We identified a tight coupling between the lakes' chemistry and occurring microorganisms. During stratification, phototrophic sulfur-oxidation by green and purple sulfur bacteria (GSB and PSB) dominated microbial processes in the lake and consumed most reduced sulfur species (RSS) before these reached oxygenated water layers. However, after a mixing event the GSB and PSB populations diminished, and the homogeneous, anoxic water column was dominated by a bloom of chemotrophic, sulfur-oxidizing SUP05 clade *Gammaproteobacteria*. This study represents the first report of a community shift from phototrophic to chemotrophic sulfide oxidation during holomictic conditions in a stratified lake.

Introduction

Stratified, sulfidic lakes offer a physically and chemically well-defined environment, enabling the development of complex, yet stable microbial communities along light, oxygen, sulfide and depth gradients (Sorokin, 1970; Guerrero *et al*., 1985; Eckert and Trüper, 1993; Tonolla *et al*., 2003; Martínez-Alonso *et al*., 2008). Only few of those lakes are permanently stratified or meromictic. In the great majority, referred to as holomictic lakes, the different water layers mix at least once a year. Nonetheless, during stratification the water columns of mero- and holomictic lakes display similar features and usually are divided in an oxic surface layer - the epilimnion, and an anoxic bottom layer - the hypolimnion (Jørgensen *et al*., 1979). The interface between these layers is the chemocline. It is the zone of highest chemical reactivity in the lake, typically accompanied by elevated microbial activity.

In the anoxic hypolimnion and the underlying sediments, sulfate-reduction coupled to organic matter remineralization is a key process (e. g. Bowman *et al*., 2000 Tonolla *et al*., 2003; Koizumi *et al*., 2004). It results in the production of hydrogen sulfide, which diffuses upwards toward oxygenated water layers. Where sulfide and light are present but oxygen is absent, anoxygenic phototrophic green and purple sulfur bacteria (GSB and PSB) oxidize sulfide and form extensive blooms with cell densities up to 10^7 cells ml^{-1} (Guerrero *et al*., 1985; Pedrós-Alió and Guerrero, 1993; Tonolla *et al*., 1998; 1999; 2003; Casamayor *et al*., 2000). Chemotrophic sulfur-oxidizers (e.g. *Alphaproteobacteria*, *Epsilonproteobacteria*) often occur in layers above or together with GSB and PSB at the chemocline (Tonolla *et al*., 1999; 2003; Lehours *et al*., 2007; Gregersen *et al*., 2009; Klepec-Ceraj *et al*., 2012). In the oxic epilimnion, the microbial community is generally clearly distinct from the communities in underlying anoxic waters (Casamayor *et al*., 2000; Klepec-Ceraj *et al*., 2012; Garcia *et al*., 2013).

Lake Rogoznica is a shallow stratified sea-water lake on the Croatian Adriatic coast. The hydrography and chemistry of Lake Rogoznica has been studied extensively throughout the last two decades. Conditions generally resembled those in other holomictic sulfidic lakes, like Lake Kinneret (Eckert and Trüper, 1993), Lake Pavin (Lehours *et al*., 2007) or Lake Estanya (Martínez-Alonso *et al*., 2008; Casamayor *et al*., 2012). Most of the year the water column is stratified (Ciglenečki *et al*., 2005). The epilimnion is similar to waters of the adjacent Adriatic Sea in temperature and salinity, but highly enriched in nutrients and dissolved organic carbon (DOC; up to 6 mg l^{-1}) (Kršinić *et al*., 2000; Ćosović *et al*., 2000; Ciglenečki *et al*., 2005). This is assumed to be

a consequence of extensive organic matter remineralization coupled to microbial sulfate reduction in anoxic lake sediments and the hypolimnion, where sulfide concentrations reach up to 5 mmol l^{-1} (Ciglenečki *et al*., 2005; Bura-Nakić *et al.,* 2009; Kamyshny *et al*., 2011). The depth of the chemocline, where oxic and sulfidic water layers meet, is not stagnant and increases from winter to summer months (Ciglenečki *et al*., 2005). In autumn (October-November), a wind and temperature driven perturbation occurs, typically leading to the oxygenation of the entire water column during holomixis (Bura-Nakić *et al*., 2009). However, in particularly warm years (e.g. 1941, 1997, 2000 and 2011), oxygen solubility in the epilimnion is lowered due to higher water temperatures and autumn perturbation leads to anoxia in the entire water column (Ciglenečki *et al*., 2005; Bura-Nakić *et al*., 2009). A milky turbidity caused by the formation of colloidal elemental sulfur (S^0) accompanies anoxic holomixis (Bura-Nakić *et al*., 2009). Furthermore, mass mortality of pelagic and benthic eukaryotes during these anoxic events was reported (Barić *et al*., 2003).

While the diversity of benthic metazoans, as well as zoo- and phytoplankton communities in Lake Rogoznica are well investigated (e.g. Kršinić *et al*., 2000; Barić *et al*., 2003; Svensen *et al*., 2008; Burić *et al*., 2009), only limited data on the lakes microbial diversity is available (Krstulović and Šolić, 2001; Šestanović *et al*., 2005). Previous studies described not further classified PSB populations at the chemocline (Ciglenečki *et al*., 1998) and abundant *Synechococcus*-related *Cyanobacteria* in the epilimnion (Šestanović *et al*., 2005). Increased total cell numbers and abundance of not further classified PSB during and after anoxic holomixis in 1997 were reported (Krstulović and Šolić, 2001). A recent sulfur isotope study suggested co-occurrence of phototrophic sulfur oxidation, chemotrophic sulfur oxidation and sulfate reduction (Kamyshny *et al*., 2011) in Lake Rogoznica, but no details about involved microorganisms are available.

In this study, we aimed to characterize the microbial community of Lake Rogoznica during both stratified and mixed conditions, with a special focus on microorganisms involved in the sulfur cycle. We hypothesized that the microbial diversity in the epilimnion resembles the diversity in the photic zone of adjacent Adriatic Sea (Manti *et al*., 2012, Šantić *et al*., 2013). The *Synechococcus*/*Prochlorococcus*-type *Cyanobacteria* and SAR11-clade related *Alphaproteobacteria* were expected to represent a major fraction of the bacterioplankton. In anoxic waters, a microbial community with sulfate-reducing character, as indicated by sulfur isotope analysis

(Kamyshny *et al*., 2011) and described in other stratified sulfidic lakes (Tonnola *et al*., 2000; 2004; Lehours *et al*., 2007), was expected. At the chemocline, the relative importance of PSB, GSB and chemotrophic sulfur-oxidizers had to be investigated. Especially the response of the microbiome to anoxia during holomixis was of high interest, as the anoxic episodes in Lake Rogoznica resemble phenomena observed in oceanic redoxiclines, like those occurring in the Baltic Sea (Rheinheimer *et al*., 1989) or of the coast of Namibia (Lavik *et al*., 2009) and were not previously investigated in a stratified lake.

Microbial diversity was investigated via the 16S rRNA approach, by performing 16S rRNA gene (Sanger- and 454-pyrotag) sequencing and catalyzed reporter deposition – *in situ* fluorescence hybridization (CARD-FISH). The diversity of sulfur oxidizing prokaryotes (SOPs) was additionally examined by amplification and sequencing of the sulfate thiohydrolase encoding gene *soxB*. Furthermore, the physico-chemical settings of Lake Rogoznica were monitored in parallel to microbiological sampling, allowing us to identify links between microbial community composition and the chemical environment. Water temperature, salinity, pH and oxygen concentrations were recorded *in situ*, while concentration of reduced sulfur species (RSS) and dissolved and particulate organic carbon (DOC/POC) concentrations were determined in the laboratory.

Material and methods

Sampling location and sample collection

Lake Rogoznica (43°32′N, 15°58′E) is small (~10 300 m² surface area), shallow (15 m), holomictic sea-water lake (Fig. S1) located between high limestone cliffs on the Dalmatian Peninsula *Gradina*, at about 100 m distance to the Adriatic Sea. No riverine input and no surface connection to the open sea is present, and water is exchanged with the Adriatic by subsurface circulation through the underlying porous karst (Žic and Branica, 2006).

In order to investigate the microbial community of Lake Rogoznica and its relation to chemical settings, the lake was sampled eight times between June 2011 and May 2012. During each sampling campaign, water from six to eight depths (surface [~30-50cm], 5 m, 7 m, 9 m, 10 m, 11 m, 12 m and 13 m; Table S1) was collected with horizontally lowered, 5 L Niskin bottles (General Oceanics, USA) and divided into samples for microbiological and chemical analysis. Supplementary samples for further

analyses of PSB cells were obtained on the 30th of September 2013 from 10 m water depth.

Chemical and hydrographical data

RSS concentrations were determined by cathodic stripping linear sweep voltammetry (LSV) on unfiltered water samples (Ciglenečki *et al*., 1997; Bura-Nakić *et al*., 2009 and references therein). Particulate organic carbon (POC, size fraction >0.7 µm) and dissolved organic carbon (DOC, size fraction <0.7 µm) concentrations were determined by a high temperature, catalytic oxidation method (HTCO; Dafner and Wangersky, 2002) with the TOC-VCPH-5000 solid sample TOC analyzer (Shimadzu, Japan) coupled to the solid sample combustion unit SSM-5000A (Shimadzu, Japan), according to protocols published in Penezić *et al*. (2010) and Dautović *et al*. (2012). Dissolved oxygen concentrations (O_2) and hydrographical data, including temperature (T), salinity (S) and pH, were recorded with the HQ40D multimeter probe (Hach Lange, Germany) in parallel to water sampling.

Total cell counts and CARD-FISH analyses

Samples designated for microscopic analyses were fixed by addition of formaldehyde solution (final concentration 2%) and subsequent incubation at 4°C for up to 12 h. After fixation, sample aliquots of 10 to 100 ml were brought on polycarbonate membrane filters (pore size 0.2 µm, type GTTP; Millipore, Germany), air dried and stored at -20°C. Total cell counts (TCC) were determined by epifluorescence microscopy on agarose embedded filter sections after 4',6-diamidino-2-phenylindole (DAPI) staining, while abundances of selected microbial taxa were determined by CARD-FISH on agarose embedded filters as described in Perntahler *et al*. (2002). Applied oligonucleotide probes are listed in Table S2.

DNA isolation

For DNA based microbial community analysis, 120 ml lake water were brought on polycarbonate filter membranes (0.2 µm pore size, Whatman, UK) immediately after sampling. Filters were stored at -80°C in ~1 ml sucrose buffer (40 mmol l^{-1} ethylenediaminetetraacetic acid [EDTA], 50 mmol l^{-1} Tris-HCl and 0.75 mol l^{-1} sucrose) in 2.2 ml sample tubes (Eppendorf, Germany). DNA was extracted according to a phenol/chloroform procedure published in Massana *et al*. (1997). Briefly, filters were

treated with lysozyme (1 mg ml^{-1}) at 37°C for 45 min, and proteinase K (0.2 mg ml^{-1}) and SDS (1%) at 55°C for 1 h. Extraction was performed twice with 750 µl of phenol : chloroform ($CHCl_3$): isoamyl alcohol (IAA) (25 : 24 : 1, pH 8) solution and once with 750 µl of $CHCl_3$: IAA (24 : 1) solution. Finally, 1/10 volume of sodium-acetate was added to the aqueous phase and DNA was precipitated for 30 min at -20°C with 1 ml of isopropanol. After a centrifugation step (20 min at 4°C and 20,000 g) the DNA pellet was washed with 500 µl of 70% ethanol. After a second centrifugation step (5 min at 4°C and 20,000 g) the pellet was dissolved in 80 µl of deionized (MQ) water.

16S rRNA gene clone libraries

For an initial community diversity analysis, bacterial 16S rRNA genes were amplified from isolated DNA for samples from three to six water depths retrieved on the 20th of June 2011, the 9th of October 2011 and the 8th of May 2012 with *Bacteria*-specific primers GM3F and GM4R (Muyzer *et al*., 1995, Table S3). For every sample, four PCR reactions containing 1x Green GoTaq® Flexi Buffer (Promega, USA), 1.5 mmol l^{-1} $MgCl_2$, 200 µmol l^{-1} of each dNTP, 0.2 µmol l^{-1} of each primer, 0.625 U of GoTaq® DNA Polymerase (Promega, USA) and approximately 25 ng of DNA template were performed. After an initial denaturation at 95°C for 5 min the samples were amplified for 30 cycles (denaturation at 95°C for 30 s, annealing at 48°C for 1.5 min, elongation at 72°C for 2 min) followed by a final elongation at 72°C for 10 min. Replicate PCR products were pooled and purified with the PureLink PCR Purification Kit (Invitrogen, USA) according to the manufacturer's instructions and eluted in 30 µl MQ water. The purified PCR products were cloned using the pGEM®-T Vector System (Promega, USA) or TA Cloning® Kit (Invitrogen, USA) and Subcloning Efficiency™ DH5α Competent Cells according to the manufacturers' instructions. Selected clones were sent for commercial Sanger sequencing (Macrogen, the Netherlands).

Retrieved sequences were quality clipped with the Sequencher Version 4.6.1. Software package (GeneCodes Corporation, USA) and phylogenetic analysis was performed with the ARB software package (Ludwig *et al*., 2004). Sequences were aligned against the SILVA 16S rRNA SSU reference database release 115 with the SINA (SILVA Incremental) aligner (Pruesse *et al*., 2012). Nearly-full-length 16S rRNA gene sequences from this study and reference sequences from the SILVA database were used for phylogenetic reconstruction with the ARB RaxML maximum-likelihood method (Stamatakis *et al*., 2008). To the generated tree, partial 16S rRNA gene

sequences were added using maximum-parsimony criteria, while disabling changes to the general tree topology. Sequences were grouped in operational taxonomic units (OTUs) based on 98% sequence identity (SI).

16S rRNA gene 454-pyrosequencing

To obtain a more detailed picture of 16S rRNA diversity, DNA samples from three water depths (surface, 10 m, 13 m) of three sampling dates (20th of June 2011, 9th of October 2011, 8th of May 2012) were sent to the MR DNA Molecular research lab (Texas, USA) for amplification and 454-pyrosequencing of bacterial 16S rRNA genes. Tag-encoded FLX amplicon pyrosequencing (bTEFAP) was performed as described in Dowd *et al*. (2008). Briefly, bacterial 16S rRNA genes were amplified with modified primers 28F (Lane, 1991) and 519R (Turner, 1999; Table S3) in a single-step PCR using HotStarTaq Plus Master Mix Kit (Qiagen, USA). Primer 28F was extended to contain sample specific barcodes, so amplicons from multiple samples could be sequenced in one run. Amplification was performed under following conditions: a 5 min denaturation step at 95°C, followed by 30 cycles of 40 sec denaturation at 94°C, 40 sec annealing at 53°C and 1 min elongation at 70°C, finalized by a 10 min elongation step at 70°C. Following PCR, amplicons from all samples were mixed in equal concentrations and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, USA) and sequenced with the Roche 454 FLX titanium system.

Retrieved 454-pyrosequencing reads were adapter and quality clipped with the 'sff_extract -c' option of the COMAV Bioinformatics toll sff_extract (http://bioinf.comav.upv.es/sff_extract). Thereafter, sequences were analyzed with the Next Generation Sequencing pipeline of the SILVA Database project (Quast *et al*., 2013). Briefly, sequences were quality checked, excluding all reads containing >2% ambiguities or homopolymers and reads shorter than 200 base pairs. Remaining reads were aligned against the SILVA SSU SEED alignment using the SILVA Incremental Aligner - SINA (Pruesse *et al*., 2012). Reads with an alignment identity <50% were excluded from further analysis, while remaining reads were de-replicated, clustered in OTUs at 98% SI with Cd-hit (Li and Godzik, 2006; Fig. S2) and assigned to a taxonomic path based on a local nucleotide BLAST (Camecho *et al*., 2009) search against the SILVA 16S rRNA SSU reference database, release 111. Analysis statistics are provided in Table S4.

Sorting and identification of PSB cells

For a definite phylogenetic identification of PSB cells, an additional sample from Lake Rogoznicas' chemocline was collected on the 30th of September 2013. The sample was preserved with glycerol (12% final concentration) and stored at -20°C. For analysis, 500 µl of the glycerol-fixed sample was diluted with sterile-filtered lake water (1 : 1) and DAPI was added to this solution to a final concentration of 1 µg ml^{-1}. From this sample, cells were sorted with a MoFlo flow cytometer (Beckman Coulter GmbH, Germany) equipped with two argon ion lasers. To ensure sterile conditions, the sample line was rinsed with ~1% bleach in autoclaved MQ water prior to sorting. As sheet fluid, an autoclaved, sterile filtered (0.2 µm and 0.1 µm pore size in-line filter cartridge; Sartorius Stedim, Germany) 0.075% NaCl solution was used. A nozzle with an orifice diameter of 70 µm was used. Preceding measurements, the instrument was calibrated with Fluoresbrite^{TM} multifluorescent 1 µm polystyrene beads (Polysciences, USA). During cell sorting, one laser was used for the detection of side angle light scatter (SSC) and autofluorescence at a wavelength of 488 nm (400 mW). SSC was detected trough a 488/6 nm band pass filter, while autofluorescence was detected by 530/40 nm, 600/120 nm and 670/40 nm band pass filters. The second laser was tuned to multiple line UV output (86 mW) for the simultaneously detection of DAPI signal with a 450/50 nm band pass filter. Sorting was performed at 98.200 Hz at amplitude of 11.04 V and a drop delay of 39 8/16 droplets. Sort control, online and past analysis were performed with the Summit software, version 4.3 (Beckman Coulter GmbH, Germany). Based on bivariate dot plot analyses of SSC (488/6) vs. autofluorescence (530/40) and SCC (488/10) vs. DAPI signal (450/50), objects with high SCC signal and high autofluorescence were selected and sorted out, under exclusion of events with low DAPI signal. The number of sorted objects was 1000 and 5000 events per tube in 10 replicates each.

Sorted cells were used for microscopy and 16S rRNA gene amplification and sequencing. Briefly, for 16S rRNA gene amplification three replicate PCR reactions, containing ~100 sorted cells each, were prepared. PCR reactions and amplification conditions were as described above for 16S rRNA gene clone libraries. The purified PCR product (PureLink PCR Purification Kit; Invitrogen, USA) was used as direct template for Sanger-sequencing with a Sequencer 3130xl Genetic Analyzer (Applied Biosystems, Foster City, USA). Obtained sequences were quality-checked and a consensus sequence was generated with the Sequencher Version 4.6.1. software package (GeneCodes Corporation, USA). For microscopy, 10 µl containing ~5000

sorted cells were spotted on a polycarbonate membrane filter (0.2 µm pore size, Whatman, UK) and visualized under phase-contrast on an AxioSkop 2 mot plus epifluorescence microscope (Carl Zeiss, Germany). Images were taken with an AxioCam MRm camera (Carl Zeiss, Germany) and processed with the Axio Vision 4.7. software (Carl Zeiss, Germany).

soxB *gene diversity*

Diversity of the sulfur-oxidation marker gene *soxB* encoding for sulfate thiohydrolase was investigated by functional gene amplification, cloning and sequencing. *soxB* gene sequences were successfully obtained for surface samples from the 20th June 2011 and the 9th of October 2011, and a 10 m sample from the 9th of October 2011. For each sample, amplification was performed in triplicate reactions using gene-specific primers soxB432F and soxB1446R (Petri *et al*., 2001, Table S3). Each 20 µl PCR reaction contained 1x *Taq* PCR buffer, 2 µl of 3 mg ml^{-1} BSA, 320 µM of each dNTP, 2 µM of each primer, 0.04 U of *Taq* polymerase (Eppendorf, Germany) and 1 µl of DNA template. A two-step PCR with in total 35 amplification cycles, as described in Petri *et al*. (2001), was performed. Replicates were pooled and purified by excision of desired bands (~1000 bp) from a SYBR Green I DNA stain (Invitrogen, Germany) stained 1% agarose gel. Gel-purified PCR products were cloned with the TOPO-TA cloning kit (Invitrogen, Germany) as instructed by the manufacturer and vector inserts were Sanger-sequenced using a Sequencer 3130xl Genetic Analyzer (Applied Biosystems, USA). Again, the Sequencher Version 4.6.1. software package was used for quality clipping and generation of consensus sequences.

About 500 publically available full-length *soxB* gene sequences from isolates and environmental samples were aligned with the Fourier transformation based multiple sequence alignment tool MAFFT v6.864 (Katoh *et al*., 2002; www.ebi.ac.uk/Tools/msa/mafft/) and used for reconstruction of a phylogenetic guide-tree with the ARB RaxML maximum-likelihood method (Stamatakis *et al*., 2008). Sequences generated in this study were added to the guide try by maximum parsimony criteria, without allowing changes to overall tree topology. *soxB* gene nucleotide sequences were grouped in OTUs based on a 94% similarity cut-off, as inferred from previous studies on *soxB* gene sequence divergence in closely related strains (Petri *et al*., 2001; Meyer *et al*., 2007).

Results

Chemical and hydrographical characteristics of Lake Rogoznica

We recorded basic hydrographic parameters (salinity, temperature, pH), DOC, POC, RSS and O_2 concentrations in the water column of Lake Rogoznica during eight microbiological samplings between June 2011 and May 2012. Hydrographical profiles, as well as dissolved O_2, RSS and organic carbon concentration data (Fig. 1, Table S5) were well in line with previously reported values from respective seasons in the 1990's and 2000's (Ciglenečki *et al*., 2005; Bura-Nakić *et al*., 2009; Kamyshny *et al*., 2011). Water column stratification was stable throughout spring and summer. The annual mixing, resulting in the formation of a chemically homogeneous water column, took place in early October 2011 (Fig. 1). The summer of 2011 was characterized by little rainfall and unusually high temperatures, which led to the occurrence of an almost total anoxia after the lakes' perturbation. Micro-oxic conditions prevailed at the lakes surface (~1 m), while oxygen concentrations were close to zero in the remaining water column (Fig. 1). Anoxic holomixis lasted until mid-November, when stratification reestablished and prevailed until the end of our sampling cycle in May 2012 (Fig. 1).

During stratified periods, RSS concentrations were highest in the hypolimnion, with the highest recorded concentration of 3.75 mmol l^{-1} (1^{st} of September 2011 in 13 m depth, Table S5). Furthermore, RSS concentrations were higher during summer then during winter and spring months (Table S5). The depth of the chemocline, where both RSS and O_2 concentrations approach zero, migrated with changing seasons and was located closer to the lakes' bottom (below 8 m) during summer months (June 2011 – September 2011, May 2012), while it reestablished rather shallow (4 m) after holomixis in autumn and remained at lower depths (4-7 m) during winter and spring (November 2011 – May 2012). Above the chemocline, RSS concentrations were low or absent. O_2 concentrations increased towards the water surface, where O_2 saturation was at or above 100% during all times (data not shown), except during total anoxia in October 2011. DOC and POC concentrations were in general higher below than above the chemocline, with POC peaking at 0.5-1 m below the chemocline depth (Fig. 1, Table S5).

During total anoxia, RSS concentrations were low (0.02-0.40 µmol l^{-1}), but RSS was present in the entire water column. Oxygen was almost depleted (0.02-0.13 mg l^{-1}) in the entire water column (Fig. 1). DOC and POC concentrations were constant throughout all depths.

Abundance of microbial cells in Lake Rogoznica

To determine the total number of microbial cells (TCC) in different depth layers of Lake Rogoznica, DAPI signals in water samples on polycarbonate membrane filters were quantified. During stratification, TCC ranged between 2 to 9 x 10^6 cells ml^{-1}. Generally, TCC was lowest at the lakes' surface and increased with depth, peaking below the chemocline, along with POC concentration (Fig. 1, Table S5). During total anoxia, TCC was high (6.6-7.9 x 10^6 cells ml^{-1}) and comparable at all water depths (Fig. 1).

16S rRNA gene diversity in Lake Rogoznica

To obtain an insight into the microbial diversity of Lake Rogoznica, we constructed bacterial 16S rRNA gene clone libraries from water samples retrieved at several depths on the 20th of June 2011, the 9th of October 2011 (anoxic holomixis) and the 8th of May 2012. A total of 374 sequences from 15 samples were retrieved (Table S6).

Sequences retrieved from epilimnion samples (0-7 m depth on the 20th of June 2011 and the 8th of May 2012) dominantly affiliated with SAR11 clade-related *Alphaproteobacteria* (33% and 56%, respectively). Other abundant sequence groups were related to other *Alphaproteobacteria* (up to 22%), *Flavobacteriaceae*-related *Bacteroidetes* (up to 24%) and *Cyanobacteria* (up to 20%; Fig. 2). At and below the chemocline, sequences related to uncultured GSB (phylum *Chlorobi*) were most numerous in samples from both the 20th of June 2011 and the 8th of May 2012 (up to 97%; Fig. 2). Phylogenetic reconstruction revealed that the majority of GSB sequences (114 out of 118) were closely related to *Prosthecochloris vibrioformis* (87-96% SI; Fig. S3). In discrepancy to previous reports describing PSB (*Chromatiales*) as dominant phototrophic sulfur oxidizers in Lake Rogoznica (Ciglenečki *et al*., 1998), only three *Chromatiales*-related sequences were obtained (Fig. 2). They formed a single OTU closely related to *Halochromatium roseum* (99-100% SI; Kumar *et al*., 2007).

The majority of 16S rRNA gene sequences from samples collected during anoxic holomixis on the 9th of October 2011 were closely related to SUP05 clade *Gammaproteobacteria* (86%). The majority of remaining sequences (10%) affiliated with *Arcobacter*-related *Epsilonproteobacteria* and *Gammaproteobacteria* within the *Candidatus* Thiobios clade (Fig. 2, Fig. S3).

To deepen our insights on the bacterial diversity in Lake Rogoznica, 454-pyrosequencing of representative samples from the 20th of June 2011, the 9th of October 2011 and 8th of May 2012 was performed. The additional 154,152 16S rRNA gene

sequences confirmed and extended observations from our clone library results (Fig. 3). Sequences affiliated with SAR11 and *Roseobacter* clade *Alphaproteobacteria* alongside with *Synechococcus*-related *Cyanobacteria* and diverse *Bacteroidetes* were most frequently retrieved in surface samples from the 20th of June 2011 and the 8th of May 2012 (Fig. 3).

GSB (*Chlorobi*) accounted for up to 96% of 454-pyrotag sequences at the chemocline (Fig. 3), while sequences related to PBS in the gammaproteobacterial order *Chromatiales* represented only 0.3% of total sequence diversity in this layer. In hypolimnion waters *Chlorobi* remained the most frequently retrieved sequence type in the sample from the 8th of May 2012, while diversity was higher in the sample from the 20th of June 2011 (Fig. S2). Here, many sequences affiliated with SAR11 clade *Alphaproteobacteria* (29%), thiotrophic *Epsilonproteobacteria* (*Arcobacter, Sulfurimonas, Sulfurovum*; 16%), and diverse sulfate- and sulfur-reducing clades among the *Deltaproteobacteria* (2%). Most of the deltaproteobacterial sequences were related to different members of the family *Desulfobacteraceae*, while only few sequences related to the *Desulfobulbaceae* and *Desulfovibrionaceae.*

In samples from the 9th of October 2011, SUP05 clade *Gammaproteobacteria* made up the majority of retrieved 454-pyrotag sequences (57-87%, Fig. 3). The remaining sequences affiliated mostly with SAR11 and *Roseobacter* clade *Alphaproteobacteria* (14-17%), *Cyanobacteria* (3-4%), *Epsilonproteobacteria* (4%) and *Bacteroidetes* (3-4%; Fig. 4).

CARD-FISH analyses of bacterial abundance in Lake Rogoznica

After identifying the dominant bacterial taxa in representative water layers and seasons in Lake Rogoznica by 16S rRNA gene sequencing, we quantified these microorganisms in circa-monthly samples for the sampling period between June 2011 and May 2012 using CARD-FISH (Fig. 5, Table S7).

CARD-FISH results were well in line with retrieved 16S rRNA gene sequence data for respective dates (20th of June 2011, 9th of October 2011 and 8th of May 2012, Fig. 4), and showed the same trends in respect to water column chemistry for the remaining sampling period (Table S5 and Table S7). Overall, SAR11 and *Roseobacter* clade *Alphaproteobacteria* represented the largest fraction of cells in epilimnion waters throughout stratification (up to 54% off all cells, 8th of May 2012, Table S7), followed in abundance by *Bacteroidetes* (up to 16%) and non-PSB *Gammaproteobacteria* (up to

14%, Table S7). In the hypolimnion, the number of alpha- and gammaproteobacterial cells was generally lower (Table S7). Furthermore, *Cyanobacteria* were abundant in epilimnion waters during spring and summer months (March – September), where they accounted for 7-24% of all cells (Fig. 5), while almost no cyanobacterial cells were detected during winter (Table S7).

In anoxic waters at and below the chemocline, GSB (*Chlorobi*) accounted for the great majority (up to 89%) of detected cells, while they were always absent above the chemocline (Fig. 5, Table S7). Their small, rod-shaped cells formed long non-branching chains, as observed for many *Chlorobi* and *Prosthecochloris* species (Overmann, 2006). PSB were comparatively few in numbers (1-3%, Fig. 5, Table S7). However, their individual, spherical cells were significantly larger (~5 µm diameter, Fig. S4) then the abundant rod shaped GSB cells (~3 µm length and ~1 µm width). In consequence, PSB accounted for up to 20% of the biovolume in some samples (data non show). At and below the chemocline *Deltaproteobacteria* were next in abundance (1-12%) to the GSB. In contrast to 16S rRNA gene sequence data, only few *Epsilonproteobacteria* were detected by CARD-FISH in hypolimnion layers. In fact, only in November and December 2011 elevated numbers of epsilonproteobacterial cells were detected (up to 7%, Table S7).

During the anoxic event in October 2011, the distribution of different taxa was uniform throughout all water depths (Fig. 4), which is consistent with the chemically homogenous water column (Fig. 1). Numbers of *Cyanobacteria*, *Deltaproteobacteria*, *Betaproteobacteria*, GSB and PSB cells decreased drastically in comparison to their previous abundances in either water layer during stratification. Numbers of SAR11 and *Roseobacter* clade *Alphaproteobacteria* remained comparable to previous epilimnion abundances, but were evenly distributed in the entire water column. The abundance of *Bacteroidetes* changed only slightly in comparison to stratified conditions (Fig. 4). An increase in cell abundance and total cell numbers throughout the water column was only observed for the non-PSB *Gammaproteobacteria,* which accounted for 19-29% of all cells identified by CARD-FISH.

As SUP05 clade *Gammaproteobacteria* related sequence accounted for a significantly higher fraction of the microbial diversity in 16S RNA gene clone libraries and 454-pyrotags, then indicated by gammaproteobacterial CARD-FISH counts, hybridization with several previously published SUP05 specific oligonucleotide probes was attempted. First, hybridization with the GSO477 oligonucleotide probe (Lavik *et al*.,

2009) was tested. *In silico* analysis showed that this probe fully matched our SUP05 sequences. However, we did not obtain any signals with the GSO477 probe. Thereafter, a probe published by Marshall and Morris (2012) as GSO1448R was tested. No *in silico* analysis could be performed, as the 16S rRNA gene sequences retrieved in this study did not span the target region of this oligonucleotide probe. Hybridization with the GSO1448 probe was detected in samples from the 9th of October and the 7th of November 2011, but the number of hybridized cell did not exceed 2% of TCC (data not shown). Finally, a new probe targeting only SUP05 16S rRNA gene sequences obtained in this study was designed and test on environmental samples. However, the new GSO183 oligonucleotide probe (5'-TCTCCATGGAGTAAAGAGGG-3') also thus far failed to produce hybridization.

Phylogenetic identification and microscopic investigation of PSB cells

As only few PSB 16S rRNA sequences were retrieved in 16S rRNA gene libraries and 454-pyrotag sequences, but the large cells comprised a significant fraction of the biovolume in anoxic waters, a definite phylogenetic identification was necessary. Therefore, we amplified and sequenced the 16S rRNA gene from sorted PSB cells collected during a sampling trip on the 30th of September 2013. The obtained consensus 16S rRNA gene sequence was over 99% identical to sequences from our clone libraries and to the type strain *Halochromatium roseum* (Fig. S3). Unlike in the species description of *Halochromatium resoum*, no cell chains were observed in our samples and individual cells appeared more spherical than rod shaped. Furthermore, a large number of not further identified inclusions were visible in the sorted PSB cells (Fig. S4).

soxB *gene diversity*

To further investigate sulfur-oxidizing microorganism in Lake Rogoznica, we constructed *soxB* gene clone libraries from an epilimnion sample (20th of June 2011, surface sample) and two anoxic holomixis samples (9th of October 2011, surface and 10m). In total, 266 soxB gene sequences were retrieved and grouped into 27 OTUs based on a 94% sequence identity cut-off.

58% of the *soxB* gene sequences retrieved from holomixis samples were closely related to sequences from uncultured SUP05 clade *Gammaproteobacteria* and the sulfur-oxidizing clamp endosymbiont *Candidatus* Vesicomyosocius okutanii (Fig. S5). None of the *soxB* gene sequences retrieved from the epilimnion sample from June 2011

affiliated with this group. The majority of remaining sequences from October 2011 and almost all (95%) of the sequences from June 2011 affiliated with diverse uncultured and cultured *Alphaproteobacteria*, mainly within environmental sequences of *Roseobacter* clade relatives retrieved from a sulfidic coastal sediment (Lenk *et al*., 2012; Fig. S5). The only sequences not related to either of these two groups clustered with sequences of halophilic and alkaliphilic *Thioalkalivibrio*-relatives (Fig. S5). No GSB or PSB related *soxB* gene sequences were recovered, likely due to the absence of a chemocline sample in our clone libraries. Furthermore, it is important to note that the lack of epsilonproteobacterial *soxB* gene sequences might related to a primer mismatch at the 3' prime end, which significantly affect PCR efficiency (Meyer *et al*., 2007).

Discussion

Microbial diversity of Lake Rogoznica

Patterns of microbial diversity in Lake Rogoznica and changes in hydrographical and chemical conditions of the stratified water column are coinciding throughout the whole sampling period. Microbial community composition was tightly coupled to oxygen and sulfide concentration, but also light availability. Light penetration and oxygen consumption in the epilimnion were probably most crucial in determining the chemocline depth. Overall, the microbial diversity in Lake Rogoznica can be divided in epilimnion, chemocline and hypolimnion populations.

As hypothesized, microbial diversity in the epilimnion resembled the diversity of the central Adriatic Sea, with *Cyanobacteria*, *Alphaproteobacteria*, *Gammaproteobacteria* and *Bacteroidetes* as most abundant bacterial groups (Manti *et al*., 2012, Šantić *et al*., 2013). *Cyanobacteria* likely contributed to the high oxygen saturation levels in surface waters during spring and summer months (Casamayor *et al*., 2000; Šestanović *et al*., 2005) and caused the increase in chemocline depth during these seasons.

In accordance with the high salinity in Lake Rogoznica, the anoxygenic phototrophic sulfur-oxidizing populations in chemocline layers were dominated by halotolerant marine species (*Prosthecochloris* and *Halochromatium*; Imhoff, 2003; Kumar *et al*., 2007). However, in contrast to previous reports (Ciglenečki *et al*., 1998), the dominant anoxygenic phototrophs were not PSB, but GSB. This might be the consequence of a previous shift from a PSB to a GSB dominated community, like observed in recent years for Lago di Cadagno (Tonolla *et al*., 2005). Yet, it is also

possible that the previous description based on microscopy and pigmentation (Krstulović and Šolić, 2001) was biased towards the ~30 times larger cells of the *Halochromatium*-related PSB. Regardless, taking in account the high sulfide concentrations in anoxic water layers (up to 3.75 mmol l^{-1}), dominance of the more sulfide-tolerant GSB is feasible (Overmann, 2006).

The increase in abundance of deltaproteobacterial populations below the chemocline supports the previously postulated sulfate-reducing character of the microbial community in Lake Rogoznicas' hypolimnion (Kamyshny *et al*., 2011). The recovery of sequences from epilimnion-associated populations, like the SAR11 clade, in hypolimnion samples indicates that export of cells and organic matter from the lakes surface to bottom waters is taking place. The input of organic matter form the surface likely feeds the organotrophic sulfate-reducing community in Lake Rogoznicas' anoxic hypolimnion and sediments.

Anoxic holomixis in October 2011 lead to a complete collapse of the anoxygenic phototrophic sulfur-oxidizing community, although sulfide and S^0 were available in the entire water column. In parallel, we recorded an increased abundance of SUP05 clade *Gammaproteobacteria*, which were not detected in any water layer during stratification. These organisms formed a short-lasting bloom during anoxic holomixis, occupying the sulfur-oxidation niche exempted by the decline in numbers of anoxygenic phototrophs.

Microbial sulfur cycling in Lake Rogoznica

Photo- and chemotrophic sulfur-oxidation, together with microbial sulfate-reduction are the predominant microbial processes in Lake Rogoznica. Our results on the abundance and diversity of *Deltaproteobacteria* in hypolimnion waters support findings by Kamyshny and colleagues (2011) which suggest microbial sulfate-reduction as sole source of sulfide in Lake Rogoznica. The high concentrations of RSS and organic matter imply that sulfur-compound disproportionation is not a feasible alternative sulfide source (Thamdrup *et al*., 2003; Kamyshny *et al*., 2011). The low number of sequences related to known sulfur-disproportioning *Deltaproteobacteria* among the *Desulfobulbaceae* and *Desulfovibrionaceae* are in line with this observation. Furthermore, RSS concentrations were much higher in summer then in winter, indicating higher sulfate reduction rates in summer. This is likely related to the higher water temperature and higher organic matter content in hypolimnion waters during summer (Table S5).

The availability of high amounts of sulfide, oxygen and light within Lake Rogoznica support the development of a large and diverse community of sulfur-oxidizing microorganisms. Phototrophic sulfur oxidation in depths below the oxygen penetration is in all probability the dominant metabolism during stratified periods. Both GSB and PSB were detected at and below the chemocline. The almost complete disappearance of sulfide over a narrow chemocline depth indicates that the anoxygenic phototrophs were highly active. The GSB dominated in numbers and total biovolume. However, single *Halochromatium*-related PSB cells were significantly larger and packed with storage vesicles, indicating high activity (Fig. S4). The vesicles might contain S^0 or PHA, as closely related *Halochromatium* strains store these compounds in intracellularlly (Caumette *et al*., 1988; Kumar *et al*., 2007). It is known from other studies that even small populations of anoxygenic phototrophs can account for a significant part, if not the majority of phototrophic CO_2-fixation in stratified lakes (Camacho *et al*., 2001; Musat *et al*., 2008). Therefore, no conclusions on the relative contribution of GSB versus PSB to overall sulfide-oxidation or carbon-fixation can be made based solely on their abundance and biovolume.

Although phototrophic sulfur oxidizers were more abundant during stratified conditions, chemotrophic sulfur-oxidation is likely occurring above the chemocline and probably also alongside anoxygenic phototrophic processes at the chemocline. The detection of *Epsilonproteobacteria* by both 16S rRNA sequences and CARD-FISH at and below chemocline depths supports this hypothesis. Furthermore, the *Roseobacter* clade *Alphaproteobacteria*, abundant in the epilimnion, were most probably also involved in the transformation of sulfur compounds, as indicated by the abundance of related soxB genes and the fact that members of the *Roseobacter* clade can utilize both inorganic and organic sulfur compounds (Buchan *et al*., 2005).

After the lakes' perturbation and during anoxic holomixis, anoxygenic phototrophs disappeared and the chemotrophic SUP05 clade *Gammaproteobacteria* became the dominant thiotrophic clade. Such drastic community shifts during holomixis have been reported for other stratified lakes (Cohen *et al*., 1977; Casamayor *et al*., 2000). However, as most lakes are oxygenated during holomixis, usually an epilimnion-like community was observed (Cohen *et al*., 1977; Camacho *et al*., 2000 Hollibaugh *et al*., 2001). In another karst lake (Lake Cìso, Spain; Pédros-Alìo and Guerrero, 1993) during anoxic holomictic events anoxygenic phototrophs become abundant in the entire water column, like reported for Lake Rogoznica during anoxic holomixis in 1997

(Krstulović and Šolić, 2001). Our findings differ significantly from previous report and point to the dominance of a chemotrophic sulfur-oxidizing community during anoxic holomixis.

Community shift from phototrophic to chemotrophic sulfur oxidation during anoxic holomixis

Members of the SUP05 clade were previously reported to be involved in chemotrophic sulfur oxidation in diverse marine systems with similar chemical conditions as those recorded in Lake Rogoznica during anoxic holomixis in October 2011. Such systems include anoxic fjords and inlets (Schmidtova *et al*., 2009; Zaikova *et al*., 2010), pelagic redoxiclines and oxygen minimum zones (Stevens and Ulloa, 2008; Lavik *et al*., 2009; Glaubitz *et al*., 2013), hydrothermal plumes (Sunamura *et al*., 2004), low-temperature hydrothermal fluids (Kato *et al*., 2009) and Antarctic lakes (Wilkins *et al*., 2013). However, this is the first report on the abundance of this marine clade in a shallow stratified sea-water lake from a temperate region.

A discrepancy between 16S rRNA gene sequence data, were SUP05 clade related sequences encompass almost the entire diversity, and CARD-FISH results, where only 20% of the microbial cells were identified as *Gammaproteobacteria*, is evident. As previously reported by Glaubitz and colleagues (2013), it is likely that the phylum specific 23S rRNA targeting oligonucleotide probe Gam42a does not hybridize with all members of the SUP05 cluster. Two central mismatches at the target position of the Gam42a probewere detected in a reconstructed 23S rRNA gene sequence from a SUP05 metagenome and the 23S rRNA gene sequence from the closely related endosymbiotic bacterium *Candidatus* Ruthia magnifica. However, we did not manage to increase detection with any of the three tested SUP05 specific oligonucleotide probes GSO477, GSO1448R and GSO183. Problems with hybridization of the GSO477 probe in samples with *in silico* matching sequences were reported before (Glaubitz *et al*., 2013). Therefore we excluded the GSO477 probe from further experiments after first hybridizations failed. The low numbers of cells hybridizing with probe GSO1448R are possibly caused by a target sequence mismatch, as the terminal target position of this probe on the 16S rRNA gene did not allow for *in silico* comparison to the partial and almost full-lenght sequences retrieved in our study. As in silico evaluation showed that further available SUP05 specific probes (Glaubitz *et al*., 2013) do not target the Lake Rogoznica SUP05 cluster, further optimization of the newly designed GSO183 probe,

including design of helper oligonucleotides (Fuchs *et al*., 2000), will be necessary in order to resolve the *in situ* detection deficiency within the sulfur-oxidizing gammaproteobacterial population of Lake Rogoznica.

Finally, the factors favoring the sudden bloom of SUP05 clade sulfur-oxidizers in Lake Rogoznica and causing the disappearance of anoxygenic sulfur-oxidizing phototrophs during anoxic holomixis are not entirely clear. It is possible that the low sulfide concentrations remaining available after holomixis were not sufficient to sustain the large GSB and PSB populations. Alternatively, oxygen that penetrated into hypolimnion layers during perturbation might have had a toxic effect on the anoxygenic phototrophs, in particular the oxygen-sensitive GSBs (Overmann, 2006). In this case, various chemotrophs could have profited from the disappearance of GSB and PSB. A higher affinity to low-sulfide concentration might have been the key advantage for SUP05 clade *Gammaproteobacteria* in the competition with *Epsilonproteobacteria*, which are more common in high-sulfide/high-flux environments (Macalady *et al*., 2008; Grünke *et al*., 2011). Furthermore, the SUP05 clade has a high metabolic versatility, including utilization of multiple electron donors and perhaps even organic carbon sources (Swan *et al*., 2011; Marshall and Morris, 2012). Taking in account the high DOC and POC concentrations in Lake Rogoznica, the capability of organic carbon assimilation could have served as competitive advantage to members of the SUP05 clade, as well as to members of the heterotrophic alphaproteobacterial *Roseobacter* clade, which were the two dominant chemotrophic sulfur-oxidizing clades during anoxic holomixis.

Conclusion and Outlook

This study represents the first comprehensive report on microbial diversity in Lake Rogoznica and includes the identification of microbial key-player during stratified and anoxic holomictic conditions. The microbial communities in Lake Rogoznica strongly influence water column chemistry and vice versa. The microorganisms are largely responsible for both oxygen and sulfide production and consumption. During stratified periods, the abundance and activity of oxygenic phototrophs probably determines the chemocline depth, while organic matter export and sulfate reduction determine the hypolimnic sulfide concentrations. However, after a physical mixing event, the sudden change of environmental conditions and water chemistry lead to the temporary collapse of anoxygenic phototrophic sulfur-oxidizing communities. The detected response to

anoxic holomixis, displayed in a shift from dominantly phototrophic sulfur oxidation to almost exclusively chemotrophic sulfur oxidation and a bloom of SUP05 clade *Gammaproteobacteria* is a thus far unique event for a stratified lake environment. The similar composition of Lake Rogoznicas' holomictic sulfur-oxidizing community and sulfur-oxidizing assemblages in oxygen deficient pelagic waters and mildly sulfidic hydrothermal environments underlines the habitat-independent community structuring effect of sulfide and oxygen concentrations. Further investigations should monitor the frequency of such community shifts during holomixis in Lake Rogoznica and try to resolve the conditions and mechanism allowing the SUP05 clade to outcompete other chemo- and phototrophic sulfur-oxidizers after anoxic perturbations in more detail.

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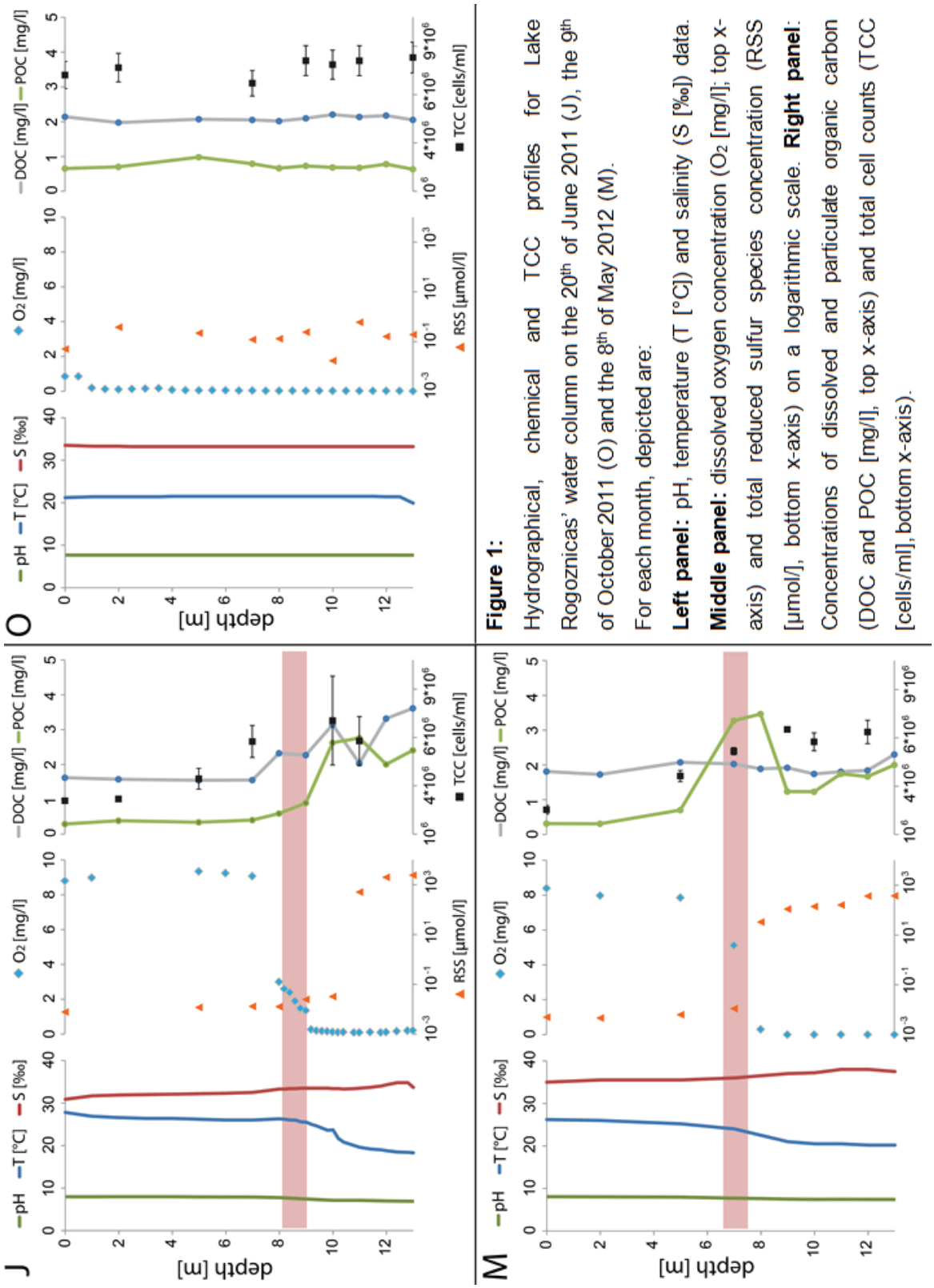


Figure 1:
Hydrographical, chemical and TCC profiles for Lake Rogoznicas' water column on the 20th of June 2011 (J), the 9th of October 2011 (O) and the 8th of May 2012 (M).
For each month, depicted are:
Left panel: pH, temperature (T [°C]) and salinity (S [‰]) data.
Middle panel: dissolved oxygen concentration (O_2 [mg/l]; top x-axis) and total reduced sulfur species concentration (RSS [µmol/], bottom x-axis) on a logarithmic scale. **Right panel**: Concentrations of dissolved and particulate organic carbon (DOC and POC [mg/l], top x-axis) and total cell counts (TCC [cells/ml], bottom x-axis).

Figure 2: Comparison of bacterial diversity in 16S rRNA gene clone libraries from different water layers sampled June 2011, October 2011 and May 2012.

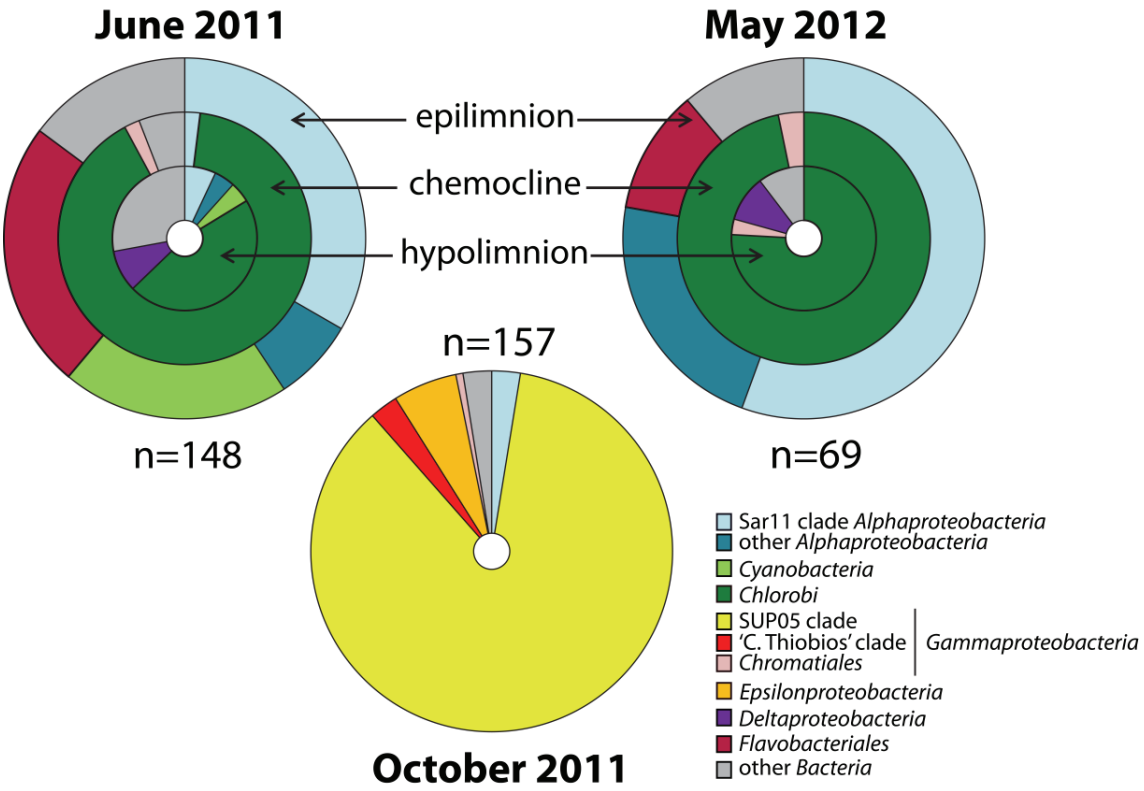


Figure 3: Diversity of 16S rRNA gene in 454-pyrotags from different water layers sampeled in June 2011, October 2011 and May 2012.

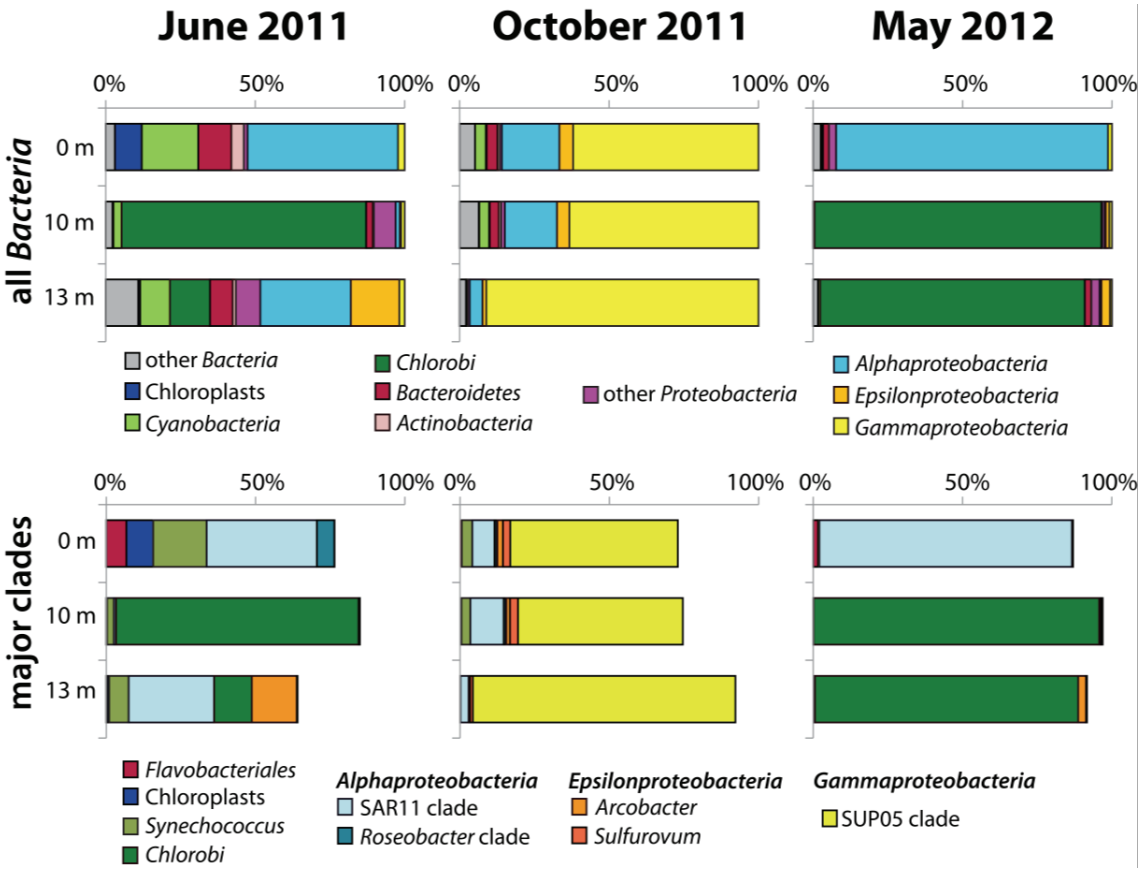
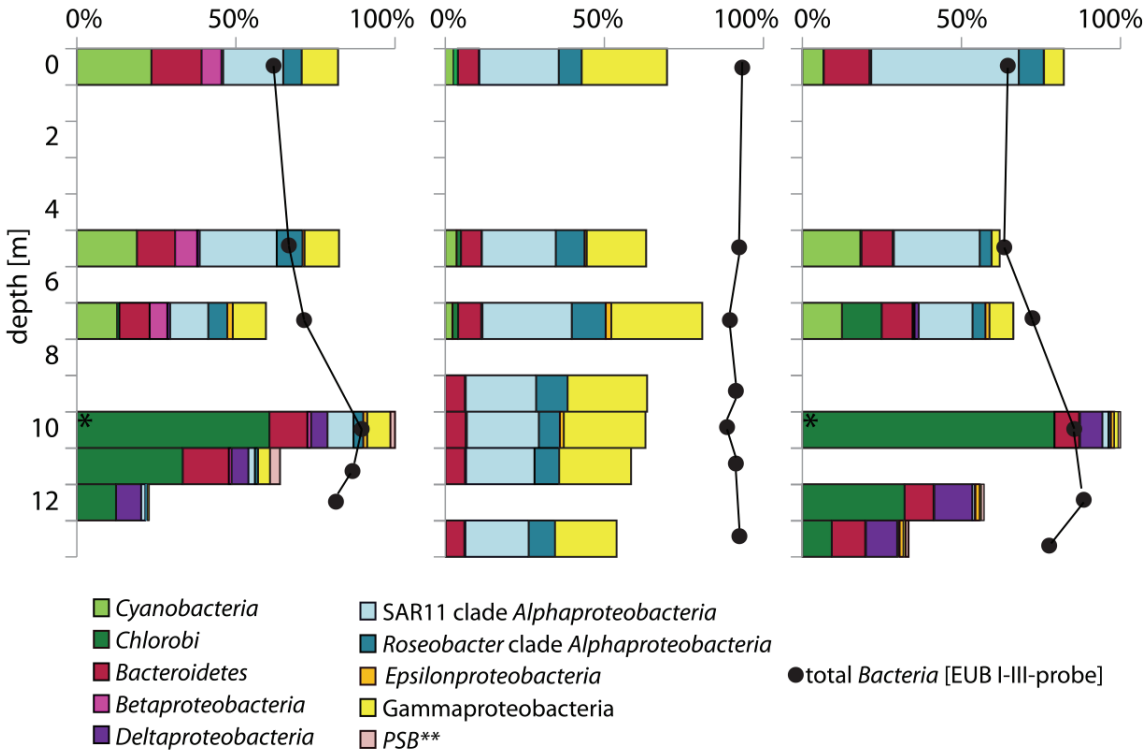


Figure 4: Abundance of different microbial taxa in water samples from different depths retrieved in June 2011, October 2011 and May 2012, as determined by CARD-FISH.



* abundance of GSB was counted higher then depicted in graph (see Table 6), for representation normalized to account for the maximal 100% with all other quantified taxa

** PSB abundances were determined based on hybridization with the Gam42a probe and presence of autofluorescence form photosynthetic pigments

Supplementary Figures and Tables

Figure S1: Map of Lake Rogoznicas' geographic location, adapted from Ciglenečki *et al*. (2006).

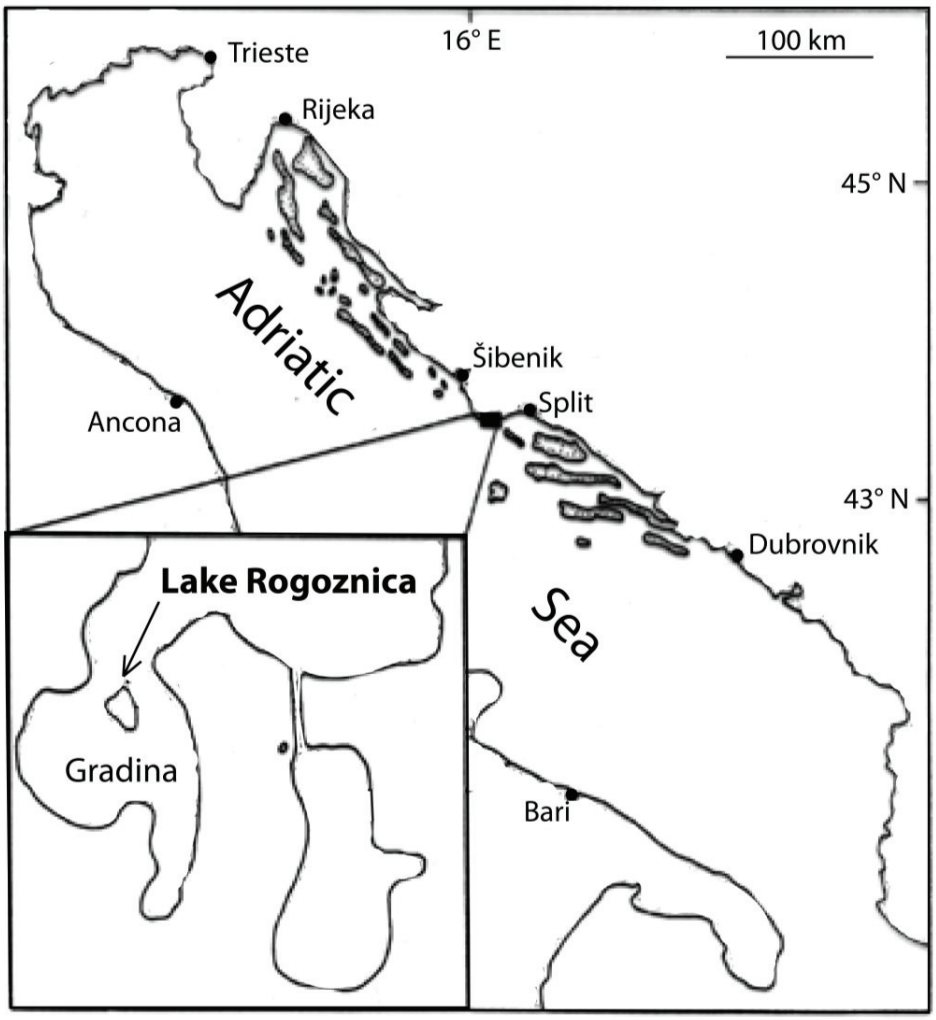


Figure S2: Rarefaction analysis of 16S rRNA gene amplicons 454-pyrosequences. OTUs are grouped at 98% SI.

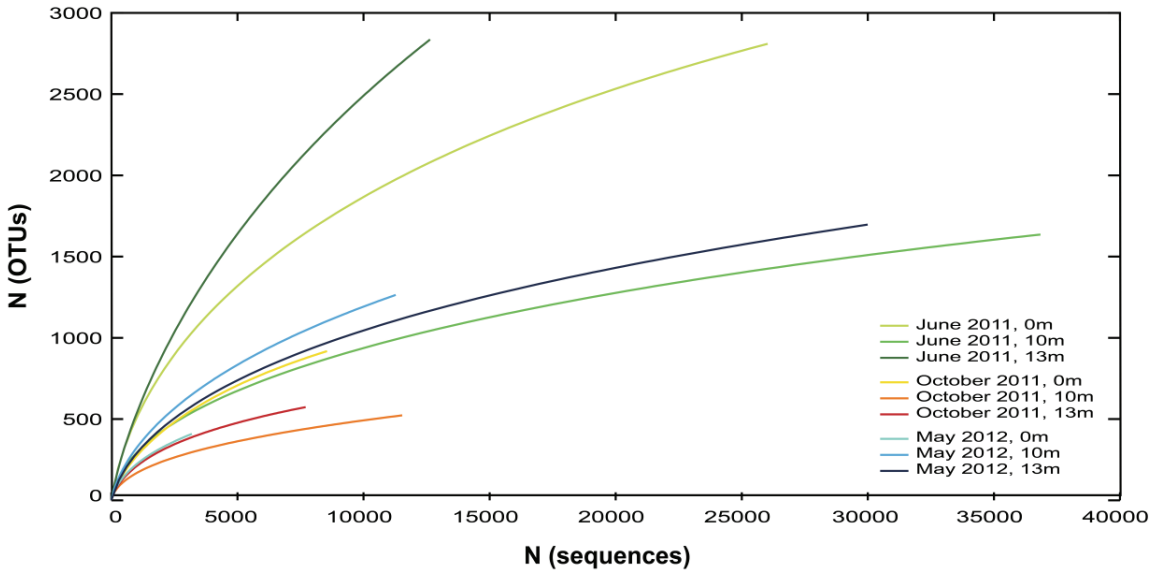


Figure S3: Phylogenetic tree illustrating 16S rRNA gene diversity in clone libraries from different samples collected on the 20th of June 2011, the 9th of October 2011 and the 8th of May 2012. Each OTU (grouped at 98% SI) is represented by one selected sequence. Number of sequences from each sampled water layer in one OTU is given by color code next to the representative sequence.

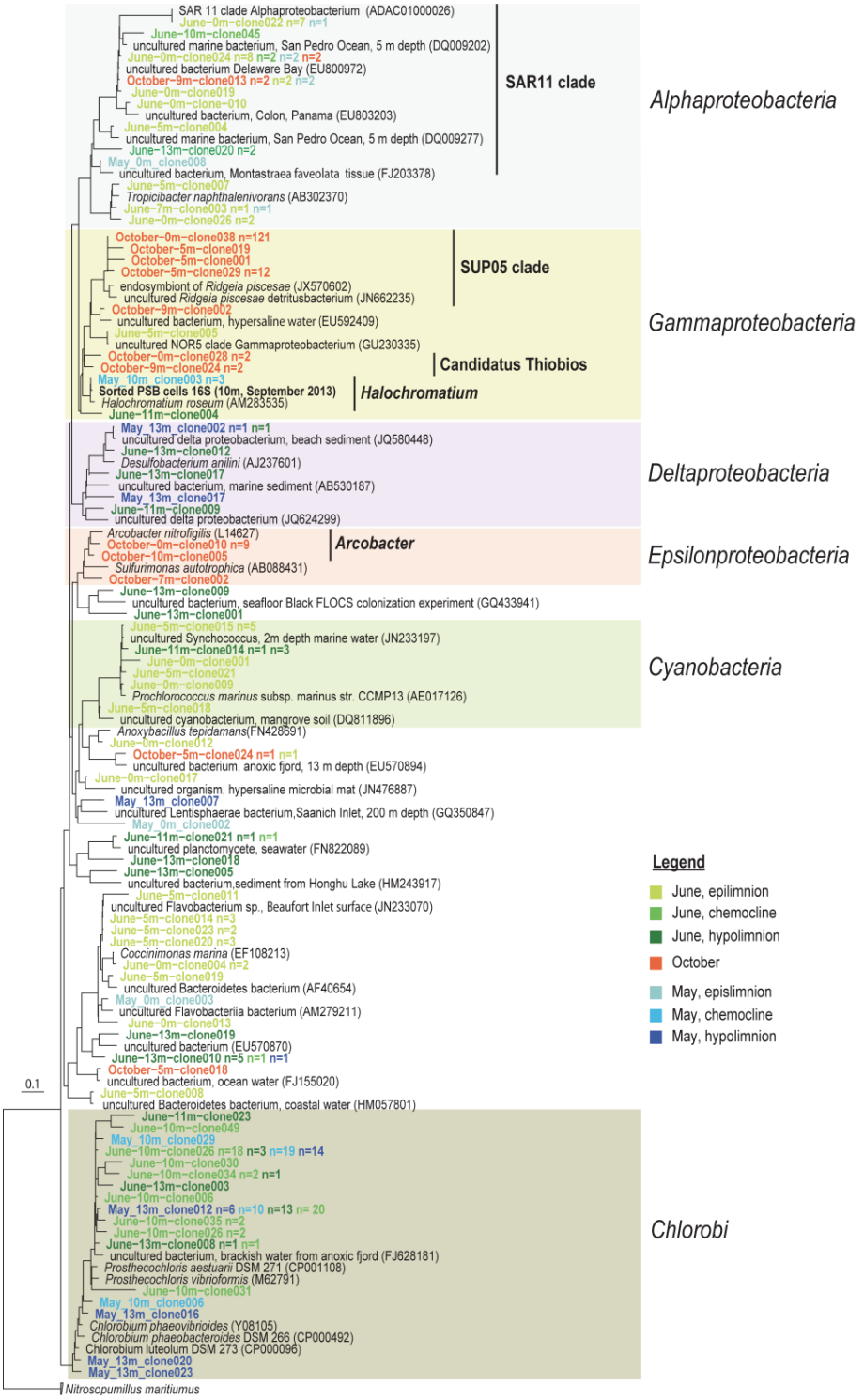


Fig S4: Sorted *Halochromatium* spp. cells with not further identified inclusions, visualized by phase contrast microscopy.

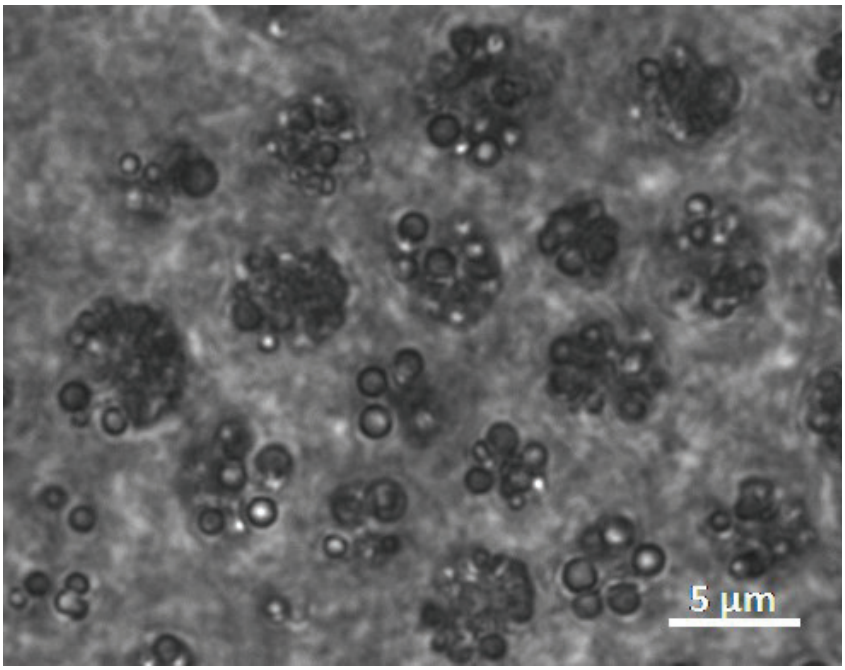


Figure S5: Phylogenetic tree illustrating the diversity of *soxB* gene sequences retrieved from a surface sample from the 20th of June 2011 and holomixis samples from the 9th of October 2011 (surface and 10 m depth). Each OTU (grouped at 94% SI) is represented by one selected sequence. Number of sequences from each sampled water layer in one OTU is given by color code next to the representative sequence.

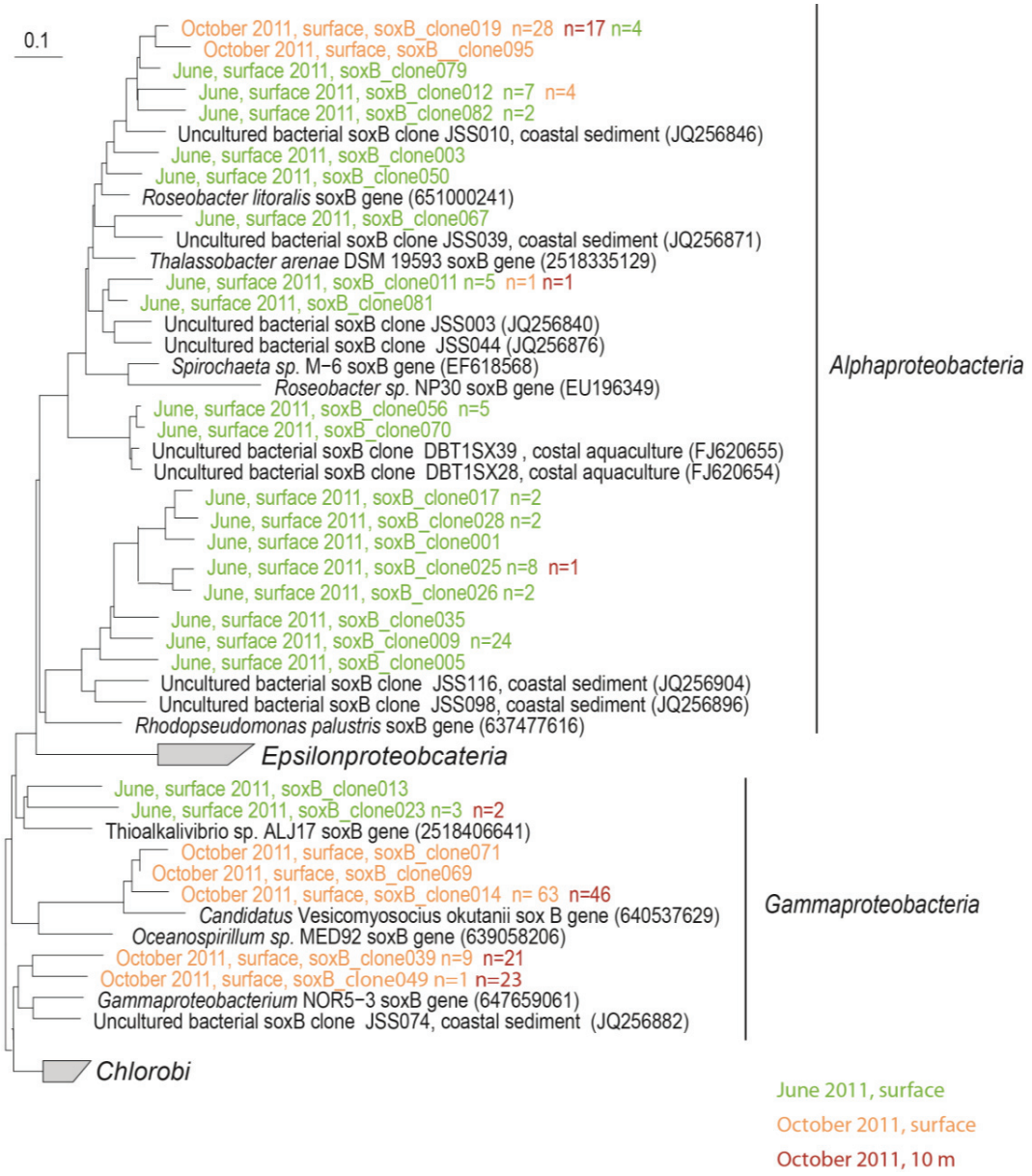


Table S1: Overview of dates and depths at which water samples from Lake Rogoznica were retrieved.

Sample	Depth							
	0 m	5 m	7 m	9 m	10 m	11 m	12 m	13 m
June 2011 (20.06.2011)	+	+	+	-	+	+	+	-
July 2011 (18.07.2011)	+	+	+	-	+	+	+	+
September 2011 (01.09.2011)	+	+	+	-	+	+	+	+
October 2011 (9.10.2011)	+	+	+	+	+	+	-	+
November 2011 (07.11.2011)	+	+	+	-	+	+	-	+
December 2011 (13.12.2011)	+	+	+	+	-	+	-	+
March 2012 (23.03.2012)	+	+	+	-	+	+	+	+
Mai 2012 (08.05.2012)	+	+	+	-	+	-	+	+
September 2013 (30.09.2013)	-	-	-	+	-	+	-	-

Table S2: List of HRP-labeled oligonucleotide probes applied in this study.

Probe name	Target group	Probe sequence ('5→'3)	% FA	Reference
EUB388 I	most *Bacteria*	GCTGCCTCCCGTAGGAGT	35	Amann *et al*., 1990
EUB388 II	*Planctomycetales*	GCAGCCACCCGTAGGTGT		Daims *et al*., 1999
EUB388 III	*Verrucomicrobiales*	GCTGCCACCCGTAGGTGT		
Non388	negative control	ACTCCTACGGGAGGCAGC	35	Wallner *et al*., 1993
CYA361	most *Cyanobacteria*	CCCATTGCGGAAAATTCC	50	Schönhuber *et al*., 1999
GSB-532	green sulfur bacteria, *Chlorobiaceae*	TGCCACCCCTGTATC	10	Tuschak *et al*., 1999
ROS537	*Roseobacter* clade	CAACGCTAACCCCCTCC	35	Eilers *et al*., 2001
SAR11-152R	SAR11 clade	ATTAGCACAAGTTTCCYCGTGT	25	Rappè *et al*., 2002
SAR11-441R (ori)		TACAGTCATTTTCTTCCCCGAC		
SAR11-542R		TCCGAACTACGCTAGGTC		
SAR11-732R		GTCAGTAATGATCCAGAAAGYTG		
SAR11-441Rmodif		TACCGTCATTTTCTTCCCCGAC		*after* Rappè *et al*., 2002
SAR11-487mod (+ helper h3)		CGGACCTTCTTATTCGGG (CGGCTGCTGGCACGAAGTTAGC)		*after* Morris *et al*., 2002
Gam42a* (+ competitor)	*Gammaproteobacteria*	GCCTTCCCACATCGTTT (GCCTTCCCACTTCGTTT)	35	Manz *et al*., 1992
GSO477	*Gammaprotoebactera* sulfur-oxidizers cluster	CTAAAGTTAACGTCAAGG	35*	Lavik *et al*., 2009
GSO1448R	*Gammaprotoebactera* sulfur-oxidizers cluster	GGTGACCGTCCTCAATAAAG	15*	Marchall and Moriss, 2012
Bet42a* (+ competitor)	*Betaproteobacteria*	GCCTTCCCACTTCGTTT (GCCTTCCCACATCGTTT)	35	Manz *et al*., 1992
Epsi549	some *Epsilonproteobacteria*	CAGTGATTCCGAGTAACG	35	Lin *et al*., 2006
Epsi914		GGTCCCCGTCTATTCCTT		Loy *et al*., 2003
Delta495a (+ competitor)	most *Deltaproteobacteria* most *Gemmatimonadetes*	AGTTAGCCGGTGCTTCCT (AGTTAGCCGGTGCTTCTT)	35	Loy *et al*., 2002 Lücker *et al*., 2007
Delta495b (+ competitor)		AGTTAGCCGGCGCTTCCT (AGTTAGCCGGCGCTTCKT)		
Delta495c (+ competitor)		AATTAGCCGGTGCTTCCT (AATTAGCCGGTGCTTCTT)		

* Hybridization performed at 35°C, instead of 46°C

Table S3: List of all primers applied for PCR amplifications in this study

Primer	Sequence (‘5→’3)	Reference
16S rRNA gene primers		
GM3	AGAGTTTGATCMTGGC	Muyzer *et al*., 1995
GM4	TACCTTGTTACGACTT	Muyzer *et al*., 1995
27F_mod	AGRGTTTGATCMTGGCTCAG	after Lane *et al*., 1991
519R_mod	GTNTTACNGCGGCKGCTG	after Turner *et al*., 1999
soxB gene primers		
soxB432F	GAYGGNGGNGAYACNTGG	Petri *et al*., 2001
soxB1446R	5CATGTCNCCNCCRTGYTG	Petri *et al*., 2001

Table S4: Analysis statistics of 16S rRNA gene 454-pyrosequence reads recovered in this study

Sample	N (sequences)	Average length	%rejected[1]	%OTUs[2]	%clustered[3]	%replicates[4]
June 2011 0m	26072	382.7	0.17 (n=44)	10.78 (n=2810)	66.42 (n=15753)	28.63 (n=7465)
June 2011 10m	36861	375.7	0.04 (n=15)	4.44 (n=1636)	46.74 (n=17230)	48.78 (n=17980)
June 2011 13m	14135	421.2	10.58 (n=1495)	20.06 (n=2836)	47.45 (n=6707)	21.91 (n=3097)
October 2011 0m	3295	421.7	3.7 (n=101)	12.41 (n=409)	51.68 (n=1703)	32.84 (n=1082)
October 2011 10m	11707	421.8	4.26 (n=433)	10.8 (n=1297)	48.96 (n=5732)	36.54 (n=4278)
October 2011 13m	31329	467.1	4.14 (n=1334)	5.42 (n=1697)	37.71 (n=11813)	52.62 (n=16485)
May 2012 0m	8992	429.8	4.92 (n=442)	10.21 (n=918)	37.98 (n=3415)	46.9 (n=4217)
May 2012 10m	13009	441.3	11.33 (n=1474)	4.02 (n=523)	36.39 (n=4734)	48.26 (n=6278)
May 2012 13m	8757	437.4	11.99 (n=1050)	6.55 (n=574)	40.12 (n=3513)	41.34 (n=3620)

Table S5: RSS, DOC and POC concentrations, TCCs and hydrographical data for all sampling dates from June 2011 – May 2012. The depth closest to the chemocline is marked in gray.

Sample		DOC [mg l^{-1}]	POC [mg l^{-1}]	RSS [µmol l^{-1}]	O_2 [mg l^{-1}]	TCC [cells ml^{-1}]	S [‰]	T [°C]	pH
20th of June 2011									
Depth [m]	**0**	1.6	0.3	$7x10^{-3}$	8.8	$2.7x10^{6}$	30.9	27.8	8.0
	5	1.5	0.3	10^{-2}	9.3	$2.8 x10^{6}$	32.2	26.2	7.9
	7	1.6	0.4	10^{-2}	9.1	$3.9 x10^{6}$	32.5	26.0	7.9
	10	3.1	2.6	$3x10^{-2}$	0.1	$5.8 x10^{6}$	33.4	23.7	7.1
	11	2.0	2.8	510	0.1	$6.9 x10^{6}$	33.5	19.8	7.1
	12	3.3	2.0	2000	0.1	$5.8 x10^{6}$	34.3	18.8	7.0
18th of July 2011									
Depth [m]	**0**	1.8	0.4	10^{-2}	8.7	$3.2 x10^{6}$	33.1	28.5	8.0
	5	1.7	0.4	$3x10^{-3}$	8.4	$3.7 x10^{6}$	33.2	27.2	8.0
	7	1.7	0.5	$4x10^{-3}$	8.5	$7.0 x10^{6}$	33.2	26.9	8.0
	10	2.3	0.1	$8x10^{-3}$	0.2	$3.8 x10^{6}$	33.6	24.8	7.4
	11	2.3	3.0	58	0.1	$6.6 x10^{6}$	33.4	20.7	7.2
	12	1.8	0.9	540	0.2	$4.7 x10^{6}$	34.3	19.0	7.0
	13	2.7	1.5	1850	0.2	$5.5 x10^{6}$	33.4	18.7	6.9
1st of September 2011									
Depth [m]	**0**	1.6	0.3	$8x10^{-3}$	6.6	$2.4 x10^{6}$	n.d.	30.0	8.0
	5	1.7	0.3	$5x10^{-3}$	6.3	$2.6 x10^{6}$	n.d.	29.8	8.1
	7	1.6	0.3	$4x10^{-3}$	6.1	$2.9 x10^{6}$	n.d.	29.7	8.1
	10	1.6	0.4	$4x10^{-3}$	4.9	$3.5 x10^{6}$	n.d.	30.0	8.0
	11	2.2	3.2	16	0	$6.2 x10^{6}$	n.d.	28.5	7.9
	12	3.1	2.8	124	0	$5.6 x10^{6}$	n.d.	25.5	7.6
	13	4.1	2.6	3900	0	$5.8 x10^{6}$	n.d.	24.9	7.2
9th of October 2011									
Depth [m]	**0**	2.1	0.7	$5x10^{-2}$	0.9	$7.0 x10^{6}$	33.5	21.2	7.7
	5	2.1	1.0	0.2	0.1	$7.4 x10^{6}$	33.2	21.5	7.7
	7	2.1	0.8	0.1	0	$6.6 x10^{6}$	33.2	21.5	7.7
	9	2.1	0.7	0.2	0	$7.8 x10^{6}$	33.2	21.5	7.7
	10	2.2	0.7	$8x10^{-2}$	0	$7.6 x10^{6}$	33.2	21.5	7.7
	11	2.1	0.7	0.6	0	$7.8 x10^{6}$	33.2	21.5	7.7
	13	2.1	0.6	0.2	0	$7.9 x10^{6}$	33.2	19.4	7.7
7th of November 2011									
Depth [m]	**0**	2.0	1.2	$6x10^{-3}$	11.9	$4.1 x10^{6}$	28.3	15.9	8.6
	5	1.9	0.6	10^{-2}	0.3	$2.7 x10^{6}$	33.4	18.8	7.5
	7	1.9	0.5	10^{-2}	0.2	$4.4 x10^{6}$	33.4	18.8	7.5
	10	1.8	0.5	0.5	0.1	$5.7 x10^{6}$	33.5	18.7	7.5
	11	1.8	0.5	0.5	0.1	$6.7 x10^{6}$	33.5	18.7	7.5
	13	1.8	0.8	25	0.1	$6.7 x10^{6}$	33.5	18.7	7.2

Table S5 (continued): RSS, DOC and POC concentrations, TCCs and hydrographical data for all sampling dates from June 2011 – May 2012. The depth closest to the chemocline is marked in gray.

Sample		DOC [mg l^{-1}]	POC [mg l^{-1}]	RSS [µmol l^{-1}]	O_2 [mg l^{-1}]	TCC [cells ml^{-1}]	S [‰]	T [°C]	pH
13th of December 2011									
Depth [m]	**0**	1.7	0.5	$3x10^{-3}$	7.0	$5.5 x10^6$	27.5	16.5	7.8
	5	1.8	1.3	7.3	0.9	$5.6 x10^6$	36.5	18.5	7.5
	7	1.8	0.9	7.1	0	$4.3 x10^6$	37.0	18.8	7.5
	9	1.7	0.8	11	0	$4.5 x10^6$	38.0	20.0	7.5
	11	1.8	0.9	16	0	$4.9 x10^6$	38.0	19.8	7.4
	13	1.5	0.9	26	0	$5.0 x10^6$	38.0	19.0	7.4
31st of January 2012									
Depth [m]	**0**	1.6	0.8	0	12.6	n.d.	28.2	6.1	8.3
	5	1.4	1.8	5.0	0.2	n.d.	35.8	17.3	7.5
	7	1.4	1.2	8.7	0.2	n.d.	35.7	17.5	7.5
	10	1.4	1.0	34	0.1	n.d.	35.7	17.5	7.4
	11	1.4	0.9	23	0.1	n.d.	35.7	17.5	7.4
	12	1.4	0.8	45	0.1	n.d.	35.8	17.7	7.4
	13	1.5	1.0	81	0.1	n.d.	35.8	17.7	7.2
23rd of March 2012									
Depth [m]	**0**	1.4	0.6	10^{-2}	11.3	$5.0 x10^6$	27.0	18.0	7.9
	5	1.5	2.2	$2x10^{-2}$	5.0	$7.7 x10^6$	35.5	20.0	7.8
	7	1.5	2.7	35	0	$7.2 x10^6$	37.0	19.5	7.6
	10	1.5	1.5	53	0	$6.3 x10^6$	37.0	19.5	7.5
	11	1.4	1.4	125	0	$7.0 x10^6$	37.0	19.5	7.4
	12	1.5	1.5	150	0	$7.7 x10^6$	37.0	19.5	7.4
	13	1.6	1.6	4000	0	$8.3 x10^6$	37.0	19.5	7.4
8th of May 2012									
Depth [m]	**0**	1.8	0.3	10-2	8.4	$2.3 x10^6$	35.0	26.2	8.2
	5	2.1	0.7	10-2	7.9	$4.0 x10^6$	35.5	25.2	8.0
	7	2.1	3.2	10-2	5.1	$5.3 x10^6$	36.0	24.0	7.7
	10	1.7	1.2	140	0	$6.4 x10^6$	37.2	20.5	7.4
	12	1.8	1.7	367	0	$5.8 x10^6$	38.0	20.2	7.4
	13	2.3	2.0	375	0	$6.3 x10^6$	7.5	20.2	7.4

n.d. – not determined

Table S6: Number of 16S rRNA gene clones retrieved per depth for different samples collected in June 2011m October 2011 and May 2012

Sample	Number of clones per depth							
	0 m	**5 m**	**7 m**	**9 m**	**10 m**	**11 m**	**12 m**	**13 m**
June 2011 (20.06.2011)	28	23	3	-	51	23	-	20
October 2011 (9.10.2011)	43	38	17	29	19	11	-	-
Mai 2012 (08.05.2012)	9	-	-	-	31	-	-	29

Table S7: CARD-FISH results in percent of TCC for all sampling dates from June 2011 – May 2012. The depth closest to the chemocline is marked in gray.

Sample		Detected with CARD-FISH probe										
		EUB I-III	SAR11-mix	Ros537	Gam42a	Bet42a	Epsy549/914	Delta495abc	CF319a	GSB-532	Cya361	PSB*
20th of June 2011												
Depth [m]	0	61%	19%	6%	11%	6%	0%	1%	16%	0%	23%	0%
	5	65%	24%	8%	11%	7%	1%	1%	12%	0%	19%	0%
	7	71%	12%	6%	10%	6%	2%	1%	10%	1%	13%	0%
	10	90%	8%	3%	7%	1%	1%	5%	12%	89%	n.d.	1%
	11	87%	2%	1%	4%	1%	n.d.	5%	14%	33%	n.d.	3%
	12	81%	1%	1%	1%	0%	n.d.	8%	n.d.	12%	n.d.	v
18th of July 2011												
Depth [m]	0	81%	5%	4%	6%	0%	0%	0%	7%	0%	13%	0%
	5	79%	5%	3%	5%	0%	0%	0%	6%	0%	21%	0%
	7	84%	4%	3%	3%	0%	0%	n.d.	6%	0%	13%	0%
	10	88%	8%	3%	6%	0%	0%	1%	6%	56%	n.d.	0%
	11	88%	1%	0%	1%	1%	0%	3%	4%	84%	n.d.	1%
	12	91%	5%	0%	1%	0%	0%	n.d.	8%	23%	n.d.	0%
	13	90%	1%	0%	0%	0%	0%	n.d.	4%	10%	n.d.	0%
1st of September 2011												
Depth [m]	0	59%	17%	10%	9%	4%	0%	2%	7%	0%	24%	0%
	5	60%	22%	10%	9%	4%	0%	2%	6%	0%	16%	0%
	7	66%	21%	9%	7%	5%	0%	2%	6%	0%	12%	0%
	10	88%	13%	6%	8%	4%	0%	3%	5%	35%	n.d.	0%
	11	83%	1%	0%	3%	1%	0%	7%	11%	86%	n.d.	3%
	12	89%	0%	0%	1%	0%	0%	6%	14%	24%	n.d.	3%
	13	83%	0%	0%	1%	0%	0%	2%	6%	11%	n.d.	3%

Table S7 (continued): CARD-FISH results in percent of TCC for all sampling dates from June 2011 – May 2012. The depth closest to the chemocline is marked in gray.

Sample		Detected with CARD-FISH probe										
		EUB I-III	SAR11-mix	Ros537	Gam42a	Bet42a	Epsy549/914	Delta495abc	CF319a	GSB-532	Cya361	PSB*
9th of October 2011												
Depth [m]	0	93%	25%	7%	27%	0%	0%	0%	7%	1%	3%	0%
	5	92%	23%	9%	19%	0%	0%	0%	6%	1%	4%	0%
	7	89%	28%	11%	29%	0%	0%	0%	7%	2%	2%	0%
	9	91%	22%	10%	25%	0%	0%	0%	6%	0%	0%	0%
	10	89%	23%	7%	26%	0%	0%	0%	6%	0%	0%	0%
	11	91%	22%	8%	23%	0%	0%	0%	6%	0%	0%	0%
	13	92%	20%	8%	19%	0%	0%	0%	6%	0%	0%	0%
7th of November 2011												
Depth [m]	0	69%	7%	10%	7%	0%	2%	n.d.	13%	0%	2%	0%
	5	78%	n.d.	5%	9%	n.d.	7%	1%	18%	0%	0%	0%
	7	82%	4%	7%	14%	1%	2%	0%	9%	0%	0%	1%
	10	91%	3%	4%	28%	0%	1%	2%	7%	0%	0%	0%
	11	92%	2%	5%	29%	0%	1%	2%	9%	0%	0%	0%
	13	87%	3%	3%	1%	0%	2%	2%	8%	0%	0%	0%
13th of December 2011												
Depth [m]	0	67%	33%	10%	14%	4%	1%	0%	13%	2%	0%	0%
	5	68%	29%	5%	6%	2%	4%	2%	11%	54%	0%	1%
	7	72%	n.d.	3%	4%	1%	3%	3%	11%	41%	0%	1%
	9	89%	17%	2%	3%	1%	2%	3%	11%	n.d.	0%	1%
	11	83%	17%	1%	3%	1%	2%	3%	10%	8%	0%	1%
	13	79%	n.d.	1%	3%	0%	1%	2%	9%	6%	0%	0%

Table S7 (continued): CARD-FISH results in percent of TCC for all sampling dates from June 2011 – May 2012. The depth closest to the chemocline is marked in gray.

Sample		Detected with CARD-FISH probe										
		EUB I-III	**SAR11-mix**	**Ros537**	**Gam42a**	**Bet42a**	**Epsy549/914**	**Delta495abc**	**CF319a**	**GSB-532**	**Cya361**	**PSB***
23rd of March 2012												
Depth [m]	**0**	69%	15%	2%	8%	0%	0%	n.d.	7%	0%	22%	0%
	5	73%	9%	1%	1%	0%	0%	3%	5%	n.d.	14%	2%
	7	79%	6%	0%	1%	0%	0%	4%	7%	45%	2%	1%
	10	84%	n.d.	0%	1%	0%	0%	6%	8%	82%	n.d.	0%
	11	92%	0%	0%	0%	0%	0%	3%	7%	67%	n.d.	0%
	12	92%	n.d.	0%	0%	0%	0%	5%	7%	33%	n.d.	0%
	13	86%	n.d.	1%	0%	0%	0%	11%	9%	11%	n.d.	0%
8th of May 2012												
Depth [m]	**0**	65%	46%	8%	6%	0%	0%	0%	14%	0%	7%	0%
	5	63%	27%	4%	3%	0%	0%	0%	10%	0%	18%	0%
	7	72%	17%	4%	7%	1%	1%	1%	10%	12%	12%	0%
	10	85%	2%	1%	1%	0%	1%	7%	8%	88%	n.d.	1%
	12	88%	1%	0%	0%	0%	1%	12%	9%	32%	n.d.	1%
	13	78%	0%	0%	1%	0%	1%	10%	11%	9%	n.d.	1%

n.d. – not determined

*PBS were not quantified by a specific PBS-probe, but as cells with signal from the Gam42a *Gammaproteobacteria* FISH-probe and autofluorescence form photopigments

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Chapter IV

Microbial lipids reveal carbon assimilation patterns on hydrothermal sulfide chimneys

Eoghan P. Reeves[§], Marcos Y. Yoshinaga[§], Petra Pjevac[§], Nadine Goldenstein, Jörg Peplies, Anke Meyerdierks, Rudolf Amann, Wolfgang Bach, Kai-Uwe Hinrichs

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E.P.R., M.Y.Y. and P.P. developed concepts and ideas, conceived and wrote the manuscript. E.P.R., P.P., A.M. and W.B. collected samples. E.P.R. performed geochemical analysis. M.Y.Y. and N.G. performed IPL and TOC experiments and analyzed the data. P.P. performed sequencing and FISH experiments and analyzed the data. J.P. run 16S rRNA gene sequence analyses tools. E.P.R. and N.G. analyzed SEM/EXD data. N.G., J.P., A.M., R.A., W.B. and K.-U.H. conceived and edited the manuscript.

Microbial Lipids Reveal Carbon Assimilation Patterns on Hydrothermal Sulfide Chimneys

Eoghan P. Reeves[1,‡]*, Marcos Y. Yoshinaga[1,‡], Petra Pjevac[2,‡], Nadine Goldenstein[1], Jörg Peplies[2,3], Anke Meyerdierks[2], Rudolf Amann[2], Wolfgang Bach[1], Kai-Uwe Hinrichs[1]

[1]MARUM Center for Marine Environmental Sciences & Department of Geosciences, University of Bremen, D-28359 Bremen, Germany [2]Department of Molecular Ecology, Max Planck Institute for Marine Microbiology, D28359 Bremen, Germany [3]Ribocon GmbH, Fahrenheitstrasse 1, D-28359 Bremen, Germany

‡These authors contributed equally to this work

*Correspondence: Present Address: Department of Earth, Atmospheric and Planetary Sciences, Massachusetts Institute of Technology, 77 Mass. Ave., Cambridge MA 02139 Phone: +1 (617) 324-3946 E-mail: ereeves@alum.mit.edu

Running Title: *Microbial lipids on hydrothermal sulfide structures*

Summary

Sulfide 'chimneys' characteristic of seafloor hydrothermal venting are diverse microbial habitats. $^{13}C/^{12}C$ ratios of microbial membrane lipids have rarely been used to investigate bacterial and archaeal carbon assimilation pathways on these structures, despite complementing gene-and culture-based approaches. Here, we integrate analyses of intact polar lipid (IPL) diversity and $\delta^{13}C$ values ($\delta^{13}C_{lipid}$) with 16S rRNA gene-based phylogeny to examine microbial carbon flow on active and inactive sulfide structures from the Manus Basin. Surficial crusts of active structures, dominated by *Epsilonproteobacteria*, yield bacterial $\delta^{13}C_{lipid}$ values higher than biomass $\delta^{13}C$ (total organic carbon), implicating autotrophy via the reverse tricarboxylic acid cycle. Our data also suggest $\delta^{13}C_{lipid}$ values vary on individual active structures without accompanying microbial diversity changes. Temperature and/or dissolved substrate effects -likely due to variable advective-diffusive fluxes to chimney exteriors -may be responsible for differing ^{13}C fractionation during assimilation. In an inactive structure, $\delta^{13}C_{lipid}$ values lower than biomass $\delta^{13}C$ and a distinctive IPL and 16S rRNA gene diversity suggest a shift to a more diverse community and an alternate carbon assimilation pathway after venting ceases. We discuss here the potential of IPL and $\delta^{13}C_{lipid}$ analyses to elucidate microbial carbon flow in hydrothermal structures when combined with other molecular tools.

Introduction

Over 300 hot spring sites have been discovered thus far on the global mid-ocean ridge system or in back-arc basins (Hannington *et al*., 2011), where the majority of high temperature fluids gush from striking sulfide 'chimney' structures. The diverse chemistries of fluids venting from these structures and the steep physical and geochemical gradients through their walls dictate microbial metabolic strategies, giving rise to diverse assortments of mesophilic, thermophilic and hyperthermophilic *Bacteria* and *Archaea* (e.g. Tivey, 2004; Takai *et al*., 2006; Schrenk *et al*., 2008; Amend *et al*., 2011; Sievert and Vetriani, 2012. Their cooler exteriors, richest in microbial biomass, are often covered with conspicuous white, orange or dark-colored biofilms (e.g. Hoek *et al*., 2003; Takai *et al*., 2008; Olins *et al*., 2013). Microbial communities on active structures have been intensively investigated using both culture-dependent and -independent approaches (e.g. Takai *et al*., 2001; Schrenk *et al*., 2003; Kormas *et al*., 2006; Reysenbach *et al*., 2006; Takai *et al*., 2008; Takai *et al*., 2009; Kato *et al*., 2010; Flores *et al*., 2011; Flores *et al*., 2012; Olins *et al*., 2013). Inactive structures, where fluid venting has ceased completely, have been shown to host very different and more diverse communities compared to active counterparts (Rogers *et al*., 2003; Suzuki *et al*., 2004; Kato *et al*., 2010; Sylvan *et al*., 2012; Sylvan *et al*., 2013; Toner *et al*., 2013). While our understanding of the diversity of chimney microbial communities and their potential biogeochemical functions has grown in recent years through these investigations, the distribution of carbon fixation pathways that operate in these habitats is still poorly understood. For example, recent studies suggest a dominance of the Calvin-Benson-Bassham (CBB) carbon assimilation pathway on active sulfide structures with abundant *Gammaproteobacteria* at Juan de Fuca Ridge (Wang *et al*., 2009; Olins *et al*., 2013). At the same time, evidence for the widespread use of the reverse tricarboxylic acid (rTCA) cycle in hydrothermal environments by ubiquitous *Epsilonproteobacteria* is increasing (Campbell and Cary, 2004; Hügler and Sievert, 2011). In this investigation, we examine the utility of membrane lipids – combined with other molecular tools – to elucidate and further probe carbon fixation pathways operating in communities inhabiting these structures and the physicochemical factors that influence associated $^{13}C/^{12}C$ signatures of this process.

Though lacking the taxonomic resolution of 16S rRNA genes, the structural diversity of intact polar lipids (IPL) reflects bacterial and archaeal distributions on sulfide chimneys (Gibson *et al*., 2013). Stable carbon isotope compositions of individual lipids

($\delta^{13}C_{lipid}$) may additionally resolve different carbon fixation pathways when integrated with other molecular observations of community diversity. Such combined approaches have already been applied to terrestrial hot spring communities (Schubotz *et al*., 2013) and sedimentary environments (Biddle *et al*., 2006; Schubotz *et al*., 2011a). IPL diversity and $\delta^{13}C_{lipid}$ variations, in the context of microbiological data, have been investigated on hydrothermal structures from the serpentinite-hosted Lost City hydrothermal field, where highly alkaline, H_2-rich and H_2S-poor low temperature fluids vent from carbonate-brucite chimneys (Kelley *et al*., 2001; Brazelton *et al*., 2006; Bradley *et al*., 2009b). However, $\delta^{13}C_{lipid}$ data from the much more widespread massive sulfide structures that vent high temperature, acidic, H_2S-rich fluids are scarce and poorly constrained. Complex bulk lipid mixtures with diverse $\delta^{13}C_{lipid}$ values (+2 to -50‰) have been reported from large sulfide chimney fragments in unsedimented systems (0.25 to 1.4 kg; Blumenberg *et al*., 2007; Blumenberg *et al*., 2012), but accompanying community diversity was not examined. Lipid extraction from whole structures also obscures the extreme habitat heterogeneity present in chimney walls (e.g. Hedrick *et al*., 1992; Takai *et al*., 2001; Tivey, 2004; Schrenk *et al*., 2008), and finer scale sampling is needed. Studies have used $\delta^{13}C$ of total organic carbon (TOC; $\delta^{13}C_{TOC}$) alone as a metric of biomass $\delta^{13}C$ in order to elucidate carbon fixation pathways in operation (e.g. Jaeschke *et al*., 2012; Olins *et al*., 2013), but this may be problematic in settings where pyrolysis of sedimentary organic matter contributes abundant thermogenic organic carbon to chimney structures (Simoneit, 1990).

In an attempt to overcome methodological limitations of individual approaches, in this study we integrate $\delta^{13}C_{lipid}$ values, associated IPL diversity and parallel 16S rRNA gene-based surveys, along with accompanying TOC ($\delta^{13}C_{TOC}$), vent fluid carbon source and mineralogical constraints on chimney habitats. We use this integrated approach to examine the nature and distribution of dominant microbial carbon fixation pathways on hydrothermal sulfide structures from the unsedimented PACMANUS site in the Manus back-arc basin. Relative to those venting at ultramafic or sedimented hydrothermal systems, fluids at PACMANUS contain abundant H_2S but low CH_4 and H_2 (Reeves *et al*., 2011). In terms of these important volatiles, the system represents one common type of fluid composition in the spectrum of global vent fluid chemistries. Our subsampling approach suggests previously unobserved fine scale heterogeneity in microbial carbon

isotope fractionation on individual active chimney exteriors, as well as a major change in such signatures upon cessation of venting. To our knowledge, this constitutes the first complete integration of $\delta^{13}C_{lipid}$ values with other molecular methods on this type of hydrothermal structure, and we discuss the potential of this approach for other more diverse systems.

Results

Chimney sample descriptions and hydrothermal fluid chemistry

Four sulfide structures were collected from the unsedimented PACMANUS vent field **(Figure 1; Supplementary Figure S1)** in the Manus back-arc basin (Binns and Scott, 1993; Reeves *et al*., 2011) and subsampled. These consisted of an active 'smoker' chimney from each of the Roman Ruins ('RMR5', 333°C) and Satanic Mills ('SM4', 345°C) vent areas, a weakly venting 'diffuser' spire (Kormas *et al*., 2006; Schrenk *et al*., 2008) from Roman Ruins ('RMR-D', temperature not determined), and an inactive (relict) chimney away from any visible fluid flow from Satanic Mills ('SM-I'). From the active structures (RMR5, SM4, RMR-D), the predominantly 'white' and 'dark' (samples with '-White' and '-Dark' suffixes) surficial crusts visible on the exteriors (denoted '-Ext-') were (**Figure 1**) subsampled from undisturbed portions. 'White' crusts were from more friable material associated with visible fluid venting or 'shimmering' surfaces (e.g. covering the exterior of RMR-D). In contrast, 'dark' crusts were associated with thicker and/or more cemented/crystalline chimney wall with no visible fluid advection. On RMR-D a thick 'orange' mat (presumably Fe oxide) overlaying the 'white' crust was also sampled (RMR-D-Ext-Orange). The inactive structure SM-I had a uniform oxide surface, and only one exterior surficial crust sample was retrieved. Larger chimney wall bulk fragments or conduits (20-40 g wet wt., labeled '-Bulk') -where contributions from interior microbial biomass are possible -were retrieved from RMR5, SM4 and SM-I and investigated for comparison to surficial crusts.

The dominant minerals present on the exteriors of the analyzed structures (SM4, RMR-D and SM-I) are consistent with those found in similar structures collected from the same areas in 2006 (Craddock, 2008), as well as from other hydrothermal systems such as the East Pacific Rise (Toner *et al*., 2013) and Southwest Indian Ridge (Tao *et al*., 2011). Samples of 'white' and 'dark' surficial crusts on SM4 both contained abundant sphalerite (ZnS) and marcasite/pyrite (FeS_2), and minor chalcopyrite ($CuFeS_2$), galena (PbS), barite ($BaSO_4$), and Fe-oxide phases. However, the 'white' crusts from both SM4

and RMR-D contained abundant mineralized filamentous structures rich in silica and sulfur **(Figure 2)**, which were not detected in any 'dark' crusts or in SM-I exterior samples. SM-I is presumed to have been a black smoker structure while active, based on the massive crystalline interiors, visibly open conduits and abundant interior chalcopyrite - a mineral characteristic of high temperature smoker conduits (Hannington *et al*., 1995; Tivey, 2004; Craddock, 2008). Its exterior contained abundant zinc sulfides and barite, similar to SM4.

Endmember fluid compositions reported in **Table 1** represent the measured compositions extrapolated to zero Mg, in order to remove the effects of accidental seawater entrainment and dilution during sample collection as per convention (Von Damm *et al*., 1985). Elevated H_2S concentrations, but very low H_2 and CH_4 concentrations were measured. Overall, the temperatures of fluids collected, and the abundance and isotopic compositions of dissolved volatiles, resemble ranges reported for the PACMANUS vent field in 2006 (Reeves *et al*., 2011). Although no fluids or temperature data could be collected from RMR-D, the weak diffusing nature of fluid emanation, lack of particulate 'black smoke' common to high temperature fluids **(Figure 1)** and observations from similar structures in 2006 (Craddock, 2008; Reeves *et al*., 2011) suggest it likely had a lower temperature than RMR5 and SM4.

Colonization densities, bacterial vs. archaeal cell and IPL abundance

Colonization densities in cell numbers per cm³ were determined for surficial crusts of active structures (RMR5, SM4, RMR-D) and a surface sample of the inactive structure SM-I. Total cell counts (TCC) ranged from 3.3 x 10^8 to 3.1 x 10^9 cells/cm³ (wet) of chimney material **(Supplementary Table S2)**. In several samples archaeal cell abundances as determined by CARD-FISH were below detection. In these cases, however, the detection limit (~1%) was rather high as autofluorescence of mineral particles interfered with more accurate quantification. *Archaea* were most abundant in the dark-colored conduit fragment from RMR5 (RMR5-Bulk-Dark, 6%). In all samples over 80% of cells were identified as *Bacteria,* except in RMR5-Ext-Dark, where they accounted for 55% with ~45% of all cells remaining unclassified **(Supplementary Table S2)**.

Combined bacterial and archaeal (total) IPL concentrations in all structures ranged from 0.39 to 56 µg/g (wet wt.) **(Supplementary Table S3)**. Overall, bacterial IPL were up to four orders of magnitude more abundant than archaeal IPL, with the latter

being most abundant in the conduit fragment from RMR5 (RMR5-Bulk-Dark), and below detection in RMR-D-Ext-White and RMR-D-Ext-Orange **(Figure 3A)**. Despite our limited sample numbers, TCC generally increased with IPL concentrations (cf. RMR5 samples) for samples of active chimneys (**Supplementary Figure 2**). The sample (SM-I-Ext) from the inactive SM-I structure is the only outlier that does not lie on the same trend.

Lipid diversity

Bacterial IPL were generally more abundant than archaeal IPL and clearly dominated by phospholipids **(Figure 3B)**, with diacylglycerol lipids (DAG) being much more abundant than mono-or dietherglycerol lipids. Phosphatidylethanolamine (PE) based IPL were present in all samples and dominated bacterial IPL in the SM4 and RMR-D samples, whereas phosphatidylcholine (PC) species dominated in RMR5. Phosphoaminopentanetetrols (APT) were present in all active samples and most abundant in RMR5-Bulk-Conduit, but represented only a trace fraction in SM-I samples. Although present in almost all active chimney exteriors, mono-and di-N-methyl derivatives of PE (PME and PDME, respectively) comprised a much larger fraction of bacterial IPL in inactive SM-I. Non-phospholipids, such as betaine lipids (BL), ornithine lipids (OL) and glycolipids (G), were present in all samples but collectively accounted for less than 20% of bacterial IPLs.

The few archaeal IPL detected **(Supplementary Table S4**) contained predominantly mono-and diglycosidic headgroups, but differed between active and inactive structures. Active structures were dominated by archaeal IPL composed of diether lipids or archaeols (AR), whereas glycerol-dibiphytanyl-glycerol-tetraethers (GDGT) were more abundant than AR and highly diverse in the inactive structure. Intact GDGT consisted of acyclic to pentacyclic (GDGT-0 to-5) and H-shaped (H-GDGT) tetraethers. For comparison with previous studies, archaeal core GDGT (not bound to headgroups) were additionally analyzed. These consisted of predominantly GDGT-0 and H-GDGT in the active samples, whereas GDGT-0 and methylated H-shaped (Me-H-GDGT) tetraethers dominated in the inactive structure **(Supplementary Table S5)**.

Bacterial and archaeal community composition based on 16S rRNA gene diversity

To obtain a more detailed picture of accompanying microbial diversity, we sequenced bacterial and archaeal 16S rRNA gene amplicons and compared those to the IPL diversity described above. In all samples of surficial crusts from active structures,

bacterial 16S rRNA sequences were predominantly affiliated to *Sulfurimonas*-and *Sulfurovum*-related *Epsilonproteobacteria*, which were close to absent in the inactive chimney samples. In surficial crusts RMR5-Ext-White and RMR5-Ext-Dark, sequences affiliated to thermophilic *Aquificae* were detected **(Figure 3C)**. The inactive chimney SM-I, in contrast, was colonized by a highly diverse bacterial community, with sequences belonging to the phyla *Actinobacteria* and *Planctomycetes,* as well as the *Alphaproteobacteria*, *Gammaproteobacteria* and *Deltaproteobacteria* classes, being most frequently retrieved **(Figure 3C)**.

Although different DNA extraction methods were tested and gene amplification with multiple primer pairs was attempted, for the majority of samples no archaeal 16S rRNA genes could be amplified. Specifically, amplification was only successful for samples from RMR-D (RMR-D-Ext-White and RMR-D-Ext-Orange) and RMR5 'white' surficial crusts (RMR5Ext-White). In all of these *Euryarchaeota* dominated, comprising over 90% of the retrieved sequence reads and mainly affiliated to uncultured representatives within the orders *Archaeoglobales*, *Thermococcales* and *Thermoplasmatales*. Sequences classified as deep-sea hydrothermal vent euryarchaeotic group 2 (DHVE2, Takai and Horikoshi, 1999; Reysenbach *et al*., 2006) within the *Thermoplasmatales* were most frequently retrieved. *Crenarchaeota*and *Thaumarchaeota*-related sequences were less frequent and mainly related to uncultured members of the Marine Group 1 (MG 1) clade **(Supplementary Table S4)**.

TOC and lipid stable carbon isotope ratios

The TOC content of surficial crusts was extremely low (0.005% to 0.035%), but highest in the inactive SM-I structure exterior **(Supplementary Table S6)**. $\delta^{13}C$ values for TOC ($\delta^{13}C_{TOC}$) in the active chimneys were high (-15.5‰ to -21.4‰, including both nonextracted and extracted) relative to $\delta^{13}C_{TOC}$ in inactive SM-I (-28.0‰). The hydrolyzed total lipid extract (TLE) of all samples was dominated by saturated and monounsaturated C16 to C19 fatty acids **(Supplementary Table S7)**, consistent with the composition of bacterial IPL side chains as revealed by negative ionization mode in the same samples. Mono-and diether glycerol lipids comprised only a minor fraction. Ether-cleavage products of archaeal lipids yielded mostly phytane derived from AR - the dominant archaeal IPL. $\delta^{13}C$ values of bacterial fatty acids ranged from ~0‰ to -34.4‰, while archaeal phytane ranged from -13.7‰ to 41.7‰ **(Supplementary Table S7)**.

Similar to $\delta^{13}C_{TOC}$, bacterial and archaeal $\delta^{13}C_{lipid}$ values were higher in active structures relative to the inactive SM-I structure, with 'white' surficial crusts having the highest values **(Figure 4)**.

Discussion

Hydrothermal structures are extremely heterogeneous microbial habitats (Tivey, 2004; Schrenk *et al*., 2008), often complicating representative sampling and investigation of *in situ* metabolic processes (e.g. Olins *et al*., 2013). In the following discussion, we demonstrate that the $\delta^{13}C$ values of bacterial and archaeal lipids represent an additional tool of sufficient sensitivity to elucidate microbial carbon assimilation on these edifices, when integrated with other molecular approaches. We use this tool to build on existing microbiological observations from active and inactive hydrothermal structures in other unsedimented back-arc geologic settings (e.g. Kato *et al*., 2010; Flores *et al*., 2012; Sylvan *et al*., 2013) and place some constraints on the dominant carbon fixation pathways likely operating on structures in these settings. In addition, we discuss the further potential of integrating IPL diversity and $\delta^{13}C_{lipid}$ analysis with available molecular approaches in other hydrothermal systems differing from the system discussed here to deepen our understanding of associated microbial carbon flow.

Carbon assimilation on active sulfide chimneys

During venting, microbial catabolic energy sources in the mixing zones on and within hydrothermal chimney walls strongly depend on the relative abundances of H_2S, CH_4 and particularly H_2 in the fluids (Tivey, 2004; Amend *et al*., 2011). As is typical for vent fluids from unsedimented back-arc systems (e.g. Takai *et al*., 2008; Reeves *et al*., 2011; Flores *et al*., 2012), RMR5 and SM4 vents had very low H_2 and CH_4 relative to hydrothermal fluids from other geologic settings, such as those at Lost City or Rainbow, but high H_2S concentrations (**Table 1**). Thermodynamic considerations indicate that H_2S oxidation is by far the largest energy source available during mixing of such back-arc vent fluids with seawater, with very little to be gained by H_2 or CH_4 oxidation (Amend *et al*., 2011). The dominance of *Epsilonproteobacteria* related to known thiotrophic genera such as *Sulfurimonas* and *Sulfurovum* (Campbell *et al*., 2006; Sievert *et al*., 2007) on all active structures sampled is therefore consistent with their widespread involvement in

reduced sulfur oxidation in hydrothermal environments (Campbell *et al*., 2006; Takai *et al*., 2006; Nakagawa and Takai, 2008; Sievert and Vetriani, 2012). Bacterial 16S rRNA gene diversity in both 'white' and 'dark' surficial crusts primarily consisted of mesophilic *Sulfurovum*-or *Sulfurimonas*-related *Epsilonproteobacteria*, similar to communities described on active chimneys from other back-arc basins (e.g. Kato *et al*., 2010; Flores *et al*., 2012). Sequences related to moderate thermophiles from the epsilonproteobacterial order *Nautiliales* (Campbell *et al*., 2006) and thermophilic members of the phylum *Aquificae* were also present, but less frequent. Such a mixture of temperature tolerances may reflect the steep temperature gradients present in the outer chimney walls (Flores *et al*., 2012), and/or versatility of the organisms (e.g. Campbell *et al*., 2006; Nakagawa and Takai, 2008; Olins *et al*., 2013).

The phospholipid classes PG, PC and PE that made up much of the IPL diversity of both 'white' and 'dark' crusts (see **Figure 3B**) are common among *Bacteria* and therefore not taxonomically specific (e.g. Goldfine, 1984; Dowhan, 1997; Sohlenkamp *et al*., 2003). However, they are nonetheless still consistent with a predominantly epsilonproteobacterial source, and as the dominant lipid classes present should reflect the bulk of the bacterial community present. All three IPL classes were abundant on active sulfide chimneys dominated by *Epsilonproteobacteria* at Lucky Strike (Flores *et al*., 2011; Gibson *et al*., 2013), and PG and PE have been reported in Canadian oil sands dominated by these *Bacteria* (Hubert *et al*., 2012). Moreover, on active chimney exteriors the fatty acids derived from these lipids (mostly C16:0, C16:1ω7, C18:0, C18:1ω9; **Supplementary Table S7**) strongly resemble profiles of cultured isolates among the *Sulfurovum*, *Sulfurimonas* and *Nautiliales* (Inagaki *et al*., 2003; Inagaki *et al*., 2004; Smith *et al*., 2008). APT-bearing IPL, considered to be diagnostic for *Aquificae* (Sturt *et al*., 2004), were only a minor component of lipid inventories in all active chimney surficial crusts **(Figure 3B)**. The unusual fatty acids most abundant in these chemoautotrophs (e.g. C20:1; Jahnke *et al*., 2001) were therefore only detected in trace amounts. Combined with few sequences relating to *Aquificae* **(Figure 3C)**, these observations are consistent with *Epsilonproteobacteria* being the primary source of abundant PC, PG, PE phospholipids on active surficial crusts.

Several factors strongly suggest that $\delta^{13}C_{TOC}$ of the surficial crusts analyzed here primarily reflects microbial biomass and can therefore be used to constrain the dominant carbon assimilation pathway in operation by comparison to $\delta^{13}C_{lipid}$ values (e.g. van der

Meer *et al*., 1998; Zhang *et al*., 2004). In settings where pyrolysis of sedimentary organic matter influences vent fluid chemistry, abundant thermogenic organic compounds could accumulate in chimney walls and skew TOC composition (Simoneit, 1990). There is, however, no evidence for thermogenic contributions to fluids at PACMANUS (Reeves *et al*., 2011). While we cannot completely exclude that TOC in this study may contain other trace allochthonous organic matter, or previous generations of microorganisms, the very low TOC contents of the samples presented here **(Supplementary Table S6)** are remarkably similar to those of active and inactive chimney exteriors from other unsedimented back-arc settings (e.g. Kato *et al*., 2010), where correlations of TOC and total nitrogen (TN) strongly suggest primarily microbial cell organic matter. TOC contents reported for chimney exteriors in sedimented systems with significant organic matter contributions to fluids, in comparison, are over an order of magnitude higher and more variable (e.g. Jaeschke *et al*., 2012).

Given that it is therefore reasonable to assume $\delta^{13}C_{TOC}$ values suffice for estimated microbial biomass $\delta^{13}C$ values, the relative positions of $\delta^{13}C_{TOC}$, $\delta^{13}C_{DIC}$ and $\delta^{13}C_{lipid}$ values allow us to discern the principal carbon fixation pathway responsible for primary productivity on active chimney surficial crusts at PACMANUS. On all active chimneys $\delta^{13}C_{TOC}$ values fall within the range predicted for autotrophic biomass formed using the rTCA pathway (**Figure 4**), which tends to produce smaller negative $\delta^{13}C$ offsets between biomass and DIC than other pathways (e.g. House *et al*., 2003, and refs. therein). Much more compelling, however, are the consistent positive offsets of bacterial $\delta^{13}C_{lipid}$ values relative to biomass from both 'white' and 'dark' surficial crusts of active hydrothermal structures. While lipids are typically ^{13}C depleted relative to biomass for many biosynthetic pathways (Hayes, 1993; Schouten *et al*., 1998), this pattern is characteristically reversed for the rTCA pathway, with $\delta^{13}C_{lipid}$ values typically being higher than biomass by up to 16‰ (van der Meer *et al*., 1998; Jahnke *et al*., 2001; Zhang *et al*., 2004). Hence, ^{13}C-enrichments among prevalent bacterial lipids (**Figure 4**) and the dominance of *Epsilonproteobacteria* strongly implicate the rTCA pathway as being dominant on active structures at PACMANUS. These results support previous gene-based evidence suggesting that the rTCA pathway is widely employed in sulfidic hydrothermal environments with abundant *Epsilonproteobacteria* (Campbell and Cary,

2004; Hügler and Sievert, 2011; Sievert and Vetriani, 2012).

The low abundances of both archaeal IPL and cells on active structures (**Figure 3A, Supplementary Tables S2 and S3**) are consistent with observations from structures in other systems with low vent fluid H_2 abundance, such as Lucky Strike (Flores *et al*., 2011; Gibson *et al*., 2013) and the Eastern Lau Spreading Center (Takai *et al*., 2008; Flores *et al*., 2012). The diversity of archaeal IPLs and core lipids in active structures in this study, however, does indicate general features of a thermophilic archaeal community. Abundant archaeal tetraethers H-GDGT and Me-GDGT were also detected in other hydrothermal vent systems (Blumenberg *et al*., 2007; Jaeschke *et al*., 2012; Gibson *et al*., 2013; Lincoln *et al*., 2013) and cultures of thermophilic methanogens (Stetter *et al*., 1981; Koga *et al*., 1993; Morii *et al*., 1998). MeGDGTs are abundant in the order *Thermococcales* (Sugai *et al*., 2004) and H-GDGTs were detected in a member of the *Thermoplasmatales*-related DHVE2 group (Schouten *et al*., 2007) present in the active structures in this study. Given the likely trace overall contribution of archaeal biomass to surficial crusts, and corresponding lack of biomass $\delta^{13}C$ constraints, it is difficult to say anything conclusive about archaeal carbon assimilation pathways operating in this system.

Variability in $\delta^{13}C$lipid values within active sulfide chimneys

Although both bacterial IPL and 16S rRNA gene diversity did not indicate large differences between microbial communities in 'white' and 'dark' surficial crusts, differences in $\delta^{13}C_{lipid}$ values were evident between these distinctive types of surficial crust. Fatty acids in 'white' surficial crusts were ^{13}C-enriched relative to corresponding biomass values by up to 15‰, approaching dissolved inorganic carbon ($\delta^{13}C_{DIC}$) values available from vent and bottom waters **(Figure 4)**. In 'dark' crusts, fatty acids were only slightly enriched relative to biomass, or overlapped the range of $\delta^{13}C_{TOC}$ on active chimneys (**Figure 4, Supplementary Tables S6 and S7**). Although sample numbers are limited ($\delta^{13}C_{lipid}$ values for both types of crust from a single structure were only obtainable from SM4), this distinctive spatial distribution of bacterial $\delta^{13}C_{lipid}$ values between crusts of very similar appearance and composition suggests variable carbon isotope fractionation by rTCA-performing *Epsilonproteobacteria* on active structure exteriors.

We hypothesize this $\delta^{13}C_{lipid}$ heterogeneity may be due to variability in the advective diffusive flux of vent fluid through chimney walls. If 'white' surfaces received more advective fluid flow relative to 'dark' areas, the observed patterns could originate from temperature and/or substrate abundance effects on $^{13}C/^{12}C$ fractionation. Temperature effects on $^{13}C/^{12}C$ fractionation during lipid biosynthesis are not fully understood (Hayes, 2001), but trends of increasing $\delta^{13}C$ offsets between microbial biomass and DIC have been observed with temperature decreases in cultured hyperthermophiles known to use the rTCA pathway (House *et al*., 2003). Significant increases (~10‰) in $\delta^{13}C_{lipid}$ values over relatively small temperature increases (10 to 35°C) have also been observed in methanotrophic *Bacteria* that utilize the CBB pathway (Jahnke *et al*., 1999). In the case of the structures examined here, obtaining very fine scale and representative *in situ* temperature distributions of both types of surficial crusts to confirm this possibility would have been difficult without disturbing or destroying them. However, silicified filamentous structures, resembling those known from terrestrial hot springs (Konhauser *et al*., 2004; Rodgers *et al*., 2004) and other seafloor hydrothermal structures (Juniper and Fouquet, 1988; Foriel *et al*., 2004), were only evident in 'white' crusts (**Figure 2**). These areas were typically 'shimmering' due to fluid emanation and either surrounded fluid orifices or covered diffusively venting surfaces, suggesting higher temperatures and fluid fluxes. Greater advective fluid transport through 'white' crusts would result in increased amorphous silica supersaturation (Tivey, 1995, 2004), favoring the observed silicification more compared to less porous, thicker-walled 'dark' areas where transport is likely to be more diffusive and no silicified filaments were found. As advective flow areas inevitably would contain higher proportions of vent fluid species such as H_2S and CO_2, effects of substrate abundance are possible but would depend on the substrate involved. In some cases decreased carbon isotope fractionation occurs when substrate was limiting (Londry *et al*., 2008; Bradley *et al*., 2009a), which would be inconsistent with decreased advective flow in 'dark' surficial crusts showing higher $\delta^{13}C_{lipid}$ offsets relative to $\delta^{13}C_{DIC}$. Finally, although archaeol-derived phytanes are non-specific within the archaeal community, their $\delta^{13}C$ pattern on active chimneys (**Figure 4**) suggests the same environmental factors (temperature, substrate availability) affecting bacterial $\delta^{13}C_{lipid}$ values may also influence carbon isotope fractionation in archaeal lipids.

To our knowledge, no previous study has directly examined variability of $^{13}C/^{12}C$ fractionation in identical lipids within an individual hydrothermal sulfide structure. However, Blumenberg *et al*. (2007) reported fatty acid mixtures with a wide range of $\delta^{13}C$ values (-29‰ to +2‰) in a large fragment of a sulfide structure from the Mid Atlantic Ridge. Based on our results, this broad range could partially reflect effects of homogenizing surficial crusts from different advective/diffusive regimes, highlighting the necessity of a finer scaled sampling strategy as applied here.

Carbon assimilation on inactive chimneys

A marked community difference between active and inactive chimneys was apparent in the near complete replacement of *Epsilonproteobacteria* by more diverse *Bacteria* on the SM-I structure, including abundant *Gammaproteobacteria*, *Alphaproteobacteria*, *Planctomycetes* and *Bacteroidetes* **(Figure 3C)**. After venting ceases, the lack of reduced substrates previously present in fluids (e.g. H_2S) is known to induce a shift in microbial community composition toward diverse seafloor weathering and heterotrophic phylotypes (Rogers *et al*., 2003; Cho and Giovannoni, 2004; Ward *et al*., 2006; Kato *et al*., 2010; Sylvan *et al*., 2012; Sylvan *et al*., 2013). Here we demonstrate for the first time that this change is also to some extent apparent in IPL diversity, in our case evidenced by a near disappearance of the thermophilic IPL indicator APT and the appearance of higher proportions of PME and PDME derivatives **(Figure 3B)**.

Compared to active structures, the distinct bacterial community composition is accompanied by lower $\delta^{13}C_{TOC}$ values and bacterial $\delta^{13}C_{lipid}$ values lower than those of TOC (**Figure 4**). This strongly suggests an accompanying change in bacterial carbon assimilation strategy from that of active chimneys. The similarity of fatty acid and TOC $\delta^{13}C$ values could indicate heterotrophy may be occurring (Hayes, 2001), for example, and PME and PDME headgroups have been attributed to heterotrophic *Gammaproteobacteria*, *Alphaproteobacteria* or *Bacteroidetes* in sediments from an asphalt volcano in the Gulf of Mexico (Schubotz *et al*., 2011a). Both the communities evident in 16S rRNA gene diversity and their $\delta^{13}C_{lipid}$ values indicate that carbon assimilation via the rTCA pathway is of no or limited relevance in the inactive SM-I structure, and if autotrophic carbon assimilation is occurring, it is mediated by another

pathway. *Gammaproteobacteria* and *Alphaproteobacteria*, in contrast to *Epsilonproteobacteria*, are suggested to predominantly use the CBB cycle for autotrophic carbon fixation (Hügler and Sievert, 2011), which results in more ^{13}C-depleted biomass with δ^{13}C values up to ~30‰ lower than DIC (House *et al*., 2003). Fatty acids produced by the CBB pathway are typically ^{13}C-depleted relative to biomass by 7.6 to 9.9‰ (Sakata *et al*., 1997), and in SM-I they are depleted by up to 6.3‰. Both the large $\delta^{13}C_{TOC}$ offset relative to $\delta^{13}C_{DIC}$, and the relatively small negative offset between $\delta^{13}C_{lipid}$ and $\delta^{13}C_{TOC}$ values are reasonably consistent with carbon fixation via the CBB pathway, suggesting it may be important for primary productivity on inactive structures.

Interestingly, the TOC content of SM-I is over twice that of the active structures **(Table S6**), and similar TOC enrichments relative to active structures have been reported in inactive structures from other unsedimented back-arc systems (Kato *et al*., 2010). IPL concentrations are also highest on the exterior of SM-I, though TCC are not especially elevated relative to the active structures **(Supplementary Figure S2)**. This discrepancy relative to active chimney surfaces may simply be due to an inherent heterogeneity in inactive structures (e.g. Sylvan *et al*., 2013) that we did not resolve with our single surficial crust sample. It could, however, also be due to the likely different turnover times of the lipid and DNA pools probed in this study, and hence the chances that these integrate disparate stages of venting and associated microbial activity. For example, the abundance of the archaeal Me-H-GDGT core lipids, thus far only described from cultures of the thermophilic methanogen *Methanopyrus kandleri* (Liu *et al*., 2012), and OH-AR **(Supplementary Table S4)**, a typical core lipid from methanogenic *Archaea* (Koga and Nakano, 2008), indicate methanogens as the most likely source for some archaeal lipids in SM-I, despite methanogenesis only being a realistic metabolic energy source during active venting (Tivey, 2004). Archaeal cell numbers were below detection and archaeal 16S rRNA genes were not amplifiable from the SM-I exterior, which is in line with other studies that also reported negligible abundances of *Archaea* on inactive sulfide chimneys in back-arc environments (Kato *et al*., 2010; Sylvan *et al*., 2013). The possibility that the detected archaeal IPL are relics of past communities therefore exists (cf. Xie *et al*., 2013), and could explain the very low phytane δ^{13}C values in SM-I, given the ubiquity of acetyl-CoA pathway in methanogens (e.g. Berg *et al*., 2010). However,

with little known about viable archaeal diversity on these and inactive structures in general, and trace IPL concentrations, further organic geochemical and microbiological investigations are needed.

Implications and applications for other active hydrothermal sulfide structures

Our observations suggest that microbes using the rTCA cycle are likely to be the dominant contributors to vent primary productivity on active hydrothermal structures that vent H_2S-rich but H_2- and CH_4-poor fluids similar to those at PACMANUS i.e. those systems dominated by S-oxidation metabolisms. These fluid characteristics are common in numerous other systems, such as the Lau Basin structures examined reported by Flores *et al*. (2012). Similar $\delta^{13}C_{lipid}$ patterns may be found in comparable vent structures from the East Pacific Rise or Mid-Atlantic Ridge, for example, where these metabolisms are predicted to dominate available energy (Amend *et al*., 2011).

In hydrothermal sulfide structures characterized by more H_2 and/or CH_4-rich fluids, or by thermogenic organic inputs to fluids, microbial communities and their metabolic and carbon assimilation pathways will undoubtedly be more diverse (Amend *et al*., 2011). Although no $\delta^{13}C_{lipid}$ data were reported for IPL from the H_2-rich Rainbow site (Gibson *et al*., 2013), carbon isotope analyses of the high concentrations of archaeal lipids observed could be used elucidate the carbon assimilation strategies employed by thermophilic *Archaea* present there. On sulfide structures from the sedimented Middle Valley system, Olins *et al*. (2013) recently suggested that carbon may be predominantly assimilated via the CBB pathway based on a large estimated offset between $\delta^{13}C_{TOC}$ and estimated $\delta^{13}C_{DIC}$ values, despite comparable expression of proxy genes for both the rTCA and CBB pathways, and more diverse microbial communities. ^{13}C-depleted thermogenic organic compounds are abundant in vent fluids at Middle Valley (Cruse and Seewald, 2006), however, and could condense in chimney walls (Simoneit, 1990), complicating the use of $\delta^{13}C_{TOC}$ values to approximate biomass $\delta^{13}C$ in sedimented systems. The ^{13}C-depleted nature of thermogenic vent fluid CO_2 from many of these same structures (-20.7‰ to -34.6‰; Cruse and Seewald, 2006) further suggests this offset may actually be smaller than would be expected for the CBB pathway. $\delta^{13}C_{lipid}$ values may provide an alternative approach to assess the relative importance of CBB or rTCA pathways at Middle Valley.

The relatively simple approach presented here, in which generic PC, PG and PE derived fatty acids serve as to examine relationships to TOC, would not be sufficient in the cases discussed above, but other targeted $\delta^{13}C_{lipid}$ approaches could be applied to aid molecular investigations of microbial carbon flow. For example, head-group specific separations of IPL, prior to cleaving of side chains, allow stable carbon isotope analyses of individual IPL classes (Schubotz *et al*., 2011a), and may help resolve diverse mixtures of carbon assimilation signatures such as those at Middle Valley. More detailed characterization of bulk organic matter (e.g. C:N ratios) may aid in detecting any skewing of chimney TOC by thermogenic organic matter. Consumption of organic compounds in vent fluids (e.g. thermogenic organic acids such as formate and acetate) could be examined by pairing their isotopic analyses with $\delta^{13}C_{lipid}$ values, similar to recent approaches in terrestrial hot springs (e.g. Schubotz *et al*., 2013).

Given the evidence suggesting further heterogeneity among similar communities presented here, our results suggest careful subsampling of multiple areas of chimney exteriors is needed, rather than homogenization of large fragments. The sensitivity demonstrated here in the form of additional hitherto unknown heterogeneity in $\delta^{13}C_{lipid}$ values, suggests, however, that such patterns may be resolvable and further probed.

Conclusions

By combining organic geochemical, stable carbon isotope and phylogenetic marker approaches, we have assessed the distribution of microbial carbon assimilation pathways on active and inactive hydrothermal sulfide chimneys, i.e. the structures that dominate many volcanically active seafloor environments. We expanded on existing microbiological observations from similar geologic settings (e.g. Kato *et al*., 2010; Flores *et al*., 2012; Sylvan *et al*., 2013) to illustrate the dominance of the rTCA pathway in vent primary productivity on active structures that emit H_2S-rich and H_2- and CH_4-poor fluids. Our data also further offer new detail through a more refined subsampling strategy, suggesting hitherto unobserved variations in $\delta^{13}C_{lipid}$ values on a single active chimney, possible due to advective-diffusive fluid flow variability through chimney walls. The different microbial community composition and apparent carbon assimilation pathway in the inactive structure highlights the critical role of hydrothermal fluids in driving carbon cycle dynamics and isotope systematics of biomass synthesis in these settings. This

study demonstrates that $\delta^{13}C_{lipid}$ values can be a valuable probe for tracking carbon flow in these settings, if integrated with other molecular approaches to circumvent technical limitations of individual methods.

Experimental procedures

Sample collection and subsampling

All sulfide structures were collected from the unsedimented PACMANUS vent field **(Figure 1; Supplementary Figure S1)** using the ROV *Quest 4000m* during R/V *Sonne* expedition SO-216 in June/July 2011 (Bach and participants, 2011). The structures were kept in closed bio-boxes during ROV ascent and frozen at -20°C upon shipboard retrieval. Surficial crust samples (~1-3 mm thick) from large surface areas of the chimneys were removed at sea (for microbial diversity and cell counts analysis) or onshore (for lipid and TOC analyses) using sterile solvent-rinsed spatulas (e.g. Reysenbach *et al*., 2006; Flores *et al*., 2011), and either frozen (-20°C) for subsequent analysis or processed immediately. As stated above, samples of 'white' and 'dark' surficial crust were removed from the active chimneys, as well as the 'orange' mat covering RMR-D 'white' crusts.

'White' and 'dark' surficial crust samples from SM4, and exterior and interior portions of RMR-D and SM-I were examined by scanning electron microscopy with energy-dispersive X-ray spectroscopy (SEM-EDS) to determine the dominant mineral phases. These samples were first washed multiple times with Milli-Q water to removal of sea-salt residues on mineral surfaces and freeze-dried to remove water. Samples that were not rinsed (in order to preserve fine morphological features, e.g. filaments) were also examined in parallel.

Vent fluids from RMR5 and SM4, accompanying temperatures, and background seawater were collected using isobaric gas-tight samplers (Seewald *et al*., 2002) and analyzed as described in further detail in Reeves *et al*., 2011. Temperatures could not be determined or fluids collected from RMR-D due to limitations in dive duration.

Microbial lipid and total organic carbon analyses

The total lipid extract (TLE) of each sample (1.3 g to 10 g wet wt.) was obtained by a modified Bligh and Dyer extraction (Bligh and Dyer, 1959; Sturt *et al*., 2004), after adding 3 g combusted sand (only to surficial crust samples) and an internal standard (phosphatidylcholinediacylglyceride (PC) C21:0/21:0). Analyses of IPLs were performed

with an aliquot of the TLE (4–5%) by normal phase ultra-high performance liquid chromatography (U-HPLC) coupled to ultra-high resolution quadrupole time-of-flight mass spectrometry (QToF-MS), both in positive and negative electrospray ionization modes (cf. Wörmer *et al*., 2013). IPL concentrations were calculated from the relative response of each analyte compared to the internal PC standard in positive mode and we did not account for differences in response factors between various lipid classes due to the lack of authentic standards (cf. Schubotz *et al*., 2009). Identification of IPL (and associated fatty acid side chains) was based on neutral loss or diagnostic fragment ion loss in both positive and negative ion mode (cf. Sturt *et al*., 2004). Qualitative analysis of archaeal core and intact tetraether lipid diversity was performed in reversed phase and positive ionization mode according to Zhu *et al*., 2013.

To determine bacterial and archaeal $\delta^{13}C_{lipid}$ values, an aliquot of each TLE (25–50%) was chemically hydrolyzed using both mild saponification (yielding fatty acids, Elvert *et al*., 2003) and ether cleavage (yielding phytane, Summons *et al*., 1998) to retrieve GC-amenable side-chain moieties. $\delta^{13}C$ values from individual lipid side chains were determined (correcting for the addition of a methyl group during derivatization of fatty acids) using gas chromatography coupled to a combustion unit and isotope-ratio-monitoring mass spectrometer (GC-irms) according to the methods of Schubotz *et al*., 2011a.

Separate representative samples of surficial crusts (RMR-D-Ext-White and SM-I-Ext) were collected, homogenized and analyzed for the concentration of total organic carbon (TOC) and its isotopic composition ($\delta^{13}C_{TOC}$, referred to as non-extracted TOC). Residual solids from Bligh and Dyer extraction of bulk chimney fragments (RMR5-Bulk-Dark and SM4-Bulk-Dark) were also analyzed for TOC and $\delta^{13}C_{TOC}$ for direct comparison with extracted lipids and the above non-extracted biomass. TOC and $\delta^{13}C_{TOC}$ were determined on decalcified samples by dual inlet mass spectrometry according to Heuer *et al*., 2010. All $\delta^{13}C$ values in this study are reported in ‰ relative to the Vienna Pee Dee Belemnite (V-PDB) standard.

DNA extraction and 454-pyrosequencing

DNA was extracted from 0.75-1 g of sample with the Ultra Clean Soil DNA Kit (MoBio Laboratories) following the manufacturer's protocol. 16S rRNA genes were

amplified with bacterial primers GM3F (Muyzer *et al*., 1995) and 907RM (Muyzer *et al*., 1998) or archaeal primers 20F (Massana *et al*., 1997) and 958RV (Klindworth *et al*., 2013) in ten parallel PCR reactions. The primers were modified for 454-pyrosequencing, so that each reverse primer contained a sample specific, error-tolerant, hexamer barcode at the 5'-end, and both forward and reverse primers were extended at the 5'-end with an asymmetric SfiI restriction site for subsequent ligation of 454-sequencing adapters. Briefly, 1x Phusion HF Buffer (Finnzymes, New England BioLabs Inc.), 250 µM of each deoxynucleoside triphosphate, 0.5 µM of forward and reverse primer, 0.4 U of Phusion DNA polymerase (Finnzymes, New England BioLabs Inc.) and 0.5–2 µL of DNA template were mixed in 20 µL PCR reactions. Gene amplification was performed under the following conditions: 3 minutes initial denaturation at 98°C, 25 or 30 cycles of 30 seconds denaturation at 98°C, 30 seconds annealing at 48°C or 54°C (for bacterial or archaeal primers, respectively) and 30 seconds elongation at 72°C, finalized by 10 minutes of elongation at 72°C. Replicate reactions were pooled and DNA was precipitated by addition of 3.3 volumes of non-denaturated, absolute ethanol and 0.15 volumes of 3 M Na-Acetate (pH 5.2). After 1 hr incubation on ice, DNA was pelleted by centrifugation (20 min at 10,000 g), re-suspended in 20 µL 1xTE buffer and visualized on a 1% LE Agarose gel with SYBR Green I Nucleotide Gel Stain (Invitrogen). DNA bands were excised and purified with the MinElute Gel Extraction Kit (Qiagen), as instructed by the accompanying protocol. 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.

From several samples, no archaeal 16S rRNA genes were amplified with the above described protocol. In an attempt to overcome the problem and minimize biases introduced by DNA extraction and primer selection (Martin-Laurent *et al*., 2001; Feinstein *et al*., 2009; Klindworth *et al*., 2013), alternative protocols were tested. DNA was extracted with the Power Soil DNA Kit (MoBio Laboratories) and a SDS-based DNA extraction protocol after Zhou *et al*., 1996 on 1 or 3 g sample sizes, respectively. Besides those listed above, a modified archaeal 20F primer (also targeting *Nanoarchaeota*; personal communication, K. Knittel) and the reverse primer Uni1390R (Zheng *et al*., 1996) were tested. Nevertheless, amplification of archaeal 16S rRNA genes from further samples remained unsuccessful.

Library construction and 454-pyrosequencing was performed by the Max Planck

Genome Center (Cologne, Germany) for library construction and 454-pyrosequencing. Briefly, amplicon pools were digested with a SfiI restriction enzyme (NEB) for 1 hour at 50°C and purified with the MinElute PCR purification kit (Qiagen). Following, 454 adaptors (A and B) were ligated with 1 U T4 DNA ligase (Roche) overnight at 6°C. A 10 min heat inactivation at 65°C, followed by size fractionation on a 2% LE agarose gel to remove unligated adapters, was performed. Obtained gel bands were cut out and purified with the Qiaquick gel extraction kit (Qiagen). Emulsion PCRs with the GS FLX Titanium LV emPCR Kit (Lib-L), and sequencing from the 3'-end (reverse primer) with the GS FLX Titanium Sequencing Kit XLR70 on a Roche 454 Genome Sequencer FLX+ instrument according to manufacturer's protocols, were performed.

Analysis of 16S rRNA gene 454-pyrosequence reads

Analysis of all retrieved 16S rRNA gene 454-pyrosequences was performed with the sff_extract tool (version 0.2.8; http://bioinf.comav.upv.es/sff_extract/index.html) and the bioinformatics pipeline of the SILVA rRNA gene database project (Quast *et al*., 2013) as described by Klindworth *et al*., 2013. Briefly, reads were extracted and clipped with the 'sff_extract-c' option of the sff_extract toll package. Thereafter, reads that passed this quality check were aligned with the SILVA Incremental Aligner -SINA (Pruesse *et al*., 2012) against the SILVA 16S rRNA SSU SEED alignment. In a further quality control step, all reads with an alignment quality below 50%, shorter than 200 base pairs or containing >2% homopolymers and/or ambiguities were excluded from the dataset. Remaining reads were dereplicated and clustered with Cd-hit at 98% sequence identity (Li and Godzik, 2006) and a taxonomic path was assigned to each cluster based on the results of a local nucleotide BLAST search (Camacho *et al*., 2009) against the SILVA 16S rRNA SSU reference database, release 111. Sequences that passed quality controls, but had an average BLAST alignment coverage and alignment identity of less than 93% are presented as 'unclassified'. An overview of results is presented in **Supplementary Table S1** and a rarefaction analysis in **Supplementary Figure S3**.

TCC and CARD-FISH sample preparation and analysis

After recovery on board, samples designated for total cell count (TCC) determination and catalyzed reporter deposition - fluorescence *in situ* hybridization (CARD-FISH) were fixed for 4 hours at 4°C with formaldehyde (2% final concentration in PBS -137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4). After fixation, the

samples were washed twice with PBS and transferred to a 2:3 PBS ethanol mixture for transport and storage at 20°C. On shore, samples were shortly centrifuged to pellet solid material and 0.1-0.2 mL of pellet were transferred to 1 mL of fresh, cooled 1:1 PBS ethanol mix for cell detachment by ultrasonication (Ishii *et al*., 2004). Shortly, the samples were sonicated for 30 seconds at low intensity (20%) with the Sonoplus HD70 sonication probe (Bandelin). 500 µL of the supernatant were removed and replaced with 500 µL of fresh 1:1 PBS ethanol. This procedure was repeated 5 times and removed supernatants were collected and pooled. Following, 10-100 µL of the sonication product (depending on particle density in the supernatant) were mixed with 5 mL PBS and brought on a polycarbonate membrane (type GTTP, 0.2 µm pore size; Millipore) by vacuum filtration.

For TCC determination, membrane filter slices were placed on glass slides and mounted with a SYBR Green I-based DNA staining solution (190 µL Mowiol, 5 µL SYBR Green I 1000x stock solution and 5 µL 1 M ascorbic acid in TAE buffer), covered with a cover slip and incubated for 10 minutes as described in Lunau *et al*., 2005. To determine the relative abundance of *Bacteria* and *Archaea,* CARD-FISH on agarose-embedded filter pieces was performed following the protocol given in Ishii *et al*., 2004. The *Bacteria*-specific probe mix EUB I-III (Amann *et al*., 1990; Daims *et al*., 1999) and the *Archaea*-specific probe ARCH915 (Stahl and Amann, 1991) were applied, as well as the negative control Non388 (Wallner *et al*., 1993). For all samples and oligonucleotide probes, hybridization was performed at 46°C for 3 hours in a 35% formamide buffer and signal amplification with Alexa 488-labeled tyramide for 30-45 minutes at 46°C. Lastly, samples were counterstained with 4',6'-diamidino-2-phenylindole (DAPI) and embedded in an anti-fading mounting medium consisting of a 4:1 mixture of Citifluor (Citifluor Ltd) and VECTASHIELD® (Vector Labs). Microscopic analyses were conducted with the epifluorescence microscope AxioSkop 2 mot plus (Carl Zeiss).

Nucleotide sequences

Sequence reads retrieved in this study have been deposited in the European Nucleotide Archive (ENA) sequence read archive (http://www.ebi.ac.uk/ena/) and can be found under accession numbers ERR435815–ERR435825 (study accession number PRJEB5213).

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FIGURES AND CAPTIONS (MAIN ARTICLE)

Figure 1: Photographs of the hydrothermal diffuser structure RMR-D (A), the active smokers SM4 (B) and RMR5 (C), and the inactive structure SM-I (D) at the seafloor and upon shipboard recovery, with sampled surficial areas denoted. In sample nomenclature, the suffix '-Ext-' denotes exterior surficial crusts (<3 mm depth) of '-White' or '-Dark' appearance, respectively, while '-Bulk-' denotes larger, bulk wall fragments or whole chimney conduits. Visibly venting fluid orifices on active structures are denoted by asterisks (*). RMR-D (A) was observed to be diffusely venting clear fluid (lacking particulate 'smoke') over the entire surface and was located among a small complex of similar diffuser-type structures. No inherent 'dark' crusts were obvious on this structure (those shown are due to ROV manipulator damage) but the 'orange' mat overlaying 'white' crusts was sampled (RMR-D-Ext-Orange). For SM4 (B) and RMR5 (C), fluids were collected from adjacent accessible orifices on the same parent structures. The inactive SM-I structure (D) was located in a complex of extinct chimneys away from any visible fluid flow. Dotted lines and arrows show the original structure configurations before shipboard fragmentation.

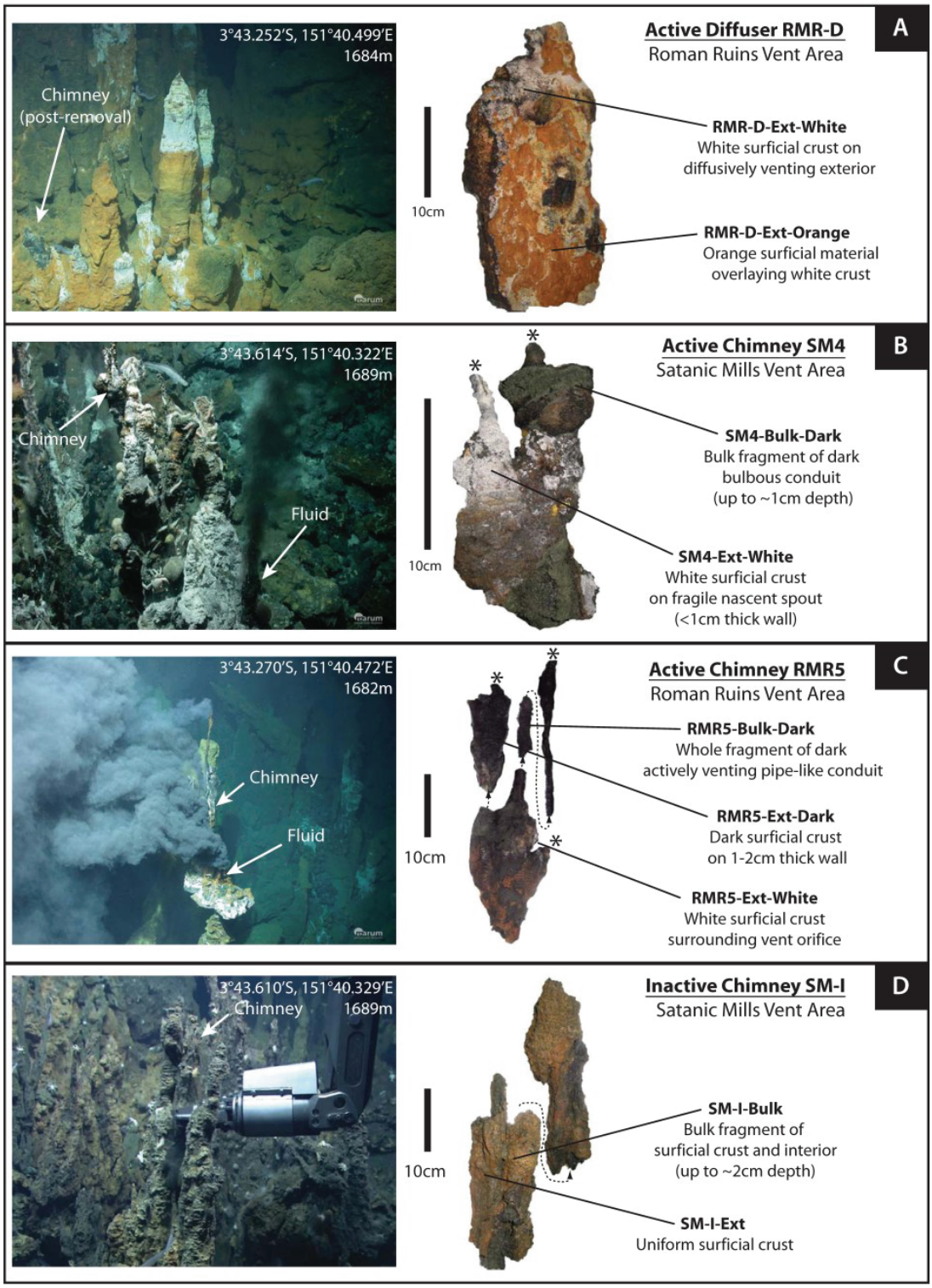

A
3°43.252'S, 151°40.499'E
1684m
Chimney
(post-removal)
10cm
Active Diffuser RMR-D
Roman Ruins Vent Area
RMR-D-Ext-White
White surficial crust on
diffusively venting exterior
RMR-D-Ext-Orange
Orange surficial material
overlaying white crust
B
3°43.614'S, 151°40.322'E
1689m
Chimney
Fluid
10cm
Active Chimney SM4
Satanic Mills Vent Area
SM4-Bulk-Dark
Bulk fragment of dark
bulbous conduit
(up to ~1cm depth)
SM4-Ext-White
White surficial crust
on fragile nascent spout
(<1cm thick wall)
C
3°43.270'S, 151°40.472'E
1682m
Chimney
Fluid
10cm
Active Chimney RMR5
Roman Ruins Vent Area
RMR5-Bulk-Dark
Whole fragment of dark
actively venting pipe-like conduit
RMR5-Ext-Dark
Dark surficial crust
on 1-2cm thick wall
RMR5-Ext-White
White surficial crust
surrounding vent orifice
D
3°43.610'S, 151°40.329'E
Chimney
1689m
10cm
Inactive Chimney SM-I
Satanic Mills Vent Area
SM-I-Bulk
Bulk fragment of
surficial crust and interior
(up to ~2cm depth)
SM-I-Ext
Uniform surficial crust

Figure 2: Scanning electron (SEM) micrographs of siliceous filamentous structures found in abundance on RMR-D-Ext-White as aggregated clusters (A) or individual rods (B and C). Elemental analysis of individual rods using Energy Dispersive Spectroscopy (SEM-EDS) are denoted in (B) and (C), with the most abundant elements annotated. Na and Cl are due to precipitated halite as these samples were not rinsed with water in order to preserve the fragile structures.

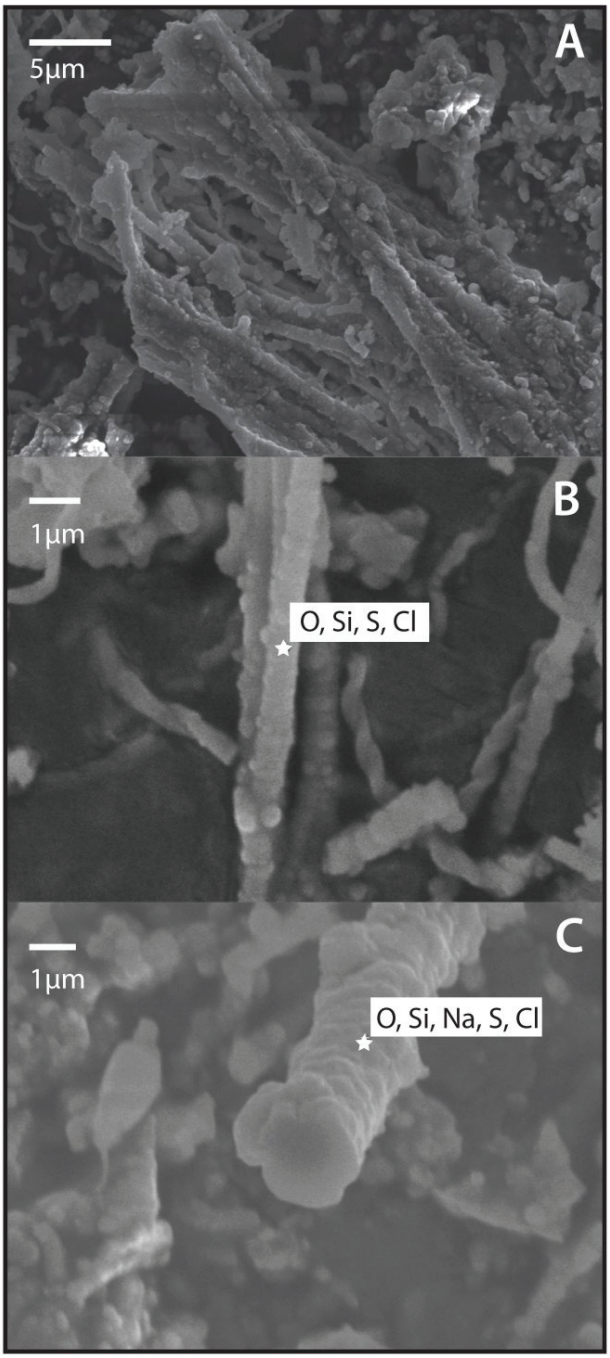


Figure 3: Proportion of bacterial versus archaeal intact polar lipids (IPL) in samples (cf. Figure 1 for sample locations) of the active and inactive structures **(A)**, along with corresponding diversity of bacterial IPL **(B)** and 16S rRNA gene-based taxonomic diversity of *Bacteria* **(C)**. IPL are abbreviated as follows: PE, Phosphatidylethanolamine; PME, Phosphatidyl-(N)-methylethanolamine; PDME, Phosphatidyl-(N,N)-dimethylethanolamine; PC, Phosphatidylcholine; APT, Phosphoaminopentanetetrol; PG*, Phosphatidylglycerol and DPG*, Diphosphatidylglycerol (*including Lyso-PG and -DPG); OL, Ornithine lipids; BL, Betaine lipids; G, Glycolipids. For full structural details of these lipids, see Sturt *et al*. (2004) and Schubotz *et al*. (2011b).

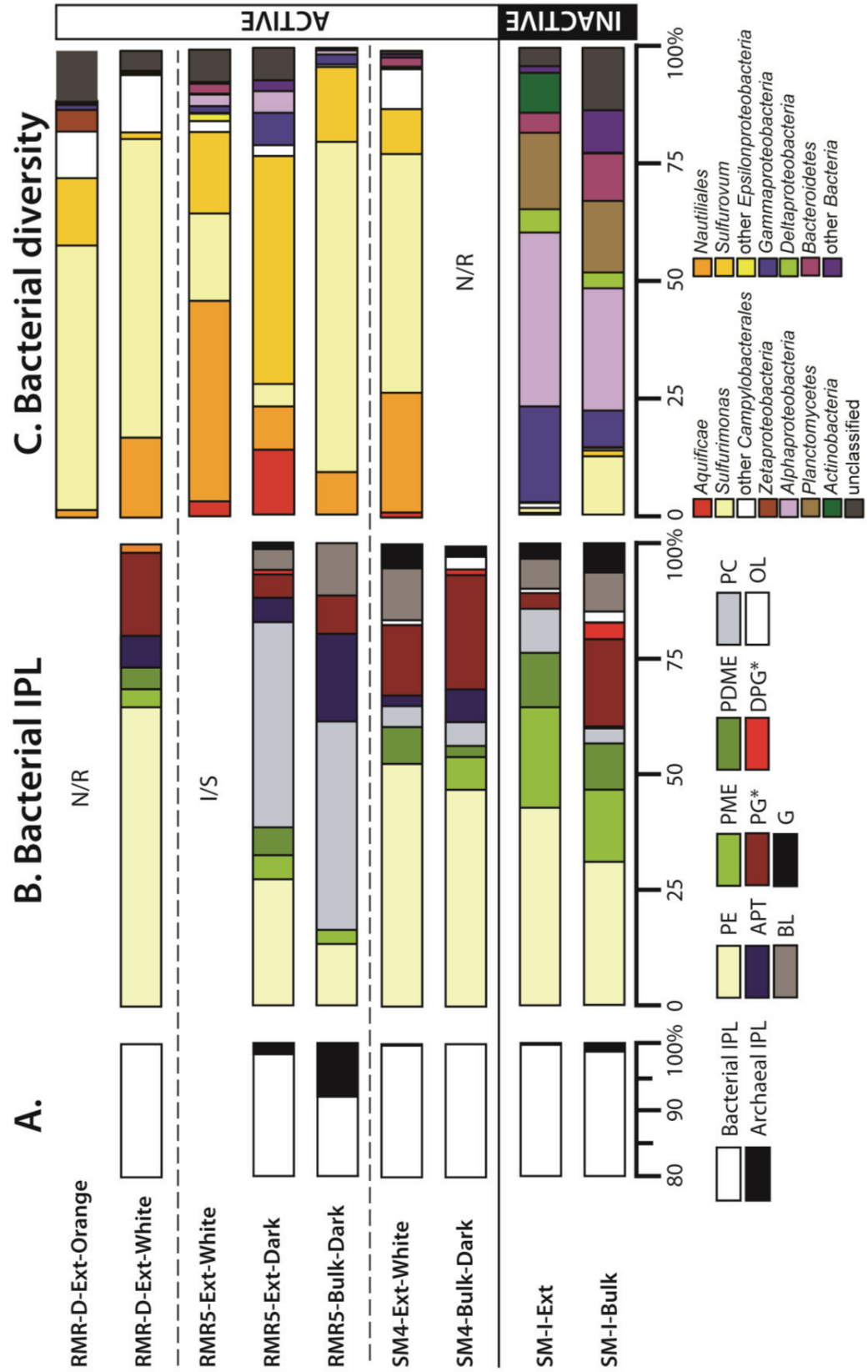
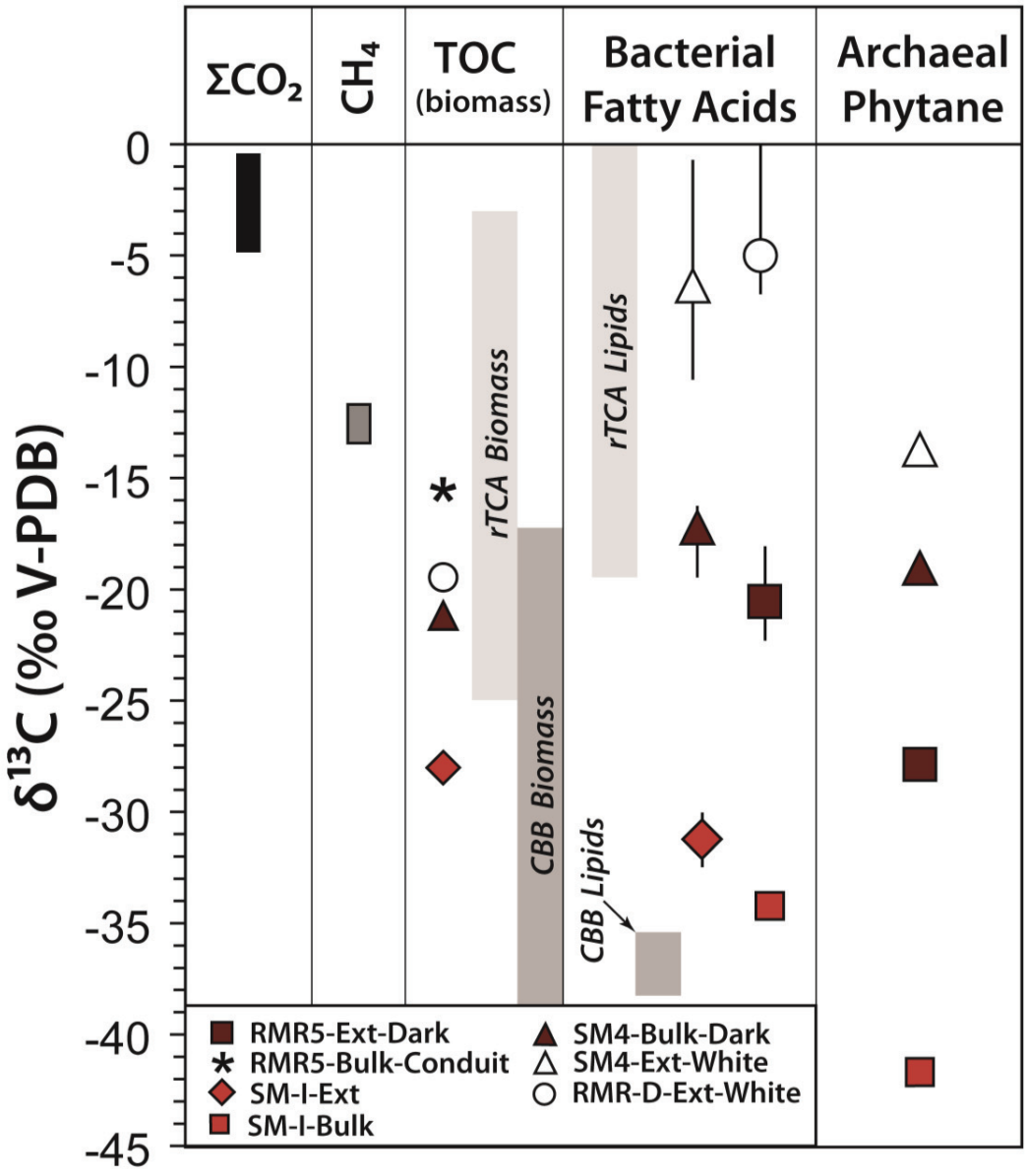

A.
B. Bacterial IPL
C. Bacterial diversity
ACTIVE
INACTIVE
N/R
I/S
N/R
RMR-D-Ext-Orange
RMR-D-Ext-White
RMR5-Ext-White
RMR5-Ext-Dark
RMR5-Bulk-Dark
SM4-Ext-White
SM4-Bulk-Dark
SM-I-Ext
SM-I-Bulk
Bacterial IPL
Archaeal IPL
PE
APT
BL
PME
PG*
G
PDME
DPG*
PC
OL
0
25
50
75
100%
80
90
100%
Aquificae
Sulfurimonas
other Campylobacterales
Zetaproteobacteria
Alphaproteobacteria
Planctomycetes
Actinobacteria
unclassified
Nautiliales
Sulfurovum
other Epsilonproteobacteria
Gammaproteobacteria
Deltaproteobacteria
Bacteroidetes
other Bacteria

Figure 4: Plot of bacterial and archaeal $\delta^{13}C_{lipid}$ values, and accompanying $\delta^{13}C_{TOC}$ values for chimney samples relative to the $\delta^{13}C$ ranges of dissolved inorganic carbon (ΣCO_2, DIC) and CH_4 in the associated fluids. For fatty acids, symbols plotted are the mass-weighted average, with the absolute range shown as lines, of all detected fatty acids. For comparison to TOC, the maximum predicted ranges of biomass $\delta^{13}C$ for both the rTCA and CBB pathways (using our $\delta^{13}C_{DIC}$ values) are plotted using data from the compilations of House *et al*. (2003) and Havig *et al*. (2011), and references therein. For comparison to bacterial $\delta^{13}C_{lipid}$ values, also plotted are the maximum predicted ranges of bacterial $\delta^{13}C_{lipid}$ values for both pathways (values from van der Meer *et al*., 1998 and Sakata *et al*., 1997), relative to our $\delta^{13}C_{TOC}$ estimates of biomass. Lipids from RMR5-Bulk-Dark were not in sufficient abundance **(see Supplementary Table S3)** to accurately quantify $\delta^{13}C_{lipid}$ values.


ΣCO2
CH4
TOC
(biomass)
Bacterial
Fatty Acids
Archaeal
Phytane
δ13C (‰ V-PDB)
0
-5
-10
-15
-20
-25
-30
-35
-40
-45
rTCA Biomass
CBB Biomass
rTCA Lipids
CBB Lipids
RMR5-Ext-Dark
RMR5-Bulk-Conduit
SM-I-Ext
SM-I-Bulk
SM4-Bulk-Dark
SM4-Ext-White
RMR-D-Ext-White

MAIN ARTICLE TABLE 1

Table 1. Endmember* concentrations and isotope compositions of hydrothermal fluids associated with the active structures

Vent	Depth	T_{max}	pH	Cl	ΣH_2S	H_2	CH_4	$\delta^{13}C_{CH4}$	ΣCO_2	$\delta^{13}C_{DIC}$
	m	°C	(25°C)	mmol/kg	mmol/L	mmol/L	mmol/L	‰ V-PDB	mmol/kg	‰ V-PDB
RMR5	1679	324	2.6	734	3.5	0.023	0.032	-13.3	19.0	-4.9
SM4	1774	345	3.0	357	18.1	0.14	0.028	-11.7	294	-2.3
Deep Seawater	1289	4	7.8	536	0	0	0	-	2.29	-0.49

*Calculated by extrapolation of measured compositions to zero Mg (see Reeves *et al*. (2011) for details). Lowest measured Mg concentrations were <10% of seawater. T_{max} = maximum temperature measured in real-time during fluid collection.
ΣH_2S = total dissolved sulfide. ΣCO_2 = total dissolved inorganic carbon (DIC)

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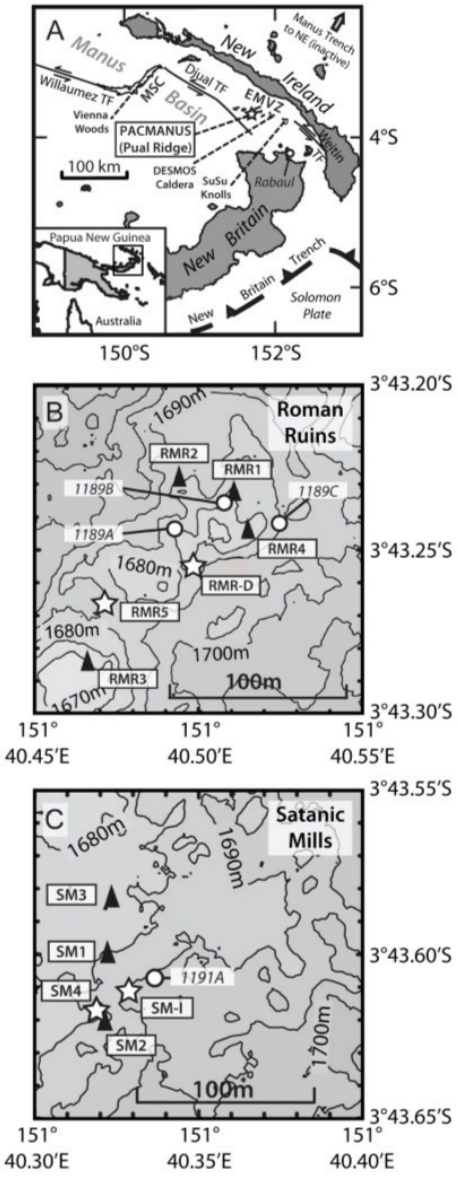
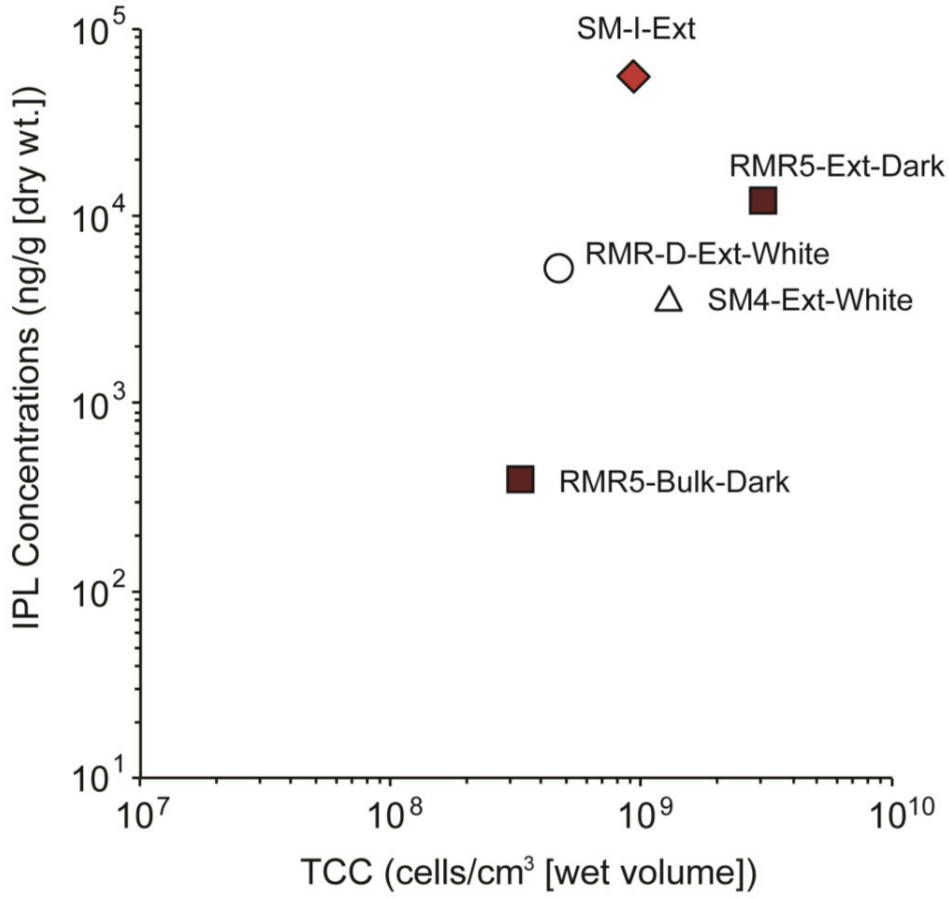
Supplementary Information

Supplementary Figures and Tables

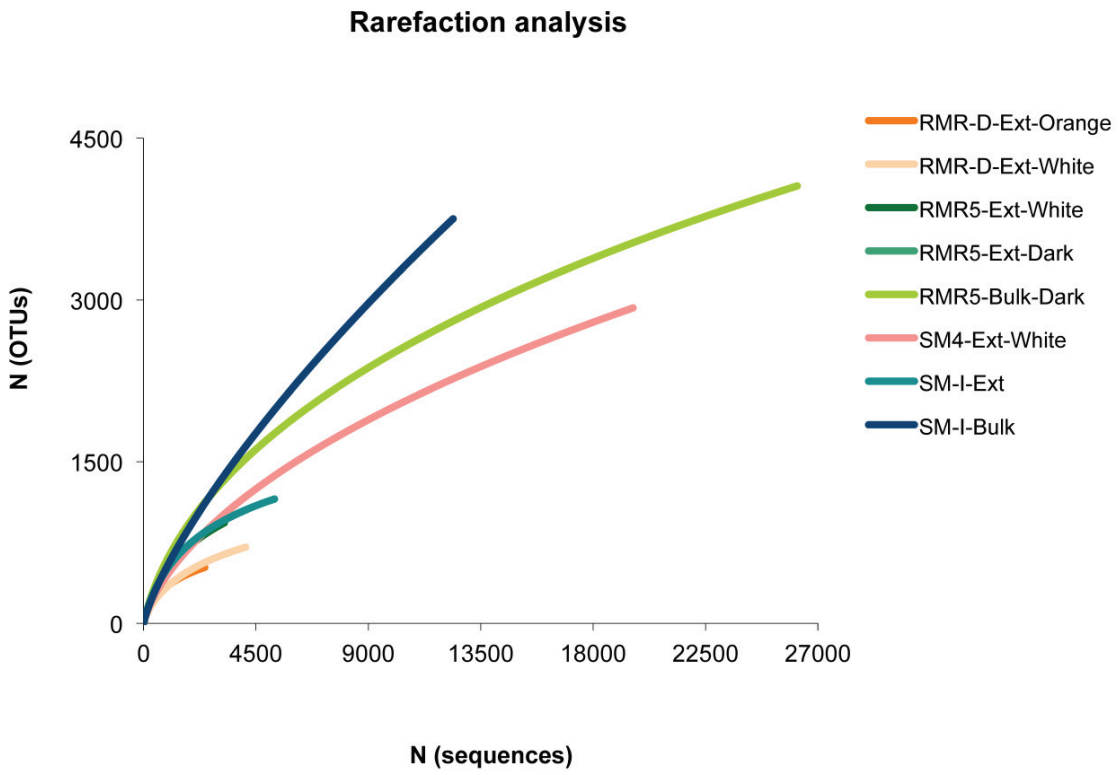
Supplementary Figure S1: Regional map of the Manus Basin showing the location of the PACMANUS vent field **(A)**. The locations (*stars*) of the RMR5 and RMR-D vent structures sampled from the Roman Ruins (RMR) area are shown in **(B)**, and SM4 and SM-I from Satanic Mills (SM) are shown in **(C)**. Vents previously sampled in 2006 and Ocean Drilling Program Leg 193 drillholes are also shown. Maps are modified from those of Reeves *et al*. (2011).



Supplementary Figure S2: Plot of IPL concentrations versus total cell counts (TCC). As samples were preserved for TCC at sea, units are necessarily cells/mL (wet weight). IPL and TCC correlate reasonably well in active structures, but the single sample for SM-I does not lie on this trend.



Supplementary Figure S3: Rarefaction analysis of 454-pyrosequences from bacterial 16S rRNA gene amplicons (OTUs clustered at 98% sequence identity).



SUPPLEMENTARY INFORMATION TABLES S1-S7

Supplementary Table S1. Statistics of the 454-pyrotag data obtained from the bioinformatics pipeline of the SILVA rRNA gene database project (Quast *et al*., 2013).

Sample	Sequences (after filtering)	Average length (bp)	OTUs*	Clustered**	Replicates***
		Bacterial 16S rRNA gene sequences			
RMR-D-Ext-Orange	2454	423.8	236	1198	1020
RMR-D-Ext-White	4088	387.1	317	1110	2661
RMR5-Ext-White	3243	421.3	563	1152	1528
RMR5-Ext-Dark	40	438.0	14	6	20
RMR5-Bulk-Dark	26174	435.9	1928	14168	10078
SM4-Ext-White	19600	519.8	2926	11575	5099
SM-I-Ext	5245	439.1	447	3054	1744
SM-I-Bulk	12397	511.3	3752	6259	2386
		Archaeal 16S rRNA gene sequences			
RMR-D-Ext-Orange	19450	388.2	1146	11122	7182
RMR-D-Ext-White	326	422.8	51	196	79
RMR5-Ext-White	662	430.8	71	402	189

* number of reference reads in sample based on 98% sequence identity

** number of reads in sample assigned to a cluster, based on 98% sequence identity

*** number of reads identical (100%) to another read within the same sample

Supplementary Table S2.
Total cell counts (TCC) and CARD-FISH quantification of relative bacterial and archaeal cell abundances

Sample	TCC cells/cm³ (wet)	CARD-FISH EUB I-III	CARD-FISH ARCH915
RMR-D-Ext-Orange	1.1×10^9	85%	<1%
RMR-D-Ext-White	4.6×10^8	88%	3.5%
RMR5-Ext-White	*no sample	*no sample	*no sample
RMR5-Ext-Dark	3.1×10^9	55%	<1%
RMR5-Bulk-Dark	3.3×10^8	81%	6.3%
SM4-Ext-White	1.3×10^9	87%	1.8%
SM4-Bulk-Dark	*no sample	*no sample	*no sample
SM-I-Bulk	9.4×10^8	87%	<1%

*Insufficient material to perform all analyses

Supplementary Table S3.
Intact polar lipid (IPL) concentrations in samples (ng/g of dry wt.)

Sample	Bacterial IPL ng/g (dry)	Archaeal IPL ng/g (dry)	Total IPL ng/g (dry)
RMR-D-Ext-Orange	n/d	n/d	-
RMR-D-Ext-White	5.4×10^3	n/d	5.4×10^3
RMR5-Ext-White	*no sample	*no sample	-
RMR5-Ext-Dark	1.2×10^4	1.9×10^2	1.2×10^4
RMR5-Bulk-Dark	3.7×10^2	28	3.9×10^2
SM4-Ext-White	3.6×10^3	6.7	3.6×10^3
SM4-Bulk-Dark	3.6×10^3	0.27	3.6×10^3
SM-I-Ext	5.6×10^4	1.4×10^2	5.6×10^4
SM-I-Bulk	1.8×10^3	21	1.8×10^3

n/d = no IPL detected in TLE.
*Insufficient material to perform all analyses

Supplementary Table S4.
Archaeal IPL and 16S rRNAgene diversity (%) detected in samples

Sample	Archaeal IPL detected	*Other Archaea*	*MG1*	*Archaeo-globales*	*Thermo-coccales*	*Thermo-plasmatales*
RMR-D-Ext-Orange	n/d	1.7%	5.4%	0.1%	18.4%	74.1%
RMR-D-Ext-White	n/d	0%	0%	48.5%	46.3%	3.1%
RMR5-Ext-White	insufficient sample	7.6%	5.0%	0.2%	41.4%	52.2%
RMR5-Ext-Dark	2G-AR ; 2G-Glycerol-AR* ;1G-GDGT ; 2G-GDGT	n/d	n/d	n/d	n/d	n/d
RMR5-Bulk-Dark	2G-AR ; 2G-Glycerol-AR*	n/d	n/d	n/d	n/d	n/d
SM4-Ext-White	2G-AR ; 1G-H-GDGT	n/d	n/d	n/d	n/d	n/d
SM4-Bulk-Dark	2G-AR	n/d	n/d	n/d	n/d	n/d
SM-I-Ext	2G-Glycerol-2Me-OH-AR*	n/d	n/d	n/d	n/d	n/d
SM-I-Bulk	1G-GDGT; 2G-GDGT; 2G-H-GDGT; PG-GDGT	n/d	n/d	n/d	n/d	n/d

AR = archaeol; **GDGT** = glycerol-dibiphytanyl-glycerol-tetraether; **1G** = monoglycosyl; **2G** = diglycosyl;
H = H-shaped; **Me** = methylated; **PG** = phosphatidylglycerol
*tentatively identified AR (according to Yoshinaga *et al*. 2011)
MG1 = Marine Group I
n/d = not detectable

Supplementary Table S5.

Archaeal core lipid composition in samples

	RMR-D -Ext-White	**RMR5 -Ext-Dark**	**RMR5 -Bulk-Dark**	**SM4 -Ext-White**	**SM4 -Bulk-Dark**	**SM-I -Ext**	**SM-I -Bulk**
			Distribution of Archaeal core lipid types				
% AR	75%	48%	55%	59%	20%	59%	14%
% GDGT	25%	52%	45%	41%	80%	41%	86%
			Distribution of Archaeal core GDGT types				
GDGT 0	100%	85%	81%	42%	34%	35%	26%
GDGT 1	-	1%	5%	3%	24%	-	-
GDGT 2	-	1%	-	3%	3%	-	-
GDGT 3	-	-	-	3%	2%	-	-
GDGT 5'	-	-	-	3%	2%	-	-
GDGT 5	-	1%	14%	2%	4%	-	-
Me GDGTs	-	1%	-	-	-	-	-
H-GDGTs	-	11%	-	42%	25%	48%	27%
Me H-GDGTs	-	-	-	2%	6%	17%	32%
2Me H-GDGTs	-	-	-	-	-	-	15%
3Me H-GDGTs	-	-	-	-	-	-	1%

GDGT = glycerol-dibiphytanyl-glycerol-tetraether (0 to 5 = number of rings, 5' = regioisomer).
For structural details, see Liu *et al.* (2012); **AR** = Archaeol; **Me** = methylated; **H** = H-shaped

Supplementary Table S6.
Amount and stable carbon isotope composition of total organic carbon (TOC) in structures.

Sample	$\delta^{13}C_{TOC}$ ‰ V-PDB	TOC wt.%	Notes*
RMR-D-Ext-White	-19.5	0.016	bulk
RMR5-Bulk-Dark	-15.5	0.013	LE
SM4-Bulk-Dark	-21.4	0.005	LE
SM-I-Ext	-28.0	0.035	bulk

*LE = lipid extracted TOC (i.e. after lipid extraction)
Bulk = TOC of non-extracted outer ~3mm surficial material

Supplementary Table S7. Stable carbon isotope compositions ($\delta^{13}C_{lipid}$, ‰ V-PDB) and relative abundance (%, in parenthesis) of major fatty acids and the archaeal phytane in the various samples.

Sample	RMR-D -Ext-White	RMR5 -Ext-Dark	SM4 -Ext-White	SM4 -Bulk-Dark	SM-I -Ext	SM-I -Bulk
*Bacterial fatty acids**						
$C_{16:1\omega7}$	b.d. (1)		-0.8 (26)	-17.9 (46)	-34.3 (46)	-32.4 (31)
$C_{16:0}$	-6.7 (24)	-18.2 (36)	-8.9 (35)	-17.4 (35)	-34.2 (38)	-30.1 (39)
$C_{17:0}$	-0.04 (5)					
$C_{18:1\omega9}$			b.d. (2)		b.d. (4)	b.d. (1)
$C_{18:1\omega7}$	-5.9 (7)	b.d. (13)	-6.2 (17)	-17.6 (16)	-34.2 (13)	-32.4 (16)
$C_{18:0}$	-4.7 (48)	-22.2 (51)	-10.4 (21)	b.d. (3)	b.d. (2)	-30.6 (13)
$C_{19:0}$	-4.3 (16)					
Archaeal phytane	-	-19.0	-13.7	-27.9	-	-41.7

* = fatty acids present in trace amounts (<1%) are not reported here
b.d. = signal too low to accurately quantify

Chapter V

Identification and activity of acetate-assimilating microorganisms in diffuse fluids venting from two hydrothermal systems

Matthias Winkel[§], Petra Pjevac[§], Manuel Kleiner, Sten Littmann, Anke Meyerdierks, Rudolf Amann and Marc Mußmann

In revision, *FEMS Microbiology Ecology*

Contributions:

M.W., P.P. and M.M. developed concepts and ideas, conceived and wrote the manuscript. M.W. performed on board experiments at Menez Gwen, CARD-FISH analysis of Menez Gwen samples, 16S rRNA gene sequencing and analysis, IRMS and nanoSIMS experiments and ammonium and nitrate concentration measurement. P.P. performed on board experiments in the Manus Basin, CARD-FISH analysis of Manus Basin samples, 16S rRNA gene sequencing and analysis, IRMS and nanoSIMS data analysis and measured sulfide concentrations. M.M. designed NautMG oligonucleotide probes. M.K. and S.L. performed nanoSIMS experiments and assisted with nanoSIMS data analysis. M.K, S.L., A.M. and R.A. conceived and edited the manuscript.

Identification and activity of acetate-assimilating microorganisms in diffuse fluids venting from two hydrothermal systems

Matthias Winkel[1§], Petra Pjevac[1§], Manuel Kleiner[2], Sten Littmann[3], Anke Meyerdierks[1], Rudolf Amann[1] and Marc Mußmann[1*]

[1] Department of Molecular Ecology, Max Planck Institute for Marine Microbiology, Bremen, Germany
[2] Department of Symbiosis, Max Planck Institute for Marine Microbiology, Germany
[3] Department of Biogeochemistry, Max Planck Institute for Marine Microbiology, Germany

§ these authors contributed equally to the study
* corresponding author:
Dr. Marc Mußmann
Department of Molecular Ecology
Max Planck Institute for Marine Microbiology
Celsiusstr. 1
Bremen, 28359, Germany
e-mail: mmussman@mpi-bremen.de

Running title:
Acetate-assimilating microorganisms in two hydrothermal systems

Key words:
Epsilonproteobacteria, Gammaproteobacteria, heterotrophy, nanoSIMS, stable isotopes

Abstract

There is accumulating evidence that diffuse hydrothermal fluids often contain organic compounds such as hydrocarbons and acetate. Microorganisms potentially consuming these compounds at hydrothermal sites are mostly known from cultivation-dependent studies only. To identify acetate-assimilating microorganisms at two distinct hydrothermal systems we combined microbial community analysis with short-term, aerobic and anaerobic incubations using ^{13}C-labeled acetate. We followed cell growth and assimilation of ^{13}C into single cells by nanoSIMS combined with fluorescence *in situ* hybridization (FISH). In 55°C fluids from the Menez Gwen system (site "Woody Crack") on the Mid-Atlantic Ridge, a novel epsilonproteobacterial group (NautMG-group) accounted for nearly all acetate-assimilating cells and represents the first aerobic, acetate-consuming member of the *Nautiliales*. In contrast, *Gammaproteobacteria* dominated the ^{13}C-acetate-assimilating community in incubations with 37°C fluids from the back-arc hydrothermal system in the Manus Basin of Papua New Guinea. Here, 16S rRNA gene sequences were mostly related to *Marinobacter*, reflecting the high content of sea water in these fluids. The instant response suggests that acetate-consumers in diffuse fluids are opportunists, which quickly exploit their food sources whenever available under the spatially and temporally highly fluctuating conditions at hydrothermal vents. Our data provide first insights into a largely under-investigated part of microbial carbon cycling at hydrothermal vents.

Introduction

In submarine hydrothermal systems, inorganic carbon is considered the primary carbon source (Shively *et al.*, 1998; Nakagawa & Takai, 2008). However, diffuse and end-member hydrothermal fluids can also contain various non-methane organic compounds (Holm & Charlou, 2001; Rogers & Amend, 2006; Konn *et al.*, 2009; Charlou *et al.*, 2010; Lang *et al.*, 2010). Organic acids, lipids and hydrocarbons are formed in the deep subsurface by serpentinization and subsequent Fischer-Tropsch-type processes under elevated temperature and pressure (Shock & Schulte, 1998; Holm & Charlou, 2001). Furthermore, simple organic compounds are formed by thermal decomposition of biomass (McCollom & Seewald, 2007), homoacetogenesis (Drake *et al.*, 2008; Lever *et al.*, 2010) or by the vent-associated macrofauna (Pimenov *et al.*, 2002). Thus, elevated concentrations (3-35 µM) of organic compounds (e.g. formate and acetate) have been measured in venting fluids from shallow and deep-sea hydrothermal systems (Amend *et al.*, 1998; Lang *et al.*, 2010).

Early cultivation-independent studies showed that radioactively labeled organic carbon, such as acetate or glucose, was consumed by unknown microorganisms in hydrothermal fluids of the Galapagos Rift (Tuttle *et al.*, 1983), the East Pacific Rise (Tuttle, 1985; Wirsen *et al.*, 1986), the Guaymas Basin (Karl *et al.*, 1988; Bazylinski *et al.*, 1989) and at the Loihi Seamount (Karl *et al.*, 1989). Beyond these studies little is known about consumption of non-methane organic carbon in hydrothermal fluids. The current knowledge on microbial groups responsible for organic compound use is limited to cultivation-dependent studies of mostly thermophilic, phylogenetically diverse strains from *Deinococcus-Thermus*, *Thermotogae*, *Proteobacteria, Deferribacterales* and *Archaea* (Pley, 1991; Marteinsson *et al.*, 1995; Huber *et al.*, 1995; Raguénès *et al.*, 1996). Genomes of the vent-associated, sulfur-oxidizing *Sulfurimonas*/*Sulfurovum*-group *Epsilonproteobacteria* (e.g. Campbell *et al.*, 2006; Sievert *et al.*, 2007; Yamamoto & Takai, 2011) also indicate a certain potential for organic carbon consumption. Hence it was proposed that these thioautotrophs might use simple organic compounds like acetate as growth supplement (Sievert *et al.*, 2008).

Our objective was to identify microorganisms assimilating organic carbon other than methane in diffuse fluids at two hydrothermal sites. In general, diffuse fluids can cover a large temperature range and usually consist of sea water mixed with hot end-member fluids (Wankel *et al.*, 2011). In particular, we hypothesized that *in situ* abundant lithotrophs may use organic carbon as supplementary carbon source (Wood *et al.*, 2004,

Sievert *et al.*, 2008). For our analysis we sampled sulfidic, diffuse fluids from two deep-sea hydrothermal systems, the Manus Basin back-arc system off the coast of Papua New Guinea and the Menez Gwen-system at the Mid-Atlantic Ridge (MAR). Diffuse fluids with *in situ* temperatures ranging from 4°C to 73°C (Manus Basin) and from 25°C to 56°C (site "Woody Crack", Menez Gwen) were sampled. We incubated fluids with ^{13}C-labeled acetate as model compound for organic carbon assimilation (Wright & Hobbie, 1966; Hoppe, 1978, Berg *et al.*, 2013) and monitored changes in the microbial community structure by 16S rRNA gene pyrotag-sequencing, clone libraries and fluorescence *in situ* hybridization (FISH). In contrast to previous environmental studies using ^{13}C-labelled tracers (Webster *et al.*, 2010; Vandieken *et al.*, 2012; Berg *et al.*, 2013) we applied lower substrate concentrations (10 and 30 µM acetate) and shorter incubation times (8-12 h) to minimize experimentally introduced bias. Finally, we identified ^{13}C-acetate assimilating populations by combining nanometer scale secondary ion mass spectrometry (nanoSIMS) with FISH. Our approach to combine 16S rRNA gene diversity analysis, single cell identification and nanoSIMS measurements provide first insights into the identity and activity of uncultured microorganisms consuming organic carbons other than methane in diffuse hydrothermal fluids.

Material and Methods

Site description and sampling

Diffuse fluids from the Menez Gwen hydrothermal vent field (37°50'N, 31°30'W) were sampled in September/October 2010 at 828 m depth during the cruise M82-3 on board of the R/V Meteor. Menez Gwen is a basalt-hosted hydrothermal system located southwest of the Azores on the Mid-Atlantic Ridge (MAR). We sampled diffusely venting fluids at the site "Woody Crack" (WC), a fissure in the basalt crust of approximately 1 m length and 0.2 m width. *In situ* temperatures ranged from 25 to 56°C with a pH of ~4.9 (Table 1). These fluids consisted of sea water that interacted with a hot vaporous phase enriched in reduced compounds (Reeves *et al*., 2011). In these fluids concentrations of H_2S, H_2 and CH_4 were elevated relative to surrounding seawater (Reeves *et al*., 2011). Besides the diffuse venting fluids, additional samples were collected from the hydrothermal plume (WC-P, 23 m above Woody Crack) and from bottom water above a patch of the vent mussel *Bathymodiolus azoricus* (WC-M, Fig. S1). For a more detailed description of the sampling site see Marcon *et al.* (2013).

Diffuse fluids from the Manus Basin (MB) back-arc spreading center off the coast of Papua New Guinea were recovered in June/July 2011 during R/V Sonne cruise SO-216. Here, we sampled the felsic-hosted hydrothermal vent fields North Su (3°47'S, 152°06'W) and Fenway (3°43'S, 151°40'W). At the North Su (NS) underwater volcano rising, diffuse fluids were recovered from two venting fissures in the sea-floor in approximately 1200 m water depth (NS-I: 16-40°C, pH ~7.1 and NS-II: 54-73°C, pH ~3.6). From the smaller vent field Fenway (FW), located north-east of North Su in the PACMANUS area, cold but shimmering diffuse fluids were sampled above a patch of vestimentiferan tube worms in 1706 m depth (3.7°C, pH ~7.3) (Fig. S1, Table 1). Based on magnesium concentrations in recovered diffuse fluid samples (Table 1), standard sea water magnesium concentration (1290 ppm) and the generally assumed absence of magnesium in end-member hydrothermal fluids, we calculated that end member fluid content in our diffuse fluids ranged from 8% (FW) to 30% (NS-II) (Table 1).

During both cruises samples were collected with the remotely controlled flow-through system (Kiel Pumping System - KIPS) (Schmidt *et al.*, 2007) mounted on to the remotely operated underwater vehicle ROV Quest (MARUM, Bremen). At Woody Crack the flasks (675 ml, Savillex, USA) of the KIPS system were pre-filled with ambient bottom sweater obtained by a CTD cast. At the Manus Basin sites flasks were pre-filled with de-ionized water, which was exchanged for ambient seawater during the ROV descent. At all sites, the pumping rate was approximately 1 l min^{-1} and pumping time per sample was set at 3 min ensuring exchange of flask volume by sample volume at least 4 times. A temperature probe, located next to the KIPS sampling nozzle, was used to monitor temperature during sampling. Fluid samples from multiple KIPS bottles were combined and divided into subsamples for microbial community analysis, stable isotope (SI) experiments, and measurements of ammonia, nitrate and sulfide concentration. Due to the limited fluid volume (<6 l) retrieved per sampling event several ROV dives were necessary to recover sufficient material for all experiments at two sites (WCa, WCb, NS-IIa and NS-IIb, see Table 1).

Ammonium, nitrate, sulfide and acetate concentration measurements

Ammonium (NH_4^+) concentrations were determined photometrically by nesslerization (Bower & Holm-Hansen, 1980). Nitrate was measured according to (Braman & Hendrix, 1989) using a CLD 60 NO_x analyzer (Eco Physics, USA). Total dissolved sulfide was determined spectrophotometrically in zinc acetate fixed samples as described in Cline,

1969. Acetate concentrations in Woody Crack fluids were only measured by HPLC in the home laboratory. Acetate in Manus Basin fluids was detected at the sampling site by *in situ* mass spectrometry (ISMS) at AMU 60 (Bach *et al*., 2011) and also measured by HPLC in the home laboratory. More detailed geochemical data will be published elsewhere by E.P. Reeves and A. Koschinsky.

16S rRNA gene-pyrotag diversity analysis in diffuse hydrothermal fluid samples

For 16S rRNA gene diversity analysis diffuse fluids (samples WCb, FW, NS-I, and NS-IIb, Table 1) were filtered on polyethersulfone (PES) membranes (0.22 µm pore size, Milipore, Darmstadt, Germany) attached to the KIPS system and stored at -20°C until further processing. DNA was extracted from PES membranes with the Ultra Clean Soil DNA Kit (MoBio Laboratories, Carlsbad, USA) as instructed in the manual. Bacterial 16S rRNA genes were amplified by PCR with the primers GM3 and 907RM (Muyzer *et al.*, 1995; 1998) in ten parallel reactions using the Phusion High Fidelity Polymerase (NEB, Ipswich, USA). PCR products were pooled, gel purified and 454-pyrosequenced at the Max Planck Genome Center (Cologne, Germany). Further details on PCR and sequencing are provided in the supplementary methods. Sequence reads >200 bp were analyzed with the bioinformatics pipeline of the SILVA database project (Quast *et al.*, 2013) as described in (Klindworth *et al.*, 2013). Details are given in the supplementary methods section. Sequencing and analysis statistics are presented in Table S1.

^{13}C-acetate and ^{15}N-ammonium incubations

Stable isotope (SI)-incubation experiments were performed onboard in 1000 ml (WCa) or 500 ml (FW, NS-I, NS-IIa) glass bottles filled with 330 ml of diffuse fluids. For oxic experiments the headspace was air, while for anoxic experiments fluids and headspace were flushed with N_2/CO_2 gas mixture for several minutes. On board fluids were pre-incubated at the measured *in situ* temperature for 1h before addition of ^{13}C-acetate and ^{15}N-ammonium addition. To diffuse fluids from Woody Crack (WCa) we added sodium 1-^{13}C-acetate (99 atom % ^{13}C, Sigma-Aldrich) at a final concentration of 10 µM. For incubations of fluids from the Manus Basin (FW, NS-I, NS-IIa) we increased ^{13}C-acetate concentrations to 30 µM to ensure sufficient labeling, since on board *in situ* mass spectrometer detected a compound with the atomic mass unit 60 that possibly was acetic acid (Bach *et al*., 2011). To all incubations we also added ^{15}N-ammonium chloride (98 atom % ^{15}N, Sigma-Aldrich) as a general activity marker at a final concentration of 10

µM. Oxic and anoxic incubations to which no ^{13}C-acetate was added were set up as living controls. As dead controls we added formaldehyde (1% final) to substrate-amended fluids. Fluids were incubated at *in situ* temperatures for 8h (55°C, WCa and 72°C, NS-IIa), 10h (37°C, NS-I) or 12 h (4°C, FW). Incubations were stopped by addition of formaldehyde (final concentration 1%) and fixed for 1h at room temperature. From each bottle 50-100 ml aliquots were filtered on a glass fiber filter (type GF, 0.7 µm pore size, Millipore, Darmstadt, Germany) for bulk SI-measurements. The remaining volume was filtered on multiple gold-palladium coated polycarbonate membranes (type GTTP, 0.2 µm pore size, Millipore, Darmstadt, Germany) for nanoSIMS and CARD-FISH analyses. All filters were air dried and stored at -20°C. For each experimental set-up duplicate (FW, NS-I, NS-IIa) or triplicate (WCa) incubations were performed.

Isotope ratio mass spectrometry (IRMS)

For bulk measurements of ^{13}C and ^{15}N, GF-filters were analyzed by gas chromatography- isotope ratio mass spectrometry (GC-IRMS). Briefly, isotope abundance in the sample was measured on released CO_2 and N_2 after flash combustion of filters in excess oxygen at 1050°C in an automated elemental analyzer (Thermo Flash EA, 1112 Series, CE Elantech, Lakewood, NJ, USA) coupled to a Delta Plus Advantage mass spectrometer (Finnigan, Thermo Fisher Scientific, Waltham, MA, USA). Filters were decalcified in a hydrochloric acid (37%) atmosphere for 24 h in a desiccator prior to combustion (Musat *et al*., 2008).

Total cell counts (TCC) and CARD-FISH

Samples for TCC and CARD-FISH were prepared from diffuse fluids and from the hydrothermal plume. Therefore, 100 ml fluid were directly formaldehyde-fixed on board (1% final concentration, overnight at 4°C) and filtered on polycarbonate membrane filters (type GTTP, 0.2 µm pore size, Millipore, Darmstadt, Germany). TCC were performed by counting at least 1000 cells per sample on 4',6-diamidino-2-phenylindole (DAPI)-stained polycarbonate membrane filters. CARD-FISH was performed on membrane filters according to (Pernthaler *et al.*, 2002; Ishii *et al.*, 2004; Teira *et al.*, 2004). Details of the applied oligonucleotide probes are listed in Table S2. For simultaneous detection of multiple microbial taxa, filters were consecutively hybridized with two probes (Pernthaler *et al.*, 2004) using Alexa488 and Alexa594 fluorochromes (Invitrogen, Karlsruhe, Germany).

Marking and mapping of hybridized cells for nanoSIMS

To combine CARD-FISH identification of single cells with nanoSIMS analysis we used correlative microscopy. To this end, the position of cell assemblages identified by CARD-FISH were marked by laser micro-dissection of filter membranes (LMD model DM6500B) (Leica, Wetzlar, Germany) using a filter set for the detection of Alexa488 (excitation maximum: 498 nm; emission maximum: 520 nm) and Alexa594 (excitation maximum: 591 nm; emission maximum: 618 nm). Fields of view with a suitable distribution of hybridized cells were marked with numbers, arrows and borders to guarantee recovery of cells during nanoSIMS analysis. Microscopic images were taken for orientation purpose during the nanoSIMS analysis and for post-processing with the Look@nanoSIMS software tool (Polerecky *et al.*, 2012).

NanoSIMS analysis

Hybridized cells within the marked areas on the filter membranes were analyzed with a nanoSIMS 50L instrument (Cameca, Gennevilliers, Cedex-France). Secondary ions $^{12}C^-$, $^{13}C^-$, $^{12}C^{14}N^-$, $^{12}C^{15}N^-$ and $^{32}S^-$ were simultaneously recorded for each individual cell using 5 electron multipliers. Samples were pre-sputtered with a Cs^+ beam of 400 to 500 pA to remove surface contaminations, to implant Cs^+ ions and to achieve a stable ion emission rate. During analysis samples were sputtered with a 0.8 to 1.8 pA Cs^+ primary ion beam focused into a spot of 50 to 100 nm diameter that was scanned over an analysis area of 5 x 5 µm to 30 x 30 µm with an image size of 256 x 256 pixel or 512 x 512 pixel and a counting time of 1 ms per pixel. The individual masses were tuned for high mass resolution (around 7000 MRP Cameca). Respective mass peaks were tuned directly on the sample. Depending on the fields of view (5 x 5 µm to 30 x 30 µm), between 20 to 100 planes were recorded.

The measured data were processed using the Look@NanoSIMS software (Polerecky *et al.*, 2012). The images of one field of view recorded during one measurement were drift corrected and accumulated. Regions of interest (ROI) corresponding to individual cells were defined using images of $^{12}C^-$, $^{12}C^{14}N^-$ and $^{32}S^-$. For each ROI $^{13}C/(^{13}C+^{12}C)$, $^{12}C^{15}N/(^{12}C^{15}N+^{12}C^{14}N)$ and $^{32}S/^{12}C$ ratios were calculated. Ratios with more than 10% trend (increase or decrease) with depth were excluded from further analysis.

Calculation of assimilation per biovolume

Assimilation of ^{13}C and ^{15}N per biovolume were calculated for 138 cells from Woody Crack (Menez Gwen), and for 112 cells from the Manus Basin (FW: 42 cells; NS-I: 66 cells; NS-IIa: 6 cells). Most cells were rod-shaped and cell volume was calculated based on measured values of cell diameters and cell lengths by adding up the respective volumes of a sphere and a cylinder. For biovolume-to-biomass conversion we used a calibration factor of 0.38 pg C μm^{-3} known for small heterotrophic *Bacteria* (Lee & Fuhrman, 1987). The calculated biomasses were correlated with $^{13}C/(^{13}C+^{12}C)$ ratio and corrected for dead control bulk measurements, assuming that only ^{13}C-acetate was present in the incubations, since we could not measure acetate in fluids (100%-labeling). The nitrogen content of cells was calculated based on a conversion factor of 3.7 for C : N ratio in heterotrophic cells (Lee & Fuhrman, 1987), correlated with $^{15}N/(^{15}N+^{14}N)$ ratio and corrected for dead control bulk measurements.

16S rRNA gene libraries and phylogenetic analyses

Bacterial 16S rRNA genes were amplified by filter-PCR (Kirchman *et al.*, 2001) or from filter-extracted DNA (Ultra Clean Soil DNA Kit, MoBio Laboratories, Carlsbad, USA) of formaldehyde-fixed samples collected at the end of ^{13}C-acetate incubations. Bacterial primers GM3F and GM4R, (Muyzer *et al.*, 1995) or primers GM5F and 907RM were applied (Muyzer *et al.*, 1998). After gel purification, PCR products were cloned and Sanger-sequenced. Further details are described in the Supplementary Methods.

Phylogenetic analysis was performed with the ARB software package (Ludwig *et al.*, 2004), based on a sequence alignment with the SINA (SILVA Incremental) aligner (Pruesse *et al.*, 2012), against the SILVA 16S rRNA SSU reference database, release 111 (Quast *et al.*, 2013). Phylogenetic trees were calculated with nearly full-length sequences (>1400 bp) using the maximum likelihood algorithm RAxML with 100 bootstraps (Stamatakis *et al.*, 2005) implemented in ARB. For calculation we applied a 50% conservation filter calculated with reference sequences for the considered phylogenetic group. Nucleotide substitutions were weighted according to the GTR model (Lanave *et al.*, 1984). Partial sequences were added after tree calculation using the ARB implemented maximum parsimony algorithm, without allowing changes in tree topology.

Nucleotide sequence accession numbers

Nucleotide sequences from this study were deposited in the EMBL, GeneBank and DDBJ nucleotide database with the accession numbers HG962423-HG962430. The 454-pyrotag sequences have been deposited at Sequence Read Archive under sample accession numbers ERS377555 - ERS377558.

Results

Geochemistry of diffuse hydrothermal fluids

Ammonium concentrations in fluids from Woody Crack (WCa) in the Menez Gwen hydrothermal vent field were between 5-8 µM. Nitrate concentrations were between 16-18 µM (Table 1). During sampling, oxygen was qualitatively detected by ISMS (personal communication, S. Hourdez). Acetate was not measured by ISMS here, but it was below 1 µM, which is the detection limit of the HPLC-based method. Hydrothermal fluids at Menez Gwen were previously shown to contain1.7 mmol/kg sulfide (Charlou *et al*., 2000) and modeling revealed 60 µmol/kg sulfide above mussel patches surrounding Woody Crack (Marcon *et al*., 2013). The 4°C and 37°C fluids (FW and NS-I) from the Manus Basin displayed low ammonium concentrations (<3 µM), whereas up to 30 µM ammonium were detected in the 72°C fluids (NS-IIa). In Manus Basin fluids oxygen ranged from 2.2 to 4.9 mg/l (Table 1) equaling saturation values from 45 to >100%. While acetic acid was possibly detected by ISMS measurements in fluids from Manus Basin (Bach *et al*. 2011), exact acetate measurements by HPLC was interfered by strong background of other, yet unknown compounds. In the low temperature fluid (FW) sulfide concentrations were close to the detection limit (~2 µM), while 14-66 µM and 113-302 µM sulfide were detected in the temperate (NS-I) and high temperature (NS-IIa) fluid (Table 1).

Microbial community in diffuse fluids from Woody Crack (Menez Gwen)

To determine the composition of the microbial community and to identify potential organic carbon consuming microorganisms in the 55°C diffuse fluid at Woody Crack, we performed total cell counts (TCC), CARD-FISH and 16S rRNA gene 454-pyrosequencing. TCC were 1.6 ± 0.3 x 10^5 cells ml^{-1}, of which 99% were identified as *Bacteria* by CARD-FISH (Table S3). Bacterial 16S rRNA gene pyrotag-sequences (6,798 reads) were dominated by *Epsilonproteobacteria* (51%, Fig. 1), which accounted for 10% of TCC (Fig. 2 and Table S3). *Gammaproteobacteria* accounted for 10% of pyrotag

sequences but made up 65% of TCC (Fig. 2 and Table S3). Besides *Gammaproteobacteria* and *Epsilonproteobacteria*, only *Alphaproteobacteria* contributed a larger number of sequences to the overall diversity (18%), while all other bacterial groups made up <5% of pyrotags (Fig. 1).

The majority (85%) of epsilonproteobacterial pyrotag sequences was related to the mesophilic, lithoautotrophic genera *Sulfurimonas*, *Sulfurovum* and *Arcobacter* within the *Campylobacterales* (Fig. 1). These genera are frequently detected in sulfidic, hydrothermal environments in the deep-sea (Campbell *et al.*, 2006; Sievert *et al.*, 2007). *Nautiliales* constituted 10% of epsilonproteobacterial pyrotags (Fig. 1). This order harbours metabolically diverse organisms including mixo- and autotrophic thermophiles (Campbell *et al.*, 2001, 2006; Miroshnichenko *et al.*, 2002). Most of the gammaproteobacterial sequences were affiliated with sequences from *Oceanospirillales* (SUP05, *Psychromonas*) and *Alteromonadales* (SAR86).

Microbial community composition in source fluids from the Manus Basin

Despite the three diffuse fluids from the Manus Basin covered a large temperature range (4°C, 37°C, 72°C) TCCs were relatively similar ranging from 1.6 to 6.2 x 10^4 cells ml^{-1}. *Bacteria* accounted for 58-85% and Archaea for 8-11% of TCC (Table S3). Of in total 10,516 bacterial pyrotag sequences 84-91% affiliated with *Proteobacteria* (Fig. 1). In the 4°C fluids (FW), *Gammaproteobacteria* made up 57% of pyrotag sequences, whereas they contributed to only 4% of TCC (Table S3). Besides numerous sequences related to the sulfur-oxidizing SUP05 clade of *Oceanospirillales* (Fig. 1), some sequences grouped with genera such as *Acinetobacter, Marinobacter* and *Alteromonas* that harbor heterotrophs (Fig. 3). *Epsilonproteobacteria* (all *Campylobacterales*) accounted for 12% of pyrotag sequences (Fig. 1) and for 4% of TCC (Table S3).

In both fluid samples from the North Su vent field (37°C, NS-I and 72°C, NS-IIb), *Epsilonproteobacteria* accounted for the major fraction of pyrosequences (78-85%), but again CARD-FISH with the probe-mix EPSY914/EPSY549 indicated abundances of only 7 to 15% of TCC (Table S3). In the 37°C fluid sample (NS-I) the majority of all epsilonproteobacterial sequences (90%) was again related to the same *Campylobacterales*, while sequences related to the *Nautiliales* represented a minor fraction (9% of epsilonproteobacterial sequences). Contrastingly, over 93% of epsilonproteobacterial sequences retrieved from the 72°C fluid (NS-IIb) were classified as *Nautiliales* (Fig. 1). *Gammaproteobacteria* were similarly abundant (5-15%) as

Epsilonproteobacteria according to CARD-FISH (Table S3) and accounted for 4-7% of pyrotag sequences in NS-I and NS-IIb fluids. Here, gammaproteobacterial sequences were also closely related to sequences of *Alteromonas* that have been detected in cold FW fluids and to *Acinetobacter* (Fig. 3).

Acetate-assimilation by Epsilonproteobacteria in diffuse fluids from Woody Crack

To identify microbial populations that actively assimilate organic carbon in fluids from Woody Crack (WCa), we followed assimilation of the model compound ^{13}C-acetate in bulk samples and individual cells in short-term experiments at *in situ* temperature (55°C). Besides ^{13}C-acetate, we also added ^{15}N-ammonium as general activity marker. TCCs only increased in oxic experiments by 2.7-2.8-fold (Table S3). Bulk uptake of ^{13}C and ^{15}N in these incubations was higher than in the dead controls (0.8 to 1.1% and 19.1 to 25.3% atom percent, respectively) (Fig. S2), while no elevated ^{13}C- and/or ^{15}N-uptake was detected in anoxic and control incubations (Table S4). These results indicated that ^{13}C-acetate and ^{15}N-ammonium was assimilated into cell material only under oxic conditions. Furthermore, within 8 h of incubation the community significantly shifted from *Gammaproteobacteria* (65% of TCC in diffuse fluids) to an *Epsilonproteobacteria*-dominated community (88.2% ± 4.2 of TTC after incubation) (Fig. 2; Table S3).

Epsilonproteobacteria grown in ^{13}C-acetate incubations were further identified by 16S rRNA gene sequencing. Almost all retrieved epsilonproteobacterial sequences were affiliated to the family *Nautiliaceae* and formed a separate branch with 94.2% sequence identity to *Nautilia profundicola* (Fig. 3). We designated this novel sequence cluster as NautMG-group. Most gammaproteobacterial sequences recovered from this experiment affiliated with the heterotrophic genus *Alcanivorax* (Fig. 3).

To quantify these *Epsilonproteobacteria* in incubations and diffuse fluids we designed the specific oligonucleotide probe Naut842 (Fig. 3). This probe targeted 84-87% of TCC in all triplicate ^{13}C-acetate incubations (Fig. 4), indicating that the large majority of epsilonproteobacterial cells grown on ^{13}C-acetate indeed belong to the novel NautMG-group. In the diffuse fluids (WCa) the NautMG-cells made up 0.8% of TCC (Table S3), in bottom waters above a nearby *Bathymodiolus*-mussel bed (WC-M) they accounted for 0.3% (not shown). No NautMG-cells were detected (not shown) in the plume obtained from 23 m above Woody Crack (WC-P). *Gammaproteobacteria* did not grow and even declined in cell numbers in ^{13}C-acetate incubations (Fig. 2, Table S3), possibly due to heat lysis.

We selected two of the triplicate incubations (WCa-R1 and WCa-R3) to confirm ^{13}C-acetate consumption by the NautMG-group using nanoSIMS. Thereof, 138 epsilonproteobacterial and gammaproteobacterial cells were identified beforehand by correlative microscopy using CARD-FISH. Almost all analyzed epsilonproteobacterial cells significantly incorporated ^{13}C and ^{15}N, whereas the few gammaproteobacterial cells found showed little or no incorporation (Fig. 5, 6 and Fig. S4). Epsilonproteobacterial cells displayed large differences in ^{13}C- and ^{15}N-assimilation per biovolume. Most cells showed both low ^{13}C (0.03 to 0.8 fmol cell^{-1}) and ^{15}N (0.7-1.7 fmol cell^{-1}) incorporation. Several cells showed high assimilation per biovolume in ^{15}N (1.7 to 4.6 fmol cell^{-1}), while they assimilated less ^{13}C (~0.5 fmol cell^{-1}). Only few cells assimilated both high ^{15}N (1.7 to 4.4 fmol µm^{-3}) and high ^{13}C (0.8 to 1.7 fmol cell^{-1}) in comparison to the average uptake of ^{15}N (1.4 ± 1.0 fmol cell^{-1}) and ^{13}C (0.4 ± 0.4 fmol cell^{-1}) (Fig. 6).

Acetate-assimilation by Gammaproteobacteria in Manus Basin diffuse fluids

Similar incubations with ^{13}C-acetate and ^{15}N-ammonium were performed with diffuse fluids from three different sources at the Manus Basin hydrothermal systems (FW, NS-I, NS-IIa). In the oxic 4°C and 37°C incubations bulk uptake of ^{13}C and ^{15}N in atom per cent was higher than in corresponding dead controls, indicating assimilation of the ^{13}C-acetate and ^{15}N-ammonium (Fig. S2). In all anoxic incubations and in the oxic 72°C incubation bulk uptake of ^{13}C and ^{15}N was not clearly higher than in the dead controls (Fig. S2 and Table S4).

In the oxic 4°C and 37°C incubations the TCC increased up to 11-fold to a final TCC of 0.8-3.7 x10^5 cells ml^{-1} (Table S3), while TCC in anoxic experiments and acetate-free controls remained stable or changed only slightly (Table S3). In both experiments observed growth could be attributed to *Gammaproteobacteria,* which accounted for 63-87% of TCC after incubations (Table S3). This was supported by a 16S rRNA gene library of 4°C fluids that were dominated by sequences of the sea water-associated and heterotrophic genera *Alteromonas* and *Marinobacter* (Fig. 3). Notably, both gene libraries from duplicates of the 37°C incubations were dominated by *Marinobacter hydrocarbonoclasticus* (98-99.7% sequence identity) and contained few sequences related to *Alteromonas marina* (99-99.6% sequence identity). Both organisms are known to grow with acetate (Gauthier *et al.*, 1992; Yoon *et al.*, 2003).

In the oxic 72°C incubation TCCs with up to 3.2 x 10^4 cells ml^{-1} were only slightly higher than in sampled diffuse fluids, while TCCs in anoxic and in substrate-free controls

even decreased (Table S3). These cells could not be further identified by FISH most likely because of an over-fixation with formaldehyde at 72°C. The 16S rRNA gene library of the nanoSIMS-analyzed replicate exclusively contained *Acinetobacter*-related sequences (*Gammaproteobacteria*) with 94-99.9% sequence identity to *Acinetobacter* species (Fig. 3). Archaeal 16S rRNA genes could not be amplified from the 72°C incubations.

The incorporation of ^{13}C and ^{15}N into single gammaproteobacterial cells was confirmed by nanoSIMS and CARD-FISH (Fig. 5 and 6). In the 4°C fluids (FW) ^{13}C-acetate assimilation by gammaproteobacterial cells (n=32) was low, with 0 to 0.15 fmol cell^{-1} ^{13}C and 0.02 to 0.57 fmol cell^{-1} ^{15}N incorporated. The few found epsilonproteobacterial cells (n=4) assimilated no or very little ^{13}C and ^{15}N (Fig. 5 and 6). In the 37°C incubation (NS-I) ^{13}C-acetate assimilation varied strongly between individual gammaproteobacterial cells (n=59), and ranged between 0.01 to 0.94 fmol cell^{-1} for ^{13}C and 0.02 to 1.28 fmol cell^{-1} for ^{15}N (Fig. 6). In the 72°C incubation only very few intact cells (n=5), not further identifiable by FISH, were slightly enriched in ^{13}C and ^{15}N (Figs. S3 and S4).

Discussion

In our study we confirmed that microbial communities in diffuse hydrothermal fluids assimilate non-methane organic carbon during short-term incubations over a temperature range from 4 to 72°C. We combined nanoSIMS analysis of ^{13}C-acetate assimilating cells with 16S rRNA gene sequencing and FISH. The molecular methods allowed us to detect community shifts and sufficient SI-labeling in cells after incubation periods of only from 8 to 12 h. The added acetate levels were close to those measured previously at other hydrothermal sites (Lang *et al.*, 2010) or in pelagic redoxclines (Albert *et al.*, 1995; Ho *et al.*, 2002). By using this approach we avoided extended incubation times and high substrate levels as generally applied for stable isotope-probing of acetate-assimilating microbes in environmental studies (Boschker *et al.*, 1998; Pester *et al.*, 2010; Vandieken *et al.*, 2012; Miyatake *et al.*, 2013; Berg *et al.*, 2013). Because of the dynamic nature of diffuse fluid systems (Perner *et al.*, 2013) our approach provides a snapshot of the physiological properties of particular microbes as our experimental set-up offered a rather narrow niche, leading to stimulation of very specific populations and not of the broad microbial communities.

Acetate-assimilation in diffuse fluids of the Manus Basin

In the 4°C and 37°C diffuse fluids from the Manus Basin the microbial communities shifted within 8 to 12h to clearly *Gammaproteobacteria*-dominated communities. These *Gammaproteobacteria* accounted for the majority of acetate-assimilation. The detection of typical sea water *Gammaproteobacteria* such as *Alteromonas* and *Marinobacter* in the 4° and 37°C incubations well reflects the high content of sea water in the studied diffuse fluids. These organisms probably represent common members of heterotrophic sea water communities that are stimulated by increased substrate availability and/or increased temperatures in the periphery of hydrothermal discharges. In particular, *Marinobacter* strains are metabolically flexible and use organic substrates including acetate (Kaye & Baross, 2000; Handley *et al.*, 2009). Moreover, the dominance of *Marinobacter* in the 37°C (NS-I) incubations is highly consistent with the frequent detection of *Marinobacter* in other diffuse fluids, in sea water surrounding spots of hydrothermal discharge (Huber *et al.*, 2007; Kaye *et al.*, 2011) and other samples of hydrothermal origin (Rogers *et al.*, 2003; Santelli *et al.*, 2008). Their competitive advantage over other acetate-consuming microorganisms in our 37°C-experiments and their widespread occurrence in hydrothermal habitats suggests that the *Marinobacter*-group is probably an important heterotroph at many hydrothermal sites.

Under thermophilic conditions (NS-IIa, 72°C), we observed slight growth of acetate-assimilating organisms, which could not be identified as FISH was difficult on these samples. Since sequences of the *Acinetobacter*-group were the only 16S rRNA phylotype recovered in the incubation analyzed by nanoSIMS, presumably these *Gammaproteobacteria* were selectively stimulated upon acetate addition. While mesophilic *Acinetobacter*-strains have been repeatedly isolated from hydrothermal sites (Jeanthon & Prieur, 1990; La Duc *et al.*, 2007), our experiments suggest that the *Acinetobacter*-group possibly also harbors at least thermo-tolerant members. The previous detection of *Acinetobacter*-like organisms implicated in degradation of hydrothermally-formed aromatic hydrocarbons in 68°C-brine pools further supports our hypothesis (Wang *et al*., 2011).

A novel group of aerobic and thermophilic Nautiliales assimilated acetate

In contrast, the microbial community in incubations of diffuse fluids from Woody Crack shifted to *Epsilonproteobacteria,* which were dominated by a yet-undescribed group phylogenetically affiliated with *Nautiliales*. So far, all known *Nautiliales* are thermophilic

and only occur at hydrothermally active sites. Although these organisms were relatively rare in the diffuse fluids they multiplied by 291-fold within 8 h at 55°C, equaling rapid generation times of approximately one hour. They constituted the most active acetate-assimilating microorganisms in oxic experiments, but did not grow in anoxic experiments or acetate-free controls. To date heterotrophic *Nautiliales* are not known to grow with acetate (Campbell *et al.*, 2001; Miroshnichenko *et al.*, 2002; Pérez-Rodríguez *et al.*, 2010). Moreover, most *Nautiliales* strains grow under anoxic conditions (Miroshnichenko *et al.*, 2002; Alain *et al.*, 2009; Pérez-Rodríguez *et al.*, 2010), whereas *Nautilia lithotrophica* tolerates oxygen (Miroshnichenko *et al.*, 2004). Thus, the NautMG-group identified here is the first example of an aerobic, acetate-consuming member of the *Nautiliales*. Although we detected the NautMG-group as the only epsilonproteobacterial phylotype after the incubation, individual cells displayed remarkable differences in ^{13}C and ^{15}N uptake. The most parsimonious explanation is that during the multiple division cycles the cellular ^{13}C and ^{15}N content accumulated from generation to generation resulting in incrementally higher labeling of the daughter cells. Interestingly, we also observed some cells with high ^{15}N but relatively low ^{13}C uptake (Fig. 6) possibly reflecting a different growth stage of this subpopulation.

While the NautMG-group was relatively rare in the source diffuse fluids, these organisms might be more abundant in other, more difficult to access compartments of the Menez Gwen system. It was repeatedly hypothesized that meso- and thermophiles found in diffuse fluids, such as thermophilic *Epsilonproteobacteria* (Huber *et al.*, 2003), are sessile organisms detached from the subsurface by venting fluids (Summit & Baross, 2001; Takai *et al.*, 2004). Furthermore, these organisms could also thrive at the crack rim or in mussel beds, where conditions are more favorable. In support of this hypothesis, we found several sequences of the NautMG-group (98% sequence identity) in bottom waters above mussel beds, but not in the plume above Woody Crack.

Rapid growth as adaptation to fluctuating conditions in hydrothermal fluids

Apparently, none of the *in situ* dominant sulfur-oxidizing clades (*Sulfurimonas*, *Sulfurovum*, SUP05) were stimulated in our sulfide-containing incubations, although even strict autotrophs may use acetate as supplementary carbon source (Wood *et al.*, 2004). Although sulfide and nitrate were clearly present in all incubations, no activity was detected in anaerobic incubations. Presumably temperatures, oxygen- or acetate-

concentrations in our incubation experiments were not in the range preferred by these groups and/or their enzymatic machinery was tuned for lithoautotrophic growth.

There is accumulating evidence that geochemical properties, temperature and the microbial community composition of diffuse fluids at hydrothermal systems are highly dynamic in time and space (Perner *et al.*, 2013). Consequently, microorganisms experience quick changes in their immediate vicinity and therefore require strategies to efficiently exploit resources in a narrow time window when conditions are favorable. In such a fluctuating environment, an opportunistic, copiotroph lifestyle with short lag-phases and rapid growth, is more competitive to exploit a temporarily available resource. In two incubation experiments with elevated temperatures (37°C, 55°C) we observed a quick response of different microbial populations within a couple of hours after adding acetate. These microorganisms apparently did not require a significant lag phase to adapt to the incubation conditions, suggesting that they were already metabolically active during sampling. Therefore, we propose that the rapid growth of specific populations upon acetate addition well reflects an adaptation to the fluctuating environment of hydrothermal fluids.

In conclusion, our results complement earlier findings by Tuttle *et al.* (1983) and Karl *et al.* (1989), who observed significant assimilation of acetate in diffuse fluids by unknown microorganisms in long-term incubations. The *Marinobacter*-group and a yet-undescribed group of *Epsilonproteobacteria* could rapidly exploit organic carbon when available. Further studies on different compartments with higher organic carbon content such as mussel beds are desirable to quantify the general importance of organic carbon turnover at hydrothermal vent sites. Future SI-labeling experiments should test a wider range of temperatures, oxygen concentrations and other organic carbon source such as formate, which is the major abiotically formed organic acid in hydrothermal fluids (McCollom & Seewald, 2001; Lang *et al.*, 2010). SI-labeling experiments could also be combined with transcriptomics and proteomics to identify the involved metabolic pathways.

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Table 1: List of sampling sites and chemical characteristics of sampled diffuse fluids.

Station	Sample	Location	Depth [m]	Temp. [°C]	pH	NH_4 [µM]	NO_3^- [µM]	H_2S [µM]	Mg^{2+} [ppm]	O_2 [mg/l]
Woody Crack (Menez Gwen)										
M83-2-702	Woody Crack plume (WC-P)[1]	N 37°50.671 E 31°31.150	805	9	n.d.	66.9	22.9	n.d.	n.d.	+[6]
M83-2-719	Woody Crack (WCa)[1,2]	N 37°50.673 E 31°31.154	828	46-56	5.0	7.8	16.3	+[4]		+[6]
M83-2-736	Woody Crack (WCb)[1,3]	N 37°50.673 E 31°31.158	828	25-49	4.9	5.3	17.4	+[4]		+[6]
M83-2-754	Woody Crack (WCc)[5]	N 37°50.673 E 31°31.154	828	49-68	4.6	n.d.	n.d.	+[4]		+[6]
M83-2-761	Mussel bed (WC-M)[1]	N 37°50.675 E 31°31.155	828	9.3	7.0	n.d.	n.d.	n.d.	n.d.	+[6]
Manus Basin										
SO216-29	Fenway[1,2,3,5] (FW)	S 03°43.697 E 151°40.350	1706	3.7	7.2	2.6	n.d.	<5	1110-1185	4.9
SO216-21	North Su[1,2,3,5] (NS-I)	S 03°47.955 E 152°06.080	1200	16-40	7.1	1.9	n.d.	14-66	1080-1098	3.6
SO216-45	North Su[1,2,5] (NS-IIa)	S 03°47.998 E 152°06.057	1155	54-73	3.6	10.9	n.d.	113-302	874-941	n.d.
SO216-19	NorthSu[3] (NS-IIb)	S 03°47.998 E152°06.051	1155	59-73	3.1	30.0	n.d.	n.d.	1053-1097	2.2

[1] used for CARD-FISH analysis

[2] used for aerobic ^{13}C-acetate incubations, molecular and isotopic analysis (CARD-FISH, 16S rRNA gene libraries, IRMS, nanoSIMS)

[3] used for 16S rRNA gene pyrotag analysis

[4] samples with typical sulfide odour (detailed data will be published by E.P. Reeves)

[5] used for acetate-free and anoxic control experiments

[6] detected by ISMS, but not quantified

n.d. – not determined

Figure 1: Diversity of the bacterial community analyzed by 16S rRNA gene pyrotags in diffuse fluids from Woody Crack (WCb), Fenway (FW), North Su-I (NS-I) and North Su–IIb (NS-IIb).

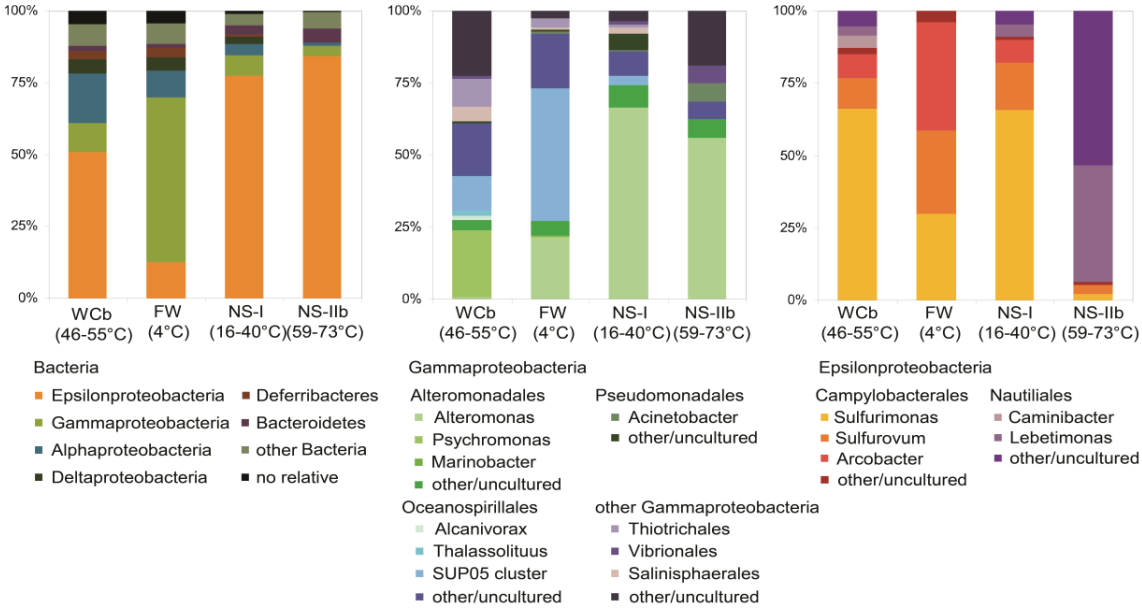


Figure 2: Relative abundances (CARD-FISH) of *Gamma-* and *Epsilonproteobacteria* and total cell counts (TCC) in diffuse fluids and in incubation experiments (WC, FW and NS-I). Relative abundance (bars) and TCC (symbols) are given as mean values. Left bars: relative abundance in diffuse fluids. Right bars: relative abundance in incubation experiments.

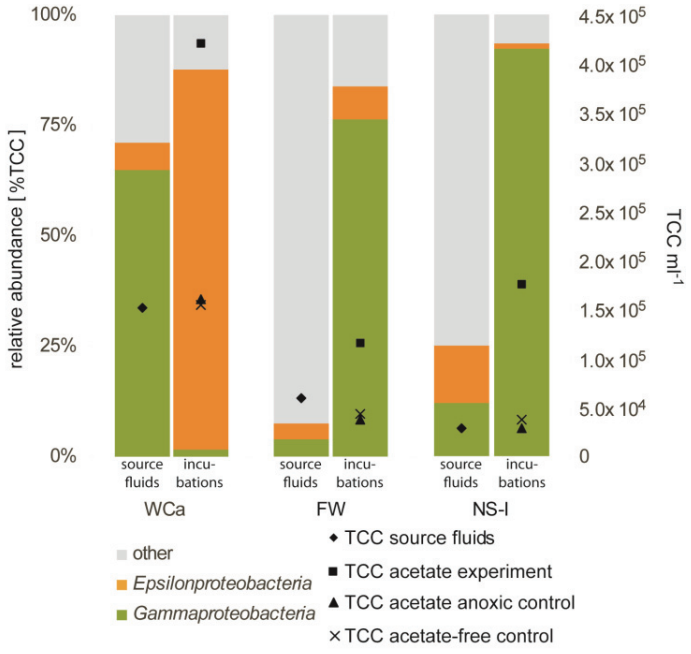


Figure 3: Phylogenetic reconstruction of 16S rRNA sequences of *Gammaproteobacteria* and *Epsilonproteobacteria* in diffuse fluids (pyrotags) from Woody Crack (Menez Gwen) and Manus Basin and from ^{13}C-acetate incubation experiments (clones). The dark gray rectangle indicates the NautMG-group targeted by probe Naut842. The light dark rectangle indicates groups targeted by competitors to probe Naut842. Numbers of sequences per OTU (97% sequence identity cut-off) are given in parenthesis. Scale bar represents 10% estimated sequence divergence.

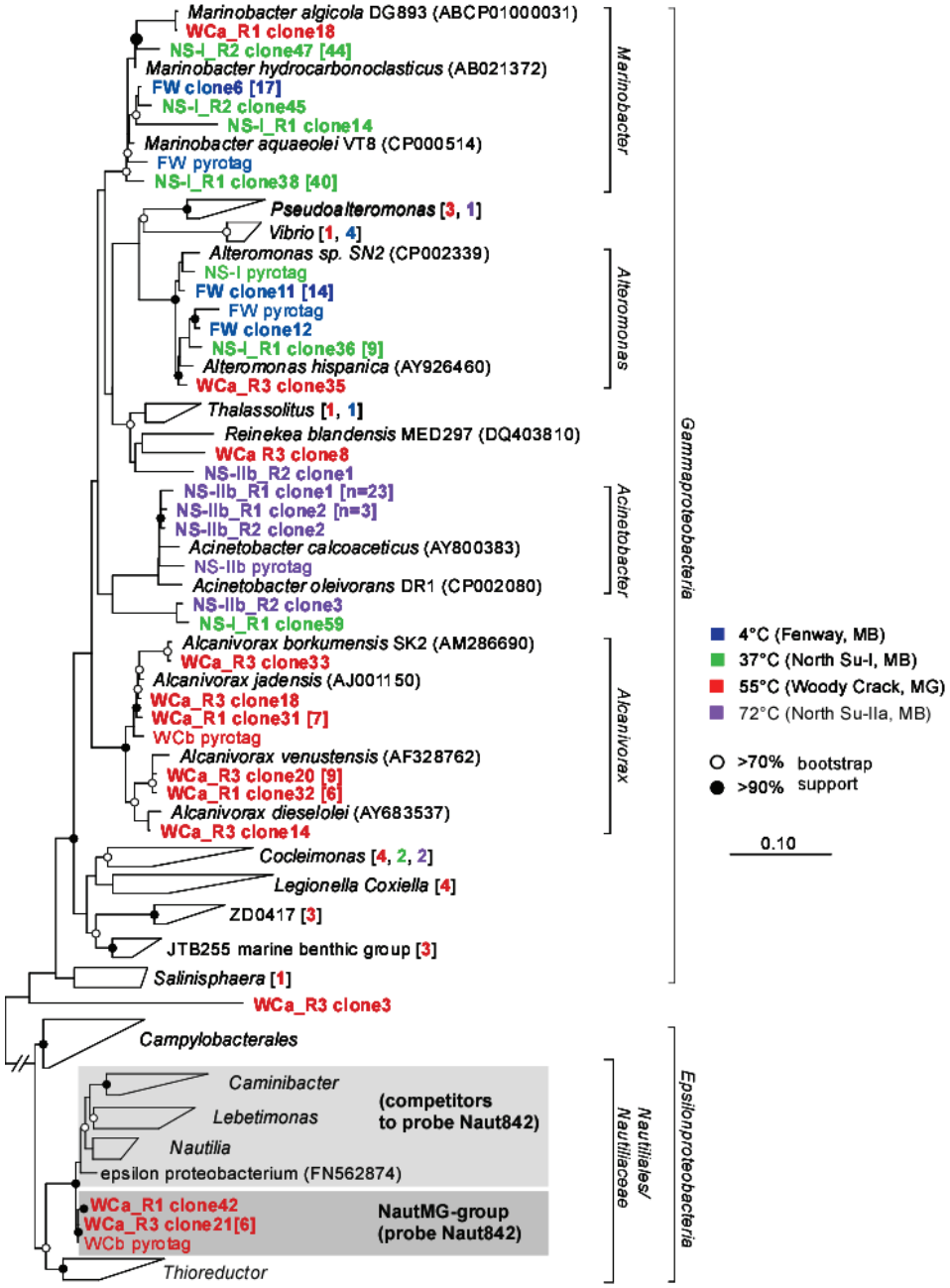


Figure 4: Relative abundance of *Gammaproteobacteria*, *Epsilonproteobacteria* and of the NautMG-group in diffuse fluids and in ^{13}C-acetate incubation experiments of Woody Crack. Images show detection of NautMG-cells (green fluorescence) by probe Naut842 in diffuse fluids (left) and in incubation experiments (right) using CARD-FISH. Scale bar represents 5 µm.

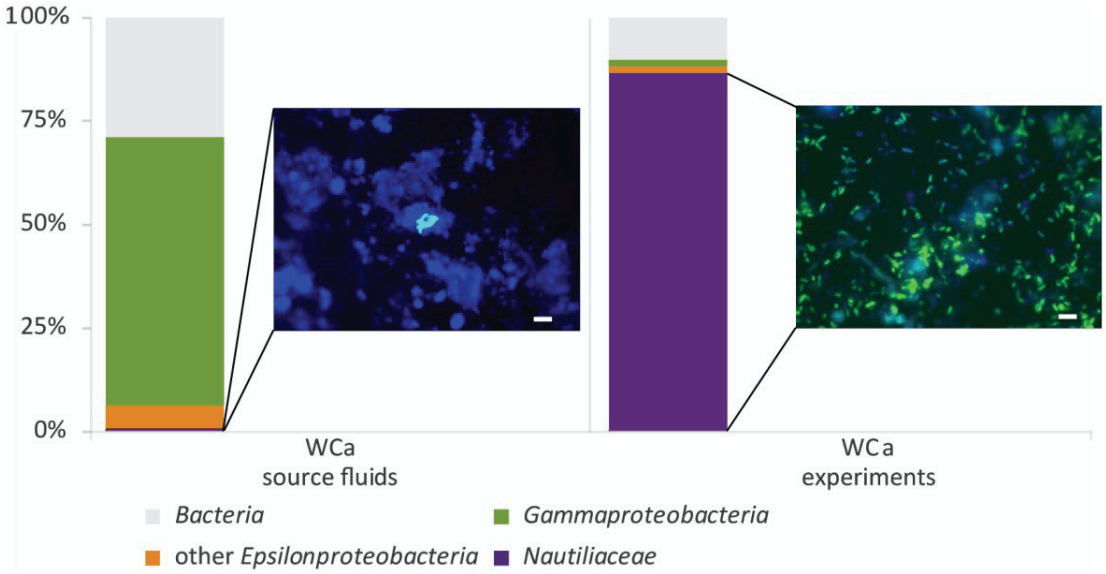


Figure 5: NanoSIMS images of ^{13}C-acetate and ^{15}N-ammonium assimilation by single cells in incubation experiments at Menez Gwen and Manus Basin. Upper row: Woody Crack (WCa, 55°C). Middle row: Fenway (FW, 4°C). Lower row: North Su (NS-I, 37°C). *Epsilonproteobacteria* were detected by green fluorescence and *Gammaproteobacteria* by red fluorescence.

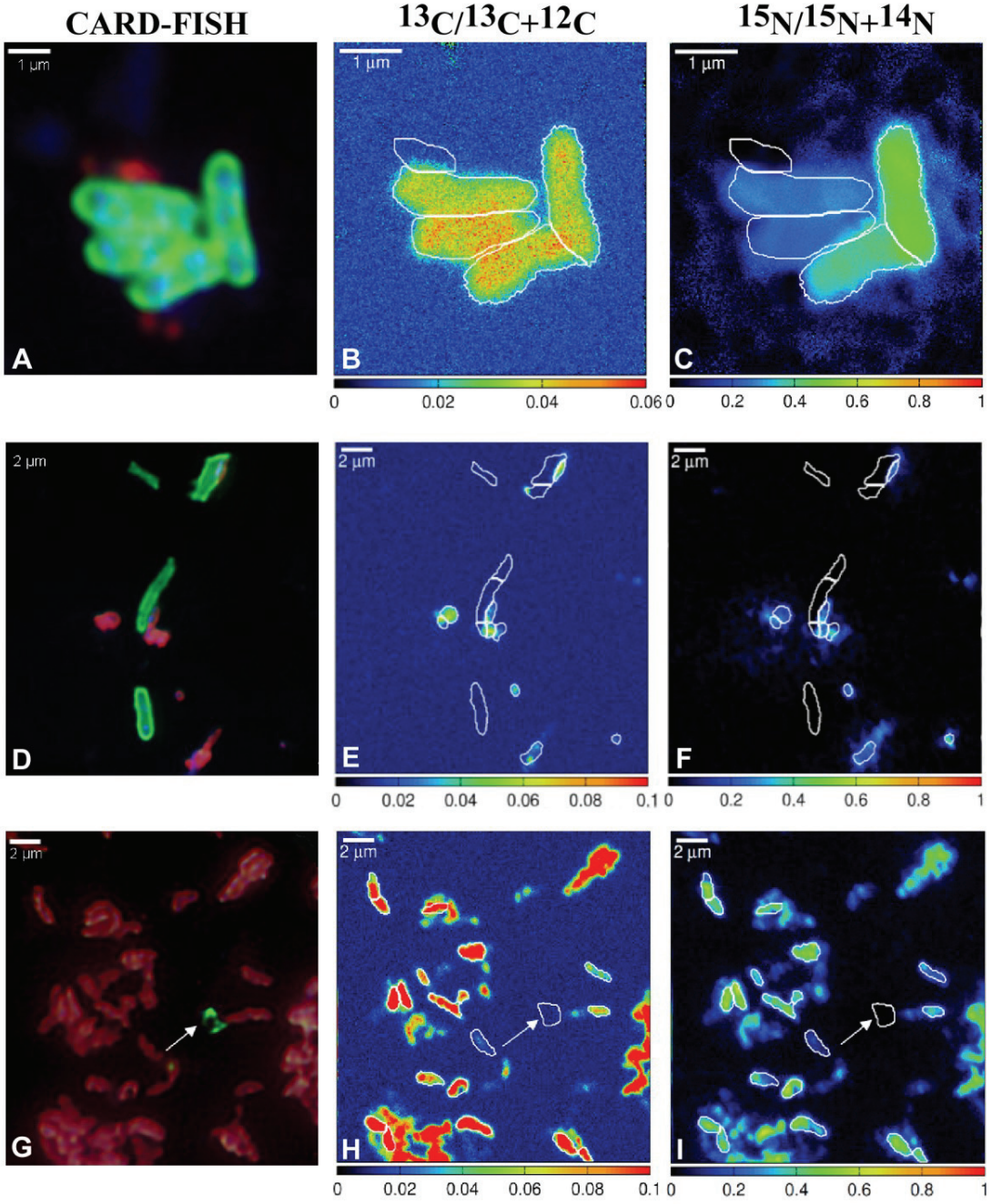
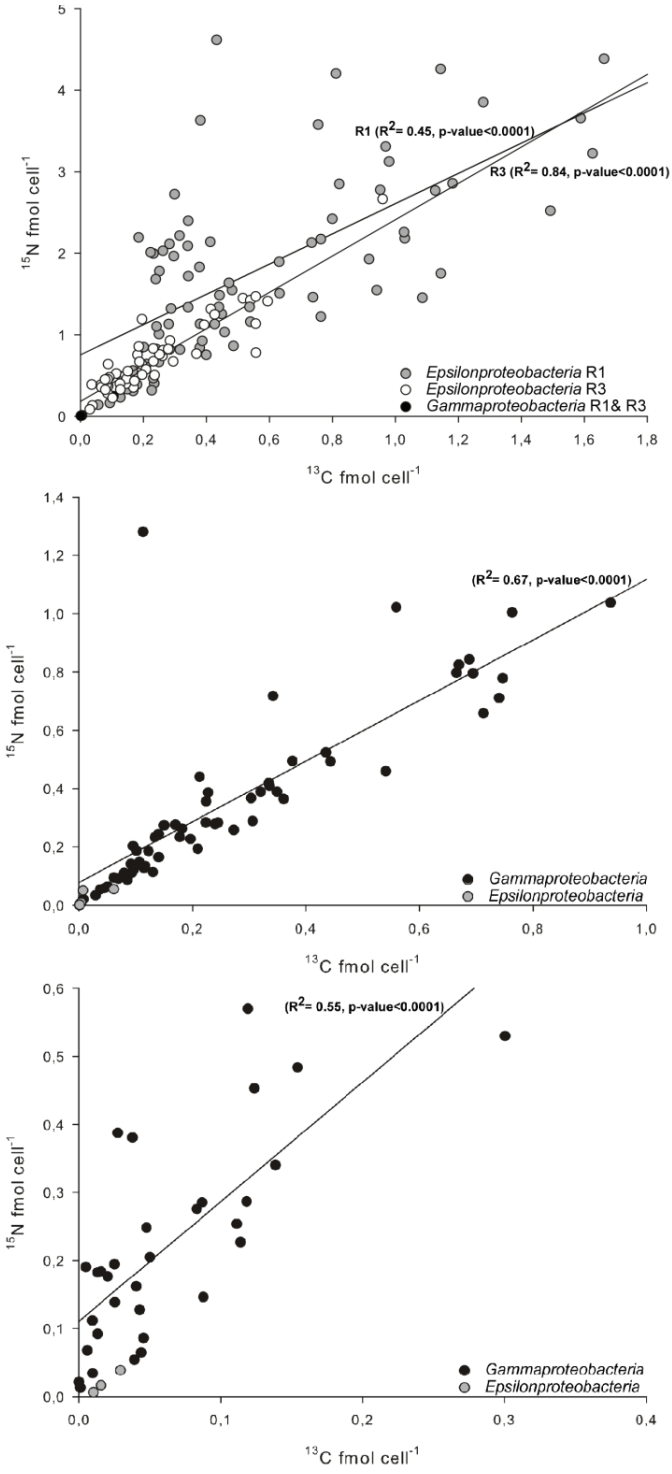


Figure 6: ^{13}C-acetate versus ^{15}N-ammonium assimilation by single cells of *Gammaproteobacteria* and *Epsilonproteobacteria*. Upper row: Woody Crack (WCa, 55°C). Middle row: Fenway (FW, 4°C). Lower row: North Su-I (NS-I, 37°C). Significance level of linear regression analysis was <0.05.



Supplementary information

Construction of 16S rRNA gene libraries and phylogenetic analysis

Bacterial 16S rRNA genes for samples WCa, FW and NS-I were directly amplified from polycarbonate filters. Therefore a filter piece of 2-3 mm was put in a 100µl PCR mix, containing 1x PCR buffer (500 mM KCl, 100 mM Tris-HCl pH 8.3 at 25°C, 15 mM WC^{2+}) (5 Prime, Hamburg, Germany), 0.2 mM of each deoxynucleoside (Roche, Basel, Schweiz), 0.5 µM of each primer (Biomers, Ulm, Deutschland), 0.3 ml^{-1} BSA (Fluka, Buchs, Schweiz) and 0.01 U $µl^{-1}$ *Taq*-polymerase (5 Prime, Hamburg, Germany). For sample NS-IIa, DNA was first extracted from polycarbonate filters with the Ultra Clean Soil DNA Kit (MoBio Laboratories, Carlsbad, USA) and 20 µl PCR reactions with 1µl DNA template and same ingredients as given above were set up. The bacteria specific 16S rRNA gene primers GM3F and GM4R (Muyzer *et al*., 1995), as well as GM5F and 907RM (Muyzer *et al*., 1998) were used for library construction. The thermocycler conditions were 94°C for 5 minutes (denaturation), followed by 25-40 cycles of 94°C for 1 minute (denaturation), 44°C for 1.5 minutes (annealing) and 72°C for 1 minute (elongation), concluded with a final elongation step at 72°C for 10 minutes.

All PCR products were run on a 1% LE agarose gel and stained with 1x SYBR green I solution (Invitrogen, Karlsruhe, Germany) dissolved in 1x TAE buffer. DNA bands of desired size were excised with a sterile scalpel, dissolved in pre-warmed PCR water and ligated directly or after gel purification with QIAquick gel extraction kit (QIAgen, Hilden, Germany) into the pCR®4-TOPO® vectors of the TOPO TA cloning kit (Invitrogen, Karlsruhe, Germany) or pGem-T-easy vector of the Promega cloning kit (Promega, Mannheim, Germany). Chemically competent *E. coli* cells, strain TOP 10, were transformed with the ligation products, as described in the manuals. Clones were screened for inserts or the right size with M13F and M13R vector primers (Yanisch-Perron *et al.,* 1985). PCRs were performed in 20 µl reactions as described above. Positive screening PCR products were cleaned with the PCR Clean Up NucleoFast96 kit from Machery & Nagel according to manufacturers' protocol. Cleaned PCR products were Sanger sequenced with the internal primer 907RM (Muyzer *et al.,* 1998) using the ABO Prism BigDye Terminator v 3.0 cycles sequencing kit and an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Darmstadt, Germany).

16S rRNA gene amplicons and 454-pyrotag sequencing of bacteria in situ *fluid diversity*

Bacterial diversity in diffuse fluid samples was analyzed by 454-pyrosequencing of 16S rRNA gene amplicons. Therefore DNA was extracted with the Ultra Clean Soil DNA Kit (MoBio Laboratories, Carlsbad, USA) from a PES filter piece of pumped fluids. Bacterial 16S rRNA genes were amplified with modified GM3 (Muyzer *et al*., 1995) ad 907RM (Muyzer *et al*., 1998) primers in ten parallel PCR reactions. Primer modification included the addition of individual, error-tolerant, hexamer barcodes at the 5' end of primer 907RM, and the extension of both GM3F and 907RM primers with asymmetric SfiI-restriction sites at the 5' end (for subsequent ligation of the 454-sequencing adapters). PCR's were performed with the Phusion High Fidelity Polymerase Kit (Finnzymes, New England BioLabs Inc.), following manufacturers' instructions. Therefore, 1x Phusion HF Buffer, 250 µM of each deoxynucleoside triphosphate, 0.5 µM of each primer and 0.4 U of Phusion DNA polymerase were mixed and to each reaction 1 µl DNA template was added. The thermocycler conditions were set as follows: 3 min initial denaturation at 98°C, 30 cycles consisting of 10 sec denaturation at 98°C, 30 sec of annealing at 48°C and 30 sec elongation at 72°C, followed by a 10 min final elongation step at 72°C. Replicate reactions were pooled after amplification and, DNA was precipitated during a 60 min incubation on ice, after addition of 3.3 volumes of non-denaturated, absolute ethanol and 0.15 volumes of 3 M Na-Acetate (ph 5.2). Finally, DNA was pelleted by 20 min centrifuged at 10,000 g and the pellet was re-suspended in 20 µl TE buffer. Next, DNA was mixed with 2 µl loading dye and run on a 1% low-melting agarose electrophoresis gel. The gel was stained with SYBR Green I Nucleotide Stain and the DNA bands were visualized under UV light. Band of the desired size (~900 bp) were cut out with sterile scalpels. The obtained gel fragments were dissolved in 60 µl of pre-warmed (65°C) PCR water and purified with the MinElute Gel Extraction Kit (QIAgen) as instructed in the accompanying protocol. DNA concentrations were determined fluorometrically at 260nm, with the Qubit 2.0 Fluorometer and the Qubit dsDNA HS Assay KIT (life Technologies). Amplicones were pooled and shipped to the Max Planck Genome Center (Cologne, Germany). There, amplicon pools were digested for 1 hour at 50°C with a SfiI-restriction enzyme (NEB) and digested DNA was purified with the MinElute PCR purification kit (QIAgen). In the next step, the 454 adaptors (A and B) were ligated to the fragment, by an overnight incubation with 1 U T4 DNA ligase (Roche) at 6°C. After incubation, the ligase was inactivated by 10 min heat treatment at 65°C. To remove excess adapters by size fractionation, electrophoresis on a 2% LE agarose gel

was performed. Gel bands were excised and purified with the QIAquick gel extraction kit (QIAgen). Finally, emulsion PCRs were performed with the GS FLXTitanium LV emPCR Kit (Lib-L) and sequencing with the GS FLX Titanium Sequencing Kit XLR70 on a Roche 454 Genome Sequencer FLX+ instrument according to the manufacturers' protocols.

Design and testing of specific probe for the MG-specific group

We designed an oligonucleotide probe (Naut842) targeting only the 16S rRNA of the *Nautiliaceae*-phylotype defined as NautMG-group (Fig. 5) and two unlabeled helper oligonucleotides (Fuchs *et al.,* 2000). For discrimination from other *Nautiliaceae* we designed three competitor oligonucleotides (up to 3 mismatches). The competitors bind to the target site of the Naut842 oligonucleotide probe in closely related non-target organisms (Table S2). We tested the probe *in silico* with the mathFISH web-based software tool (Yilmaz *et al.,* 2011) with parameters for FISH as described in material and methods. Calculated formamide dissociation curves were used as proxy for testing the probe. A formamide series was performed on cells from the incubations to optimize stringency conditions. Specificity of probes and competitors can be checked using the TestProbe tool at the ARB-Silva database according to Quast *et al.* (2013) (see also www.arb-silva.de/search/testprobe/).

Supplementary Tables and Figures

Table S1: Statistics on 454-pyrosequences analyzed and used in this study.

Sample	Reads retrieved	Sequences (% passed quality check)	Average length (bp)	Sequences (after quality check)		
				OTUs[1] (%)	Clustered[2] (%)	Replicates[3] (%)
WCb	7389	6798 (91.44)	416.6	1605 (21.9)	4601 (61.7)	592 (16.4)
FW	3393	3316 (94.7)	445.9	860 (25.9)	1832 (55.3)	624 (18.8)
NS-I	6889	6739 (94.5)	441.6	1043 (15.5)	3954 (58.7)	1742 (25.8)
NS-IIb	469	461 (96.2)	465.0	81 (17.6)	315 (68.3)	65 (14.1)

[1] number of OTUs (≥98% sequence identity) in the sample

[2] number of unique reads in the sample assigned to one OTU (≥98% sequence identity)

[3] reads identical to another read within the sample

Table S2: Oligonucleotide probes applied in this study.

Probe name	Target group	Sequence (3'-5')	position[1]	% FA	Reference
EUB338	**most *Bacteria***	GCTGCCTCCCGTAGGAGT	338-355	0-50[2]	(Amann *et al*., 1990)
EUB338 II	***Plantomycetales***	GCAGCCACCCGTAGGTGT	338-355	0-50[2]	(Daims *et al*., 1999)
EUB338 III	***Verrucomicrobiales***	GCTGCCACCCGTAGGTGT	338-355	0-50[2]	(Daims *et al*., 1999)
EUB338 IV	***Lentisphaerae***	GCAGCCTCCCGCAGGAGT	338-355	0-50[2]	(Arnds, 2009)
NON338	**complementary to EUB338**	ACTCCTACGGGAGGCAGC	-	0-50[2]	(Wallner *et al*., 1993)
Gam42a	***Gammaproteobacteria***	GCCTTCCCACATCGTTT	1027-1043	35	(Manz *et al*.,1992)
cGam42a	**competitor for Gam42a**	GCCTTCCCACTTCGTTT	1027-1043	35	(Manz *et al*., 1992)
EPSY549	***Epsilonproteobacteria***	CAGTGATTCCGAGTAACG	549-566	35	(Lin *et al*., 2006)
EPSY914	***Epsilonproteobacteria***	GGTCCCCGTCTATTCCTT	914-932	35	(Loy, 2003)
Naut842	**NautMg-group of *Nautiliales* (Fig. 3)**	CTGCGTGACTGCAGGACTGT	842-862	30	this study
Naut_helper1	**helper**	CTACTAGTGGTTGTGGGGGG	822-842	30	this study
Naut_helper2	**helper**	ATGGCTACTAGTGGTTGTGGGG	820-842	30	this study
cNaut842_1	**competitor for Naut842 to other *Nautiliales*, e.g. *Lebetimonas* sp.**	CTGCGTGACTGAAGGACTGT	842-862	30	this study
cNaut842_2	**competitor for Naut842 to other *Nautiliales*, e.g. *Lebetimonas* sp.**	CTGCGTGACTGAAGGACTAT	842-862	30	this study
cNaut842_3	**competitor for Naut842 to other *Nautiliales*, e.g. *Caminibacter, Nautilia***	CTGCGTGACTGAGGGACTAT	842-862	30	this study

[1] *E. coli* numbering according to (Brosius *et al*., 1978)

[2] Probe is specific over a range of formamide concentrations

Table S3: TCC and CARD-FISH results in diffuse fluids and in incubation experiments. Mean values and standard deviations are given, when experiments were performed in triplicates.

Sample	TCC [cells ml^{-1}]	x-fold change of TCC	CARD-FISH [%]				
			EUB I-IV	Arch915	Gam42a	EPSY549/914	Naut842
Woody crack (Menez Gwen), 55°C							
Diffuse (source) fluid (WCa)	1.6 ± 0.3 $x10^5$		99		65	10	0.8
Oxic ^{13}C-acetate incubation	4.4 ± 0.2 $x10^5$	2.7/2.8	93.7 ± 3.6		1.5 ± 0.2	86.3 ± 2.5	84/87
Source fluid (WCb)	1,26 $x10^5$						
Anoxic ^{13}C-acetate incubation	1,32 $x10^5$	1.0					
Acetate-free control	1,28 $x10^5$	1.0					
Fenway (FW. Manus Basin) 4°C							
Diffuse fluid	6.2 ± 0.5 $x10^4$		58	8	4	4	
Oxic ^{13}C-acetate incubation	0.8/1.6 $x10^5$	1.3/2.5	99		63/87	1.6/12	
Anoxic ^{13}C-acetate incubation	4.0/4.2 $x10^4$	0.6/0.7					
Acetate-free controls	4.5/4.9 $x10^4$	0.7/0.8					
North Su-I (NS-I, Manus Basin) 37°C							
Diffuse fluid	3.3 ± 1.4 $x10^4$		83/85	11	9/12	7/15	
Oxic ^{13}C-acetate incubation	1.8/3.7 $x10^5$	5.5/11.3	99		78/108	1.1/1.3	
Anoxic ^{13}C-acetate incubation	2.9/3.5 $x10^4$	0.9/1.1					
Acetate-free controls	3.8/4.2 $x10^4$	1.2/1.3					
North Su IIa (NS-IIa, Manus Basin) 72°C							
Diffuse fluid	2.2 ± 0.8 $x10^4$		62/64		5/15	8/11	
Oxic ^{13}C-acetate incubation	2.9/3.5 $x10^4$	1.4/1.6	1.0/3.8	0.5/0	0.6/0	0.2/0	
Anoxic ^{13}C-acetate incubation	1.2/1.5 $x10^4$	0.6/0.7					
Acetate-free controls	1.7/2.3 $x10^4$	0.8/1.0					

Table S4: Bulk assimilation of ^{13}C and ^{15}N as perceptual isotope ration in oxic and anoxic incubations and acetate-free control incubations (% $^{13}C/C_{total}$, % $^{15}N/N_{total}$) determined by IRMS.

Sample	oxic		anoxic		control[3]	
	$^{13}C/^{12}C$	$^{15}N/^{14}N$	$^{13}C/^{12}C$	$^{15}N/^{14}N$	$^{13}C/^{12}C$	$^{15}N/^{14}N$
WC	**55°C – 8 h incubations**					
Triplicate 1[1,2]	2.20	23.58	1.07	0.63	n.d.	
Triplicate 2[2]	1.84	26.05	1.07	0.65		
Triplicate 3[1,2]	1.84	19.83	1.08	0.70		
Dead control 1	1.08	0.95	1.07	n.d.		
Dead control 2	1.08	0.63	n.d.	n.d.		
FW	**4°C – 12 h incubations**					
Duplicate 1[1,2]	2.20	22.09	1.08	0.43	1.08	0.79
Duplicate 2[2]	1.56	9.19	1.08	0.60	1.08	0.83
Dead control	1.09	0.46	1.08	0.43	1.08	0.50
NS-I	**37°C – 10 h incubations**					
Duplicate 1[1,2]	2.00	11.27	1.08	0.55	1.10	0.97
Duplicate 2[2]	10.70	45.62	1.08	0.51	1.09	0.61
Dead control	1.08	0.49	1.08	0.40	1.08	0.76
NS-IIa	**72°C – 8 h incubations**					
Duplicate 1[1,2]	1.35	4.32	1.08	0.42	1.08	0.45
Duplicate 2[2]	1.15	2.28	1.08	0.42	1.08	0.46
Dead control	1.08	1.28	1.08	0.38	1.08	0.41

[1] used for NanoSIMS analysis (only oxic incubations with ^{13}C-acetate)

[2] used for CARD-FISH/16S rRNA gene diversity analysis

[3] controls incubated with ^{13}C-bicarbonate instead of ^{13}C-acetate

n.d. – not determined

Figure S1: Upper panel: sampling sites of this study (red dots). Lower left panel: Woody Crack (WC) with associated fauna (*Bathymodiolus* mussels, crabs). Middle panel: North Su (NS) with ROV-arm holding KIPS system and a coupled temperature sensor. Right panel: tube worms at Fenway (FW).

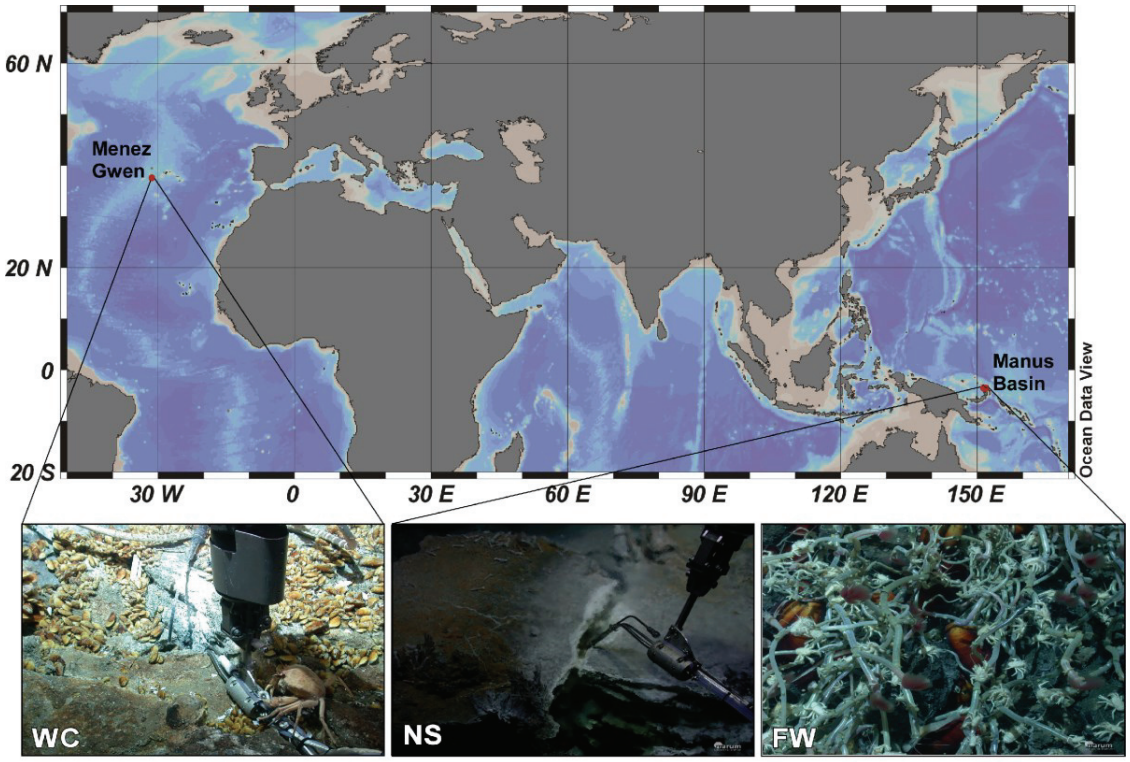


Figure S2: Bulk assimilation of ^{13}C-acetate and ^{15}N-ammonia. Each bar represents measurement of one replicate.

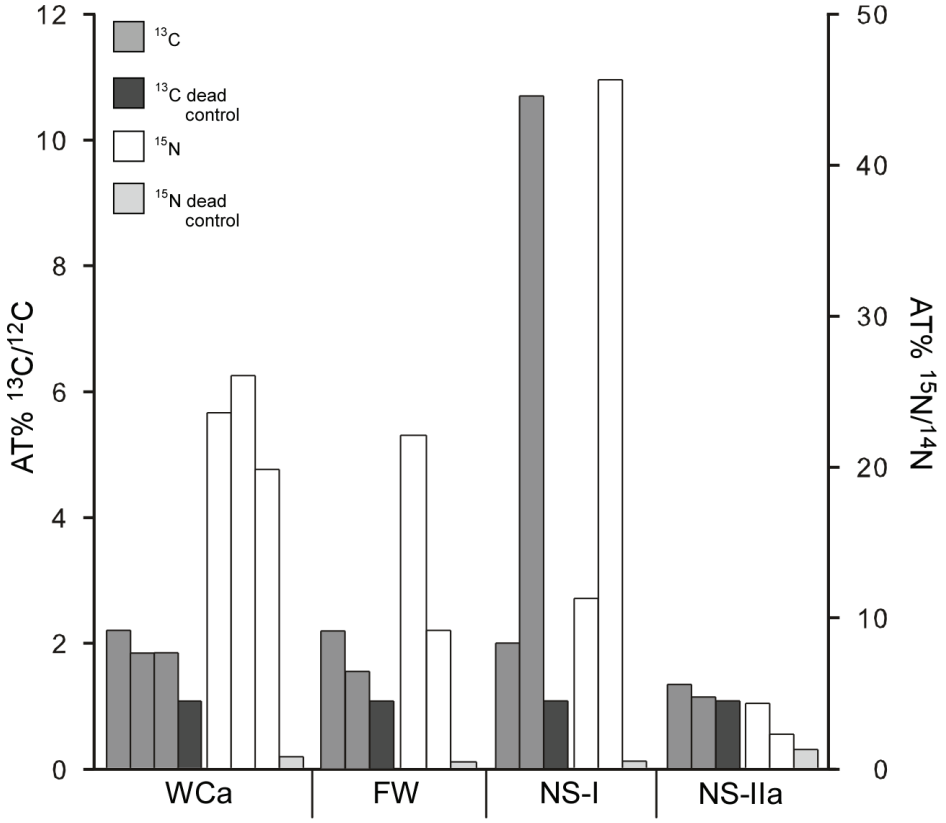
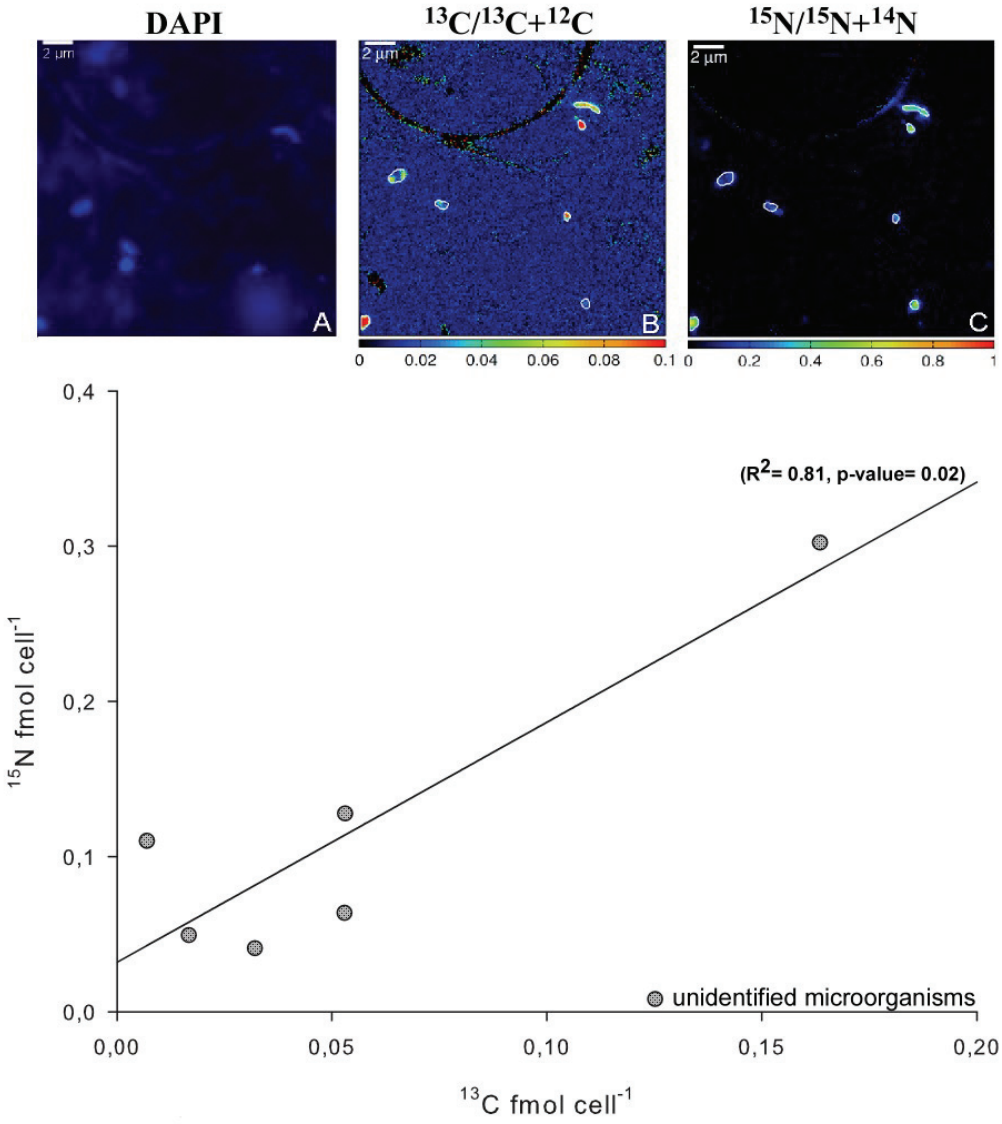


Figure S3: NanoSIMS analysis of ^{13}C-acetate and ^{15}N-ammonium assimilation by single cells in incubation experiments with North Su-IIa fluids (NS-IIa, 72°C) from Manus Basin. **(**A) DAPI staining of phylogenetically unidentified cells used for nanoSIMS analysis. B) $^{13}C/^{13}C + ^{12}C$ image. C) $^{15}N/^{15}N + ^{14}$ image. Scatter plot shows assimilation rates in amol μm^{-3} after 8 h incubation. Significance level of linear regression analysis was <0.05.



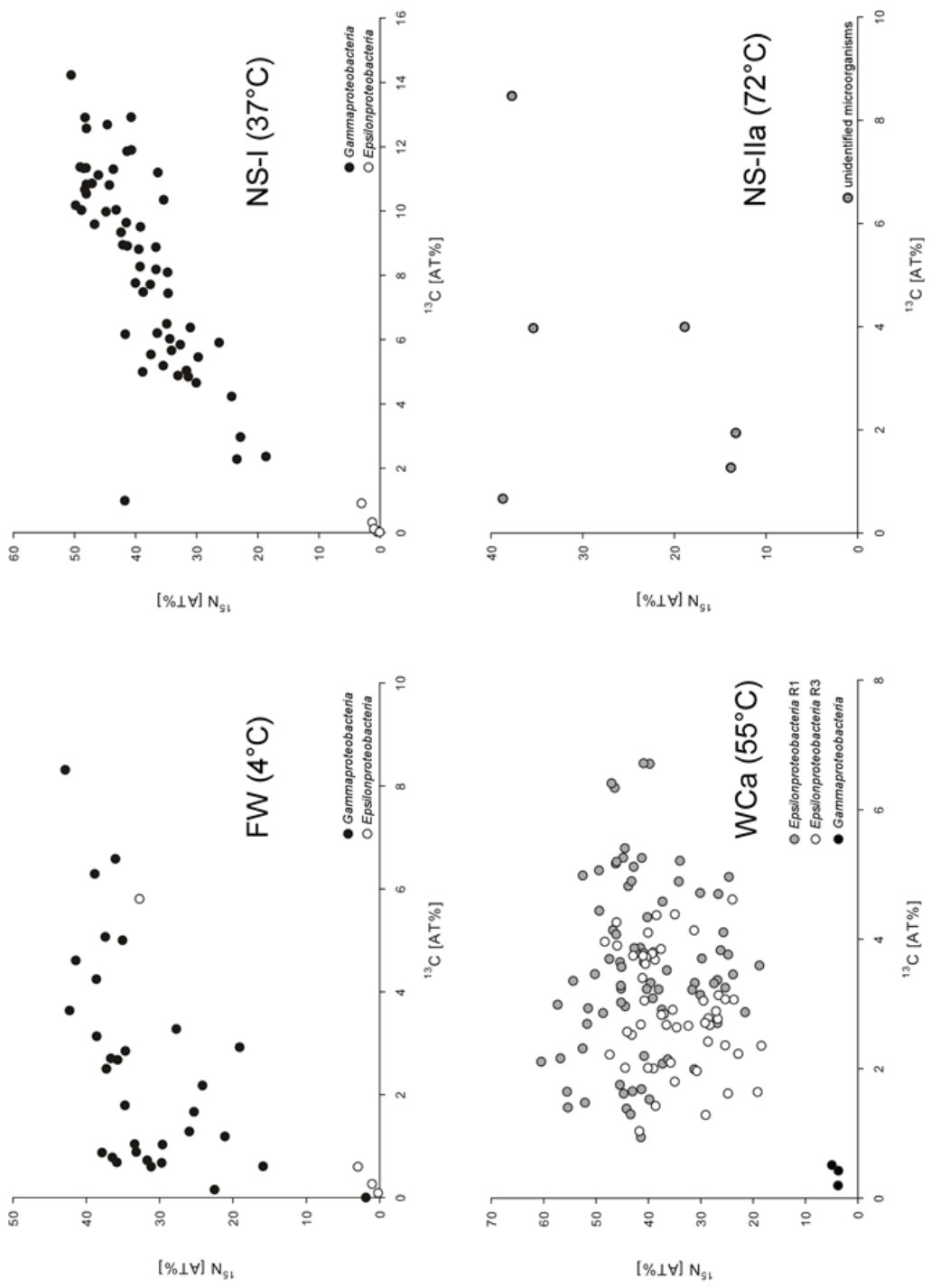


Figure S4: Comparison of atom- per cent labeling of individual cells in ^{13}C-acetate versus ^{15}N-ammonium from all incubation experiments analyzed by nanoSIMS. Values were corrected for bulk dead controls.

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Chapter VI

Sulfur-oxidizing *Epsilonproteobacteria* in S^0-rich tidal pools

Petra Pjevac, Alexey Kamyshny Jr, Marc Mußmann

Short report on additional results obtained in this thesis

Contributions:

P.P. and M.M. developed concepts and ideas. P.P. collected tidal pool samples, performed CARD-FISH and 16S rRNA gene diversity analysis. A.K. measured RSS and sulfate concentrations.

Sulfur-oxidizing *Epsilonproteobacteria* in S^0-rich tidal pools of the Janssand tidal flat (German Wadden Sea)

The Janssand tidal flat in the German Wadden Sea (introduced in more detail in **Chapter II**) has been a model study site for marine researches for many years. Studies conducted in Janssand have contributed largely to our understanding of microbial and biogeochemical processes in marine coastal systems, including processes of the sulfur cycle (e.g. Llobet-Brossa *et al*., 1998; 2002; Bosselmann *et al*., 2002; Mussmann *et al*., 2003; 2005; Kamyshny and Ferdelman, 2010; Lenk *et al*., 2011; 2012). It was previously shown that gammaproteobacterial and alphaproteobacterial SOPs are dominant in Janssand sediments (Lenk *et al*., 2011; 2012). During my thesis research, we discovered the important role of the less abundant *Epsilonproteobacteria* in oxidation of free S^0 in coastal sediments (**Chapter II**). However, the SOP diversity in S^0-rich tidal pools, formed during low tide at the Janssand sediment surface (Jansen *et al*., 2009; Kamyshny and Ferdelman, 2010), remains poorly characterized (Lenk, 2011).

Tidal pools are small, enclosed seawater ponds retained between sediment ripples at the surface of tidal flats during low tide. Pressure-driven seepage feeds some of these pools with sulfide-rich pore waters from deeper sediment layers (Røy *et al*., 2008). Highly sulfidic tidal pools typically display a milky turbidity, caused by the presence of colloidal S^0 (Fig. 3). In a previous study (Lenk, 2011), *Epsilonproteobacteria* were found to be abundant (22% of total cell counts) in such a turbid tidal pool. Additionally, *Arcobacter* spp. dominated a gradient enrichment culture from sulfidic Janssand sediments in which S^0-precipitation was observed (Lenk, 2011). *Arcobacter*-related *Epsilonproteobacteria* were previously detected in Janssand sediments (Llobet-Brossa *et al*., 1998) at low relative abundances. It was therefore hypothesized that *Arcobacter* relatives and other *Epsilonproteobacteria* are non-particle attached SOPs, originating from seeping pore waters and are involved in sulfide oxidation and S^0 formation in tidal pools (Lenk, 2011).

To further investigate this hypothesis, I used CARD-FISH to screen several turbid and clear tidal pool samples obtained between March and September 2011 for the presence of epsilonproteobacterial SOPs. Samples were fixed with 2% formaldehyde (final concentration) at 4°C overnight, and aliquots of 3-10 ml were filtered on polycarbonate membranes (0.2 µm pore size, Millipore, Darmstadt, Germany). CARD-FISH was performed as described in Pernthaler *et al*., 2002 with following oligonucleotide probes: Arc1430 (Sneidr *et al*., 1997), Epsy549 (Lin *et al*., 2006),

Epsy914 (Loy, 2003), Epsy404 (Macalady *et al*., 2006) and Epsy682 (Alm *et al*., 1996). Multiple *Epsilonproteobacteria*-specific probes were used, to these have overlapping, but not identical target groups and diversity within the pools was unknown. Furthermore, I constructed a 16S rRNA gene clone library from one turbid tidal pool sample (Pool_March_I), applying protocols given in the Experimental Procedures and Supplementary Methods of **Chapter II**. Concentrations of RSS (sulfide, polysulfide and S^0) and sulfate in three turbid and three clear tidal pools sampled in May 2011 were determined in parallel with molecular samples by A. Kamyshny

CARD-FISH results showed that *Epsilonproteobacteria* were always more abundant in turbid than in clear tidal pools, whereby relative abundances strongly varied in turbid pools (Table 3, Fig. 8). Furthermore, we confirmed high concentrations of RSS in turbid tidal pools with abundant *Epsilonproteobacteria* (Fig. 9). Additionally, the low sulfate concentrations in turbid pools, typical for pore waters from deeper sediment layers, support upwards pore water flow. This supports the hypothesis of seeping pore water as possible source of the detected *Epsilonproteobacteria* (Fig. 9; Lenk, 2011). Interestingly, only two out of 125 sequences from the tidal pool 16S rRNA gene clone library affiliated with *Sulfurimonas/Sulfurovum*-related *Epsilonproteobacteria*, and no *Arcobacter*-related sequences were recovered (data not shown). However, these results might be biased, as for example the most frequently retrieved gammaproteobacterial sequences in our clone library are related to *Photobacterium*, which is known to have fourteen 16S rRNA gene copies (Pei *et al*., 2010).

Table 3: Abundance of *Epsilonproteobacteria* as determined by CARD-FISH for Janssand tidal pools sampled between March and September 2011, and a turbid tidal pool sample analyzed previously by S. Lenk (Lenk, 2011)

Sample	Turbidity	CARD-FISH results [%]				
		Epsy549	Epsy682	Epsy404	Epsy914	Arc1430
Pool_April 2005[1]	+	n.d.	n.d.	22	n.d.	12
Pool_March[2]	+	13	11	<0.5	55	<0.5
Pool_April	+	1	n.d.	n.d.	3	n.d.
Pool_May_I	+	n.d.	n.d.	n.d.	1.5	n.d.
Pool_May_II[3]	+	n.d.	n.d.	n.d.	17	n.d.
Pool_May_III[3]	+	8	n.d.	n.d.	22	n.d.
Pool_May_IV[3]	-	<0.5	n.d.	n.d.	<0.5	n.d.
Pool_May_V[3]	-	<0.5	n.d.	n.d.	<0.5	n.d.
Pool_May_VI[3]	-	<0.5	n.d.	n.d.	<0.5	n.d.
Pool_September_I	+	2	n.d.	n.d.	4	n.d.

[1] pool analyzed and described in Lenk (2011);[2] 16S rRNA gene sequence data available (unpublished);
[3] sulfide, S^0 and polysulfide concentrations determined; n.d. - not determined

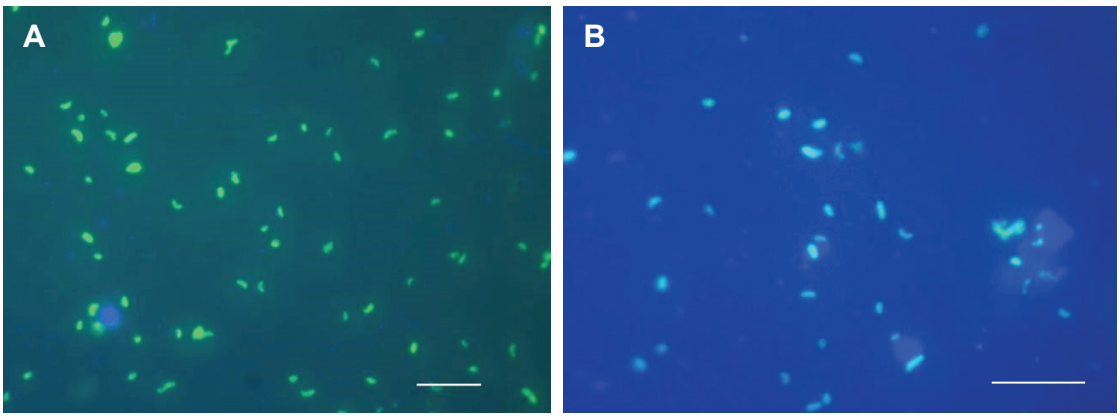

Figure 9: Epsilonproteobacterial cells, hybridized with probe Epsy914, in tidal pool samples from (**A**) Pool_May_III and (**B**) Pool_March. In green: *Epsilonproteobacteria*, in blue: cells with DAPI signal not targeted by the Epsy914 probe.

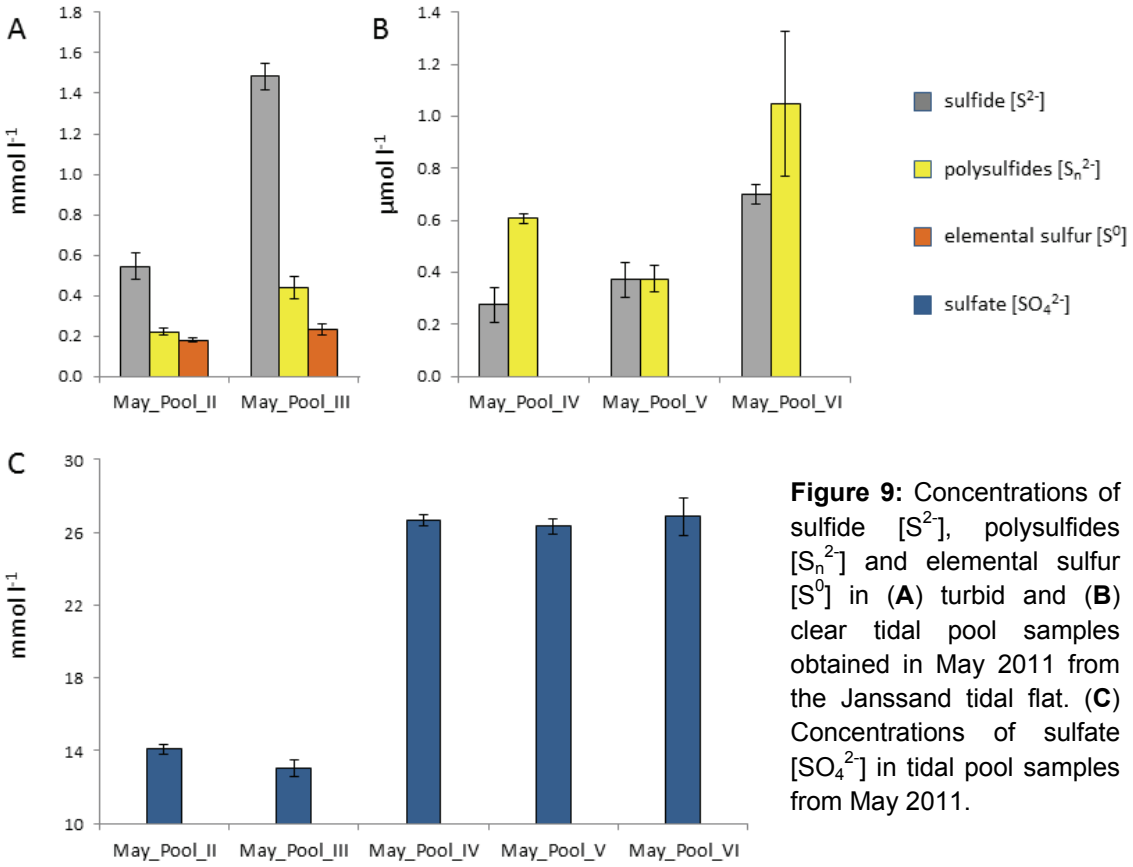

Figure 9: Concentrations of sulfide [S^{2-}], polysulfides [S_n^{2-}] and elemental sulfur [S^0] in (**A**) turbid and (**B**) clear tidal pool samples obtained in May 2011 from the Janssand tidal flat. (**C**) Concentrations of sulfate [SO_4^{2-}] in tidal pool samples from May 2011.

In summation, our results reveal an abundance of *Epsilonproteobacteria* in S^0-rich tidal pools and strongly support the hypothesis that *Epsilonproteobacteria* are important for RSS-oxidation in this environmental niche. Additional experiments are necessary to fully characterize the epsilonproteobacterial populations in S^0-rich tidal pools and to determine which of the available RSS sources they thrive on. Also, the presence and activity of other SOPs in the tidal pools need to be examined. Although the tidal pools are transient environments and probably do not largely contributing to overall sulfur-oxidation dynamics in the Janssand tidal flat, they represent an interesting ecological phenomenon. The concentration of different RSS in tidal pools changes within minutes to hours (Kamyshny and Ferdelman, 2010). Therefore, time series samplings of the pools would offer a unique possibility to study small-scale dynamics and fluctuations in sulfur-oxidizing microbial communities *in situ*. Such a study has the potential to contribute considerably to our understanding of niche differentiation, competition and succession in mesophilic, marine, sulfur-oxidizing communities.

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Chapter VII

General Discussion and Outlook

7.1. General discussion

Numerous studies have investigated the diversity of SOPs in sulfidic marine habitats. It is a well-established fact that distinct sulfur-oxidizing communities are found in different marine aquatic and sedimentary environments (e.g. Ravenschlag *et al*., 1999; Taylor *et al*., 1999; Musat *et al*., 2006; Grote *et al*., 2007; Omoregie *et al*., 2008; Lavik *et al*., 2009; Yamamoto and Takai, 2011; Lenk *et al*., 2012). However, most of the factors determining the observed differences in SOP diversity are not well defined. By identifying commonalities and differences between sulfur-oxidizing microbial communities in several distinct marine environments, my coworkers and I were able to detect some habitat-independent features that strongly influence SOP diversity and act as potential niche differentiation mechanisms.

7.1.1 *Epsilonproteobacteria* and S^0 – substrate specificity as niche differentiation mechanism among SOPs

S^0 is a major constituent of the inorganic sulfur pool in both coastal and deep-sea benthic environments (Troelsen and Jørgensen, 1982; Zopfi *et al.*, 2004; Kamyshny and Ferdelman, 2010). However, the connection between the presence of S^0 and the diversity of SOPs in benthic marine habitats has not been examined before. During my thesis research, my collaborators and I investigated the diversity of microorganisms colonizing S^0 in two coastal sediments (Janssand and Königshafen tidal flats, German Wadden Sea) and two hydrothermal systems (Guaymas Basin and Manus Basin). We found that irrespective of geographical distance and different geochemical settings in the investigated habitats, the dominant S^0-colonizing SOPs were always *Sulfurimonas*/*Sulfurovum*-related *Epsilonproteobacteria*. By measuring sulfate production on colonized particles, we were able to confirm that the *Sulfurimonas*/*Sulfurovum* group *Epsilonproteobacteria* not only colonize, but also oxidize S^0. Moreover, we verified this S^0-oxidation potential in pure culture experiments with *Sulfurimonas denitrificans*, which exhibited rapid growth with pure cyclooctasulfur (S_8). The reoccurring association between S^0/S_8 and *Sulfurimonas*/*Sulfurovum*-related *Epsilonproteobacteria* therefore reflects substrate specificity. Our findings, presented in **Chapter II**, are the first report of a potential niche differentiation mechanism among SOPs based on substrate specificity.

S^0 is the most stable form of sulfur under standard conditions, and S_8 is typically the most abundant S^0 allotrope (Kamyshny, 2009). Although over 30 stable S^0 allotropes exist (Meyer, 1976), only a few of them are relevant for SOPs (Fig. 10; Dahl and Prange, 2006). Biogenic S^0, accumulated extra- or intracellulary during sulfide oxidation by some SOPs, is built of these biologically important allotropes and contains a variable fraction of S_8 (Prange *et al*., 2002; Dahl and Prange, 2006). While anoxygenic phototrophic SOPs preferentially store organic polysulfanes, S_8 seems to be the main storage compound in many colorless sulfur bacteria (Prange *et al*., 2002; Engel *et al*., 2007). Not all SOPs are able to oxidize S_8, however. For example, the PSB *Allochromatium vinosum* only utilizes the polymeric sulfur fraction during growth on S^0 (Franz *et al*., 2007). Even *Beggiatoa* storing S_8 intracellulary do not oxidize the S_8 directly. Instead, they produce polysulfides as oxidation intermediates (Berg *et al*., 2014). S_8 is easy to access in reducing environments, where it can be chemically cleaved by sulfide and polysulfides. However, S_8 is stable in sulfide-free environments and thus the capability to oxidize S_8 can serve as competitive advantage over other SOPs in such conditions. It remains to be tested how widespread S_8 oxidation is among SOPs and which mechanisms mediate S_8 oxidation in *Sulfurimonas/Sulfurovum*-related *Epsilonproteobacteria* and potentially other S_8-oxidizing prokaryotes. Only then can the environmental importance of S_8 oxidation and the proposed substrate-specific niche differentiation mechanism be fully assessed.

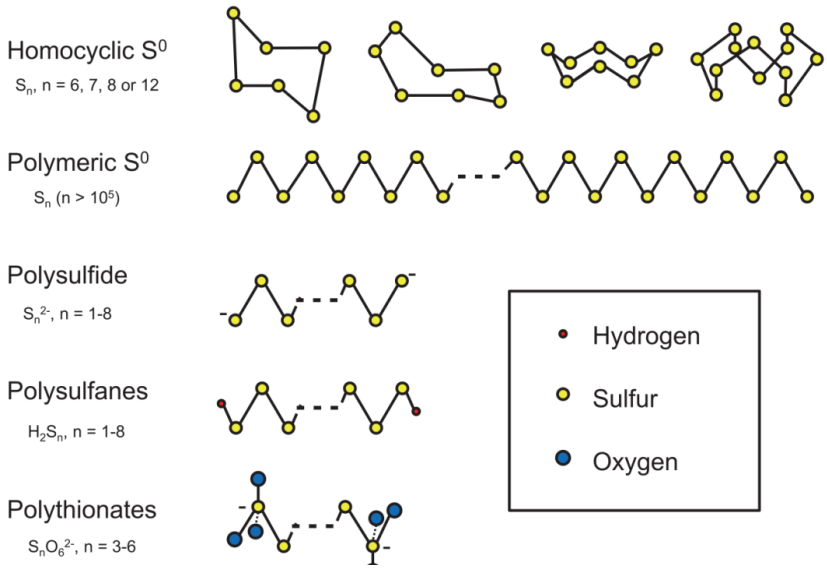

Figure 10: Allotropes of S^0 relevant for microbial sulfur cycling. Molecules and ions are represented by covalent binding models. Figure based on data from Steudel (1975), Meyer (1976) and Dahl and Prange (2006).

7.1.2 Life in a sulfide gradient – the influence of sulfide concentration on niche differentiation among chemotrophic SOPs

Microbial respiration processes establish and maintain chemical stratification in many marine environments, as favorable electron acceptors are consumed along redox gradients (Tratnyek and Macalady, 2000). For this reason, sulfide and suitable electron acceptors for sulfide oxidation, like nitrate and oxygen, usually are present in well-defined, opposing concentration gradients. The steepness of these gradients depends mainly on substrate fluxes and availability of reducible carbon (Brandes and Devol, 1995). Many such stratified marine environments have been investigated by microbiologists through the years (e.g. Trüper and Genovese, 1968; Cohen *et al.,* 1977; Eckert and Trüper, 1993; Mas and van Gemerden, 1995; Casamayor *et al*., 2000; Sorokin and Kuenen, 2005; Gregersen *et al*., 2009). Diverse photo- and chemotrophic SOPs were described to be important and abundant. In some studies, an influence of sulfide to oxygen concentration ratios on the composition of sulfur-oxidizing microbial communities was reported (e.g. Schulz *et al*., 1996; Macalady *et al*., 2008; Schmidtova *et al*., 2009; Grünke *et al*., 2011; Comeau *et al*., 2012). During my thesis research, I investigate the microbial diversity and occurrence of SOPs in marine aquatic environments with different sulfide to oxygen ratios.

My collaborators and I investigated a microbiologically poorly characterized shallow sea-water lake on the Croatian Adriatic coast, in which sulfide to oxygen ratios strongly differed between stratified and holomictic periods (**Chapter III**). In the course of this study, we detected a community shift from a mainly phototrophic sulfur-oxidizing community during stratification, to an almost exclusively chemotrophic sulfur-oxidizing community following a lake perturbation, accompanied by changes in sulfide and oxygen availability and formation of colloidal S^0. Under stratified conditions, only a narrow and steep zone of overlap between sulfide and oxygen existed. Most of the sulfide was oxidized by abundant anoxygenic phototrophic SOPs in anoxic water layers. The dominant SOPs were marine *Prosthecochloris*-related GSB and the halophilic *Halochromatium*-related PSB, previously detected in similar saline stratified environments, like marine lagoons, fjords and salt marshes (e.g. Overmann *et al*., 1991; Burke and Burton 1988; Guyoneaud *et al*., 1996; Tank *et al*., 2011; Galand *et al*., 2012). However, during holomixis characterized by very low sulfide concentrations and oxygen depletion almost in the entire water column, populations of phototrophic SOPs

diminished. Simultaneously, chemotrophic SOPs became abundant. In particular, chemotrophic sulfur-oxidizing SUP05 clade *Gammaproteobacteria* formed a large bloom. These chemotrophic SOPs were not detected in Lake Rogoznica under stratified conditions.

Members of the SUP05 clade are frequently detected in various marine environments with low sulfide concentration and microoxic to anoxic conditions, including hydrothermal-vent plumes, diffuse hydrothermal fluids, stratified fjords, inlets and pelagic oxygen minimum zones (e.g. Lavik *et al*., 2009; Schmidtova *et al*., 2009; Dick and Tebo, 2010; Zaikova *et al*., 2010; Yamamoto and Takai, 2011; Anderson *et al*., 2013; Glaubitz *et al*., 2013). Meta-omic studies revealed that these *Gammaproteobacteria* are metabolically versatile. Chemoautotrophic oxidation of reduced sulfur compounds under denitrifying conditions seems to be their key metabolism, but H_2 oxidation and oxygen respiration were also reported (Walsh *et al*., 2009; Anantharaman *et al*., 2013; Georges *et al*., 2014). An isolate from the closely related Arctic96BD-19 clade assimilates organic carbon (Marshall and Morris, 2013) - a competence not yet demonstrated, but possibly present in some members of the SUP05 clade. The capability to utilize different electron donors and acceptors, and perhaps even multiple carbon sources might have determined the success of SUP05 clade *Gammaproteobacteria* following the sudden change in environmental conditions during anoxic holomixis in Lake Rogoznica.

The second sulfidic and S^0-rich environment I examined were turbid pools forming at the surface of the Janssand tidal flat (German Wadden Sea) during low tide (**Chapter VI**). In these pools, concentrations of RSS change rapidly during a pool's short lifetime of 4-8 hours (Jansen *et al*., 2009; Kamyshny and Ferdelman, 2010), but are generally higher than concentrations measured during holomictic conditions in Lake Rogoznica. It was hypothesized that the S^0 formed in the tidal pools might be the result of microbial sulfide oxidation (Kamyshny and Ferdelman, 2010; Lenk, 2011). Preliminary CARD-FISH results indicate that *Epsilonproteobacteria*, including potentially S^0-precipitating *Arcobacter* (Lenk, 2011), are abundant in the tidal pools. In the previous study, gammaproteobacterial SOPs abundant in Janssand sediments were not detectable by CARD-FISH in a turbid tidal pool (Lenk, 2011). However, sequencing of 16S rRNA genes indicated that they are present. While the abundance of gammaproteobacterial SOPs was not determined, the abundance of *Epsilonproteobacteria* varied strongly between tidal pools. A positive correlation between

epsilonproteobacterial abundance and RSS concentrations from turbid tidal pools sampled in May 2011 was observed (Table 3 and Fig. 9, **Chapter VI)**. Consequently, the abundance of SOPs in tidal pools might be related to variations in sulfide concentration over time. However, the results reported in **Chapter VI** are only preliminary findings. Additional investigation of these pools would further our understanding of the influence of sulfide concentration on the abundance of different SOPs.

While studying S^0 oxidation and carbon assimilation in the Manus Basin (**Chapters II, IV** and **V**), I also examined the microbial diversity in different temperature diffuse hydrothermal fluids collected from this system. Some of the data is included in **Chapter II** and **V**. The distribution and diversity of SOPs, however, was not in the focus of these studies. Therefore, I reexamination the available 16S rRNA gene sequence data retrieved from diffuse hydrothermal fluids focusing on SOP diversity and distribution (Fig. 11). The analyzed diffuse hydrothermal fluids were sampled at the eastern Manus Basin and the North Su and Fenway vent fields (described in more detail in **Chapter II** and **V**). The sampled diffuse hydrothermal fluids had a temperature range of 4°C to 73°C. In all fluids, sulfide was detected with an *in situ* mass spectrometer (ISMS). While the exact *in situ* sulfide concentrations are not available yet, preliminary analysis indicates increase in sulfide concentration with temperature increase (unpublished data, S. Hourdez). *Ex situ* measured sulfide concentrations confirm this observation (FW, NS-I and NS-II fluids, see **Chapter V**). A comparison of 16S rRNA gene sequence diversity between different diffuse hydrothermal fluid samples indicates that gammaproteobacterial SOPs, in particular members of the SUP05 clade, are more frequent in the less sulfidic low temperature fluids (E, FW, FW-II), while epsilonproteobacterial SOPs dominate in the more sulfidic higher temperature fluids (NS, FW-III, NS-I, NS-III; Fig. 11). Sequence diversity in the one of the hottest fluid sample (NS-II, see **Chapter V**) is dominated by sequences related to H_2 oxidizing, S^0 reducing members of the epsilonproteobacterial order *Nautiliales*, indicating more reducing conditions prevailed in this fluid sample. The dominant SOPs in all fluid samples were either SUP05 clade *Gammaproteobacteria* or *Sulfurimonas*/*Sulfurovum*-related *Epsilonproteobacteria*.

The co-occurrence of SUP05-related gammaproteobacterial and *Sulfurimonas/Sulfurovum*-related epsilonproteobacterial SOPs has been reported in other sulfidic marine environments (Sunamura et al., 2004; Lavik *et al*., 2009; Glaubitz *et al.,* 2013). In the here analyzed diffuse hydrothermal fluids, the distribution of SUP05

clade SOPs and epsilonproteobacterial SOPs is shifting along a temperature, sulfide and pH gradient. Dominance of SUP05 clade SOPs at low sulfide concentrations and epsilonproteobacterial SOPs at high sulfide concentrations is in line with our observations from Lake Rogoznica and S^0-rich tidal pools in Janssand. Both the SUP05 clade and epsilonproteobacterial SOPs are mesophilic (e.g. Nakagawa and Takai, 2008). It is unlikely that differing temperature tolerance caused the transition from SUP05 to *Sulfurimonas*/*Sulfurovum* dominance in diffuse hydrothermal fluids between 11°C and 15°C. Especially since we have detected closely related *Sulfurimonas*/*Sulfurovum* SOPs as dominant S^0-colonizers in the Manus Basin under psychrophilic conditions (~4°C, **Chapter II**), as well as abundant SUP05 in the >20°C warm water column of Lake Rogoznica (**Chapter III**). Cultured representatives of the *Sulfurimonas*/*Sulfurovum* group grow at pH values of 4.5 to 9 and are abundant in mildly acidic hydrothermal environments (Inagaki *et al*., 2003; Takai *et al*., 2006; Yamamoto and Takai, 2011). However, no data on the pH tolerance of SUP05 relatives is available. As they have mainly been detected in neutral pH sea-water environments (Lavik *et al*., 2009; Glaubitz *et al*., 2013; Marshall and Morris, 2013) it is possible that the low pH of high temperature hydrothermal fluids had a negative effect on these microorganisms.

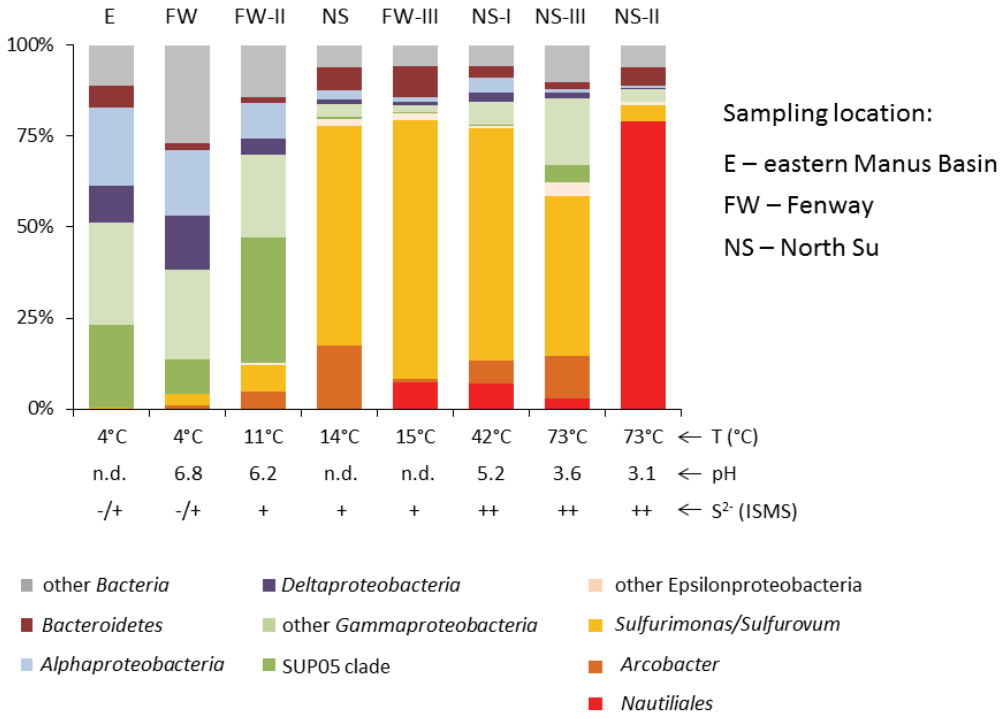

Figure 11: Diversity of bacterial 454-pyrotag 16S rRNA gene sequences in 4°C to 73°C diffuse hydrothermal fluids from different diffuse venting sites in the Manus Basin back-arc spreading center (Bismarck Sea, Papua New Guinea).

On the other hand, oxygen concentration in the low temperature diffuse fluids, which were most likely comparable to sea-water concentrations, possibly inhibited epsilonproteobacterial SOPs, as they rely on the oxygen sensitive rTCA pathway for CO_2 fixation. Oxygen does not seem to have negatively affected the SUP05 clade *Gammaproteobacteria*, although they have been thus far most commonly found in anoxic or microoxic water layers (e.g. Lavik *et al*., 2009; Glaubitz *et al.,* 2013).

In conclusion, not all factors determining and affecting the distribution of gammaproteobacterial and epsilonproteobacterial SOPs in the here discussed sulfidic marine environments could be clarified. However, the detected sulfide-concentration dependent community diversity is well in line with previous observations. Our results provide additional support for the recently proposed significance of sulfide concentration for niche differentiation between SUP05 clade and *Sulfurimonas*/*Sulfurovum*-related SOPs in marine aquatic environments (Glaubitz *et al.,* 2013).

7.1.3 Thiotrophy and carbon assimilation in hydrothermal environments

7.1.3.1 CO_2 fixation by SOPs at hydrothermal vents

Thiotrophic microorganisms have long been recognized as important primary producers in hydrothermally influenced systems, both as free-living organisms and symbionts of metazoans (e.g. Ruby *et al*., 1981; Felbeck and Somero, 1982; Wirsen *et al*., 1993; Campbell *et al*., 2003; Sievert *et al*., 2007a; Dubilier *et al*., 2008). The most abundant SOPs in marine hydrothermal vent systems are *Gammaproteobacteria*, *Epsilonproteobacteria* and *Aquificales* (Sievert *et al*., 2008b). Their distribution in hydrothermal environments is presumed to be related to differing temperature, oxygen and sulfide tolerances (Nakagawa and Takai, 2008). The *Aquificales* are thermophiles growing at temperatures of 40-80°C, while the sulfur-oxidizing *Epsilonproteobacteria* and *Gammaproteobacteria* are mostly mesophilic (Nakagawa *et al*., 2003; Campbell *et al*., 2006; Nakagawa and Takai, 2008). Isolates of *Epsilonproteobacteria* and *Aquificales* only grow under anoxic or microoxic conditions, since they apply the oxygen-sensitive rTCA pathway for CO_2-fixation (Fig. 5; Hügler and Sievert, 2011). *Gammaproteobacteria* tolerate higher oxygen concentrations, as they fix CO_2 via the oxygen tolerant CBB pathway (Nakagawa and Takai, 2008; Sievert *et al*., 2008b; Hügler and Sievert, 2011). In general, hydrothermal vent associated SOPs are metabolically versatile.

Representatives of all three groups can use various RSS and H_2 as electron donors with O_2 and NO_3 as electron acceptors (Campbell *et al*., 2006; Nakagawa and Takai, 2008; Sievert *et al*., 2008b; Petersen *et al*., 2011; Anantharaman *et al*., 2013).

Many studies have investigated the diversity and abundance of SOPs in hydrothermal environments, but the relative contribution of the different groups to vent-associated primary production is still being debated. Only recently have researchers started questioning the paradigm of CBB-mediated CO_2-fixation as the most important carbon assimilation process at vent systems. New evidence indicates that *Epsilonproteobacteria* utilizing the rTCA pathway might be the major thiotrophic primary producers in hydrothermal environments (Inagaki *et al*., 2003; Campbell *et al*., 2006; Sievert *et al*., 2008b, Hügler and Sievert, 2011). However, the few studies that attempted to quantify the contribution of CBB versus rTCA utilizing microorganisms to total primary production at hydrothermal vent sites have reported contrasting results (e.g. Campbell and Cary, 2004; Wang *et al*., 2009; Hügler *et al*., 2010; Crépeau *et al*., 2011; Xie *et al*., 2011; Olins *et al*., 2013). In an attempt to overcome possible biases introduced by the application of single analysis procedures, my collaborators and I applied a combination of methods from molecular ecology, organic geochemistry and fluid geochemistry to identified the dominant primary producers in hydrothermal sulfide deposits from an unsedimented, rock-hosted hydrothermal system in the Manus Basin (**Chapter IV**). Based on differences in ^{13}C-isotope composition of TOC and IPLs and their comparison to 16S rRNA gene diversity, we identified *Sulfurimonas/Sulfurovum*-related *Epsilonproteobacteria* as the dominant microorganisms and CO_2-fixation via the rTCA pathway as the dominant mode of primary production in active venting structures. Our results support previous hypotheses on the importance of the rTCA cycle for CO_2-fixation in similar structures from vent sites of comparable geochemistry (e.g. Kato *et al*., 2010; Flores *et al*., 2012).

Most importantly, our case study demonstrated how an integrated approach, relying on multiple independent methods, can help elucidating the question of carbon flow in hydrothermal and other environments. The heterogeneous environmental niches at hydrothermal vent sites strongly influence the distribution of vent-associated microorganisms (Nakagawa and Takai, 2008), making it challenging to determine the carbon budget in these environments. In more diverse and complex hydrothermal habitats, like sedimented hydrothermal systems and microbial mats, the selection of adequate approaches is essential in order to detect, revise and exclude methodological

biases and obtain reliable results in carbon-assimilation studies. We suggest that the independent analysis and comparison of ^{13}C-composition in biomarkers like IPLs with phylogenetic and functional marker-gene diversity interpreted in the context of geological and geochemical constraints, as introduced in our study, is a robust approach to investigated microbial carbon-assimilation in a variety of environments.

7.1.3.2 Organic carbon assimilation by SOPs at hydrothermal vents

Besides the importance of SOPs for primary production, it has been postulated that heterotrophic SOPs might significantly contribute to carbon flow in hydrothermal environments (Jannasch, 1995). Although it has been debated for over 40 years, this hypothesis has not been tested in detail to date (Sievert *et al.,* 2008b). It relies primarily on the isolation of numerous facultatively heterotrophic SOPs and facultatively sulfide-oxidizing heterotrophs from hydrothermal vents sites (Ruby *et al*., 1981; Durand *et al*., 1994; Teske *et al*., 2000; Pearson *et al.,* 2005). Besides, the capability to utilize organic carbon substrates for mixo- and heterotrophic growth has been demonstrated for close relatives of the most abundant vent-associated gammaproteobacterial and epsilonproteobacterial SOPs (Wirsen *et al*., 2002; Berg *et al*., 2013; Labrentz *et al*., 2013; Marshall and Morris, 2013). As one of the first steps towards unraveling the importance of heterotrophs at hydrothermal vents, my collaborators and I investigated the assimilation of ^{13}C-acetate into microbial biomass form diffuse hydrothermal venting fluids in the Manus Basin and Menez Gwen hydrothermal systems (**Chapter V**). Different temperature incubations from the Manus Basin all resulted with acetate-assimilation and growth of diverse heterotrophic *Gammaproteobacteria*. Some of the presumably acetate-assimilating microorganisms (*Vibrio*, *Psudoalteromonas*, *Acinetobacter*) detected are related to the heterotrophic thiosulfate-oxidizers isolated from hydrothermal vent sites (Durand *et al*., 1994; Teske *et al*., 2000). It is possible that their capability to utilize thiosulfate and maybe other RSS provides these microorganisms with a competitive advantage over non sulfur-oxidizing heterotrophs under the fluctuating conditions and erratic organic matter availability at hydrothermal vent sites. In contrast, hydrothermal vent associated *Epsilonproteobacteria* were indeed the dominant acetate consumers in venting fluids from Menez Gwen. However, they were not related to the abundant thiotrophic *Sulfurimonas/Sulfurovum* group, but to yet-uncultured *Nautiliales*. All isolated members of the epsilonproteobacterial order *Nautiliales* are anaerobic or microaerobic S^0-reducers (Campbell *et al*., 2006). We

measured acetate assimilation by *Nautiliales*-relatives under aerobic conditions, revealing that the metabolic versatility within this order is higher than initially assumed. Further analysis of the metabolic capabilities within the new *Nautiliales* cluster, including the possibility of RSS oxidation, is of interest.

Although we did not detect acetate assimilation in populations of abundant, vent-associated gammaproteobacterial and epsilonproteobacterial SOPs, our results do not exclude that these organisms can benefit from organic carbon compounds under not yet resolved conditions. In the analyzed sulfidic hydrothermal systems organic carbon sources were scares, while resources supporting an autotrophic lifestyle were plentifully. Even if the vent-fluid associated SOPs can assimilate acetate, the required enzymatic machinery might not have been expressed at the moment of sampling, since lithotrophic sulfur oxidation provided sufficient energy. However, the capability to assimilate organic carbon would be an important competitive advantage in an environment as fluctuating and dynamic as a hydrothermal vent site (Tunnicliffe and Juniper, 1990; Coumou *et al*., 2006; Perner *et al*., 2009). Further experiments, testing for example the organic carbon assimilation potential after a period of sulfide starvation could provide further insights. Finally, diffuse hydrothermal fluids are only one of the environmental niches occupied by SOPs in hydrothermal systems. While our results provide important insights into the metabolism of diffuse venting associated SOPs, heterotrophic SOP could be more important in other niches, like sulfidic sediments underlying patches of vent fauna that remain to be explored in future.

7.2 Conclusions

Reduced sulfur compound oxidation is a highly significant, ancient and widespread microbial metabolism and therefore an excellent system for studies of microbial evolution and diversification. Besides widespread substrate availability, the multiplicity of environments and the availability of distinct environmental niches within those environments unquestionably promote the great diversity of SOPs. As the majority of SOPs have partially overlapping niches, unique physiological or ecological adaptations can be advantageous in the competition for resources with other SOPs. Previously known examples for such specialization are, for example, the low-light adaptations of GSB, or the adaptation of *Aquificae* and *Acidothiobacilli* to temperature and pH extremes. The substrate-specific S^0/S_8 oxidation by *Sulfurimonas/Sulfurovum*-related *Epsilonproteobacteria* discovered during my thesis research likely represent such a

distinguishing ecological adaptation. Additionally, the increased affinity of distinct SOP groups to certain sulfide concentrations has a similar discriminatory effect. While SUP05 clade *Gammaproteobacteria* and mainly *Sulfurimonas*/*Sulfurovum*-related *Epsilonproteobacteria* remain in competition for the same environmental resources (RSS, oxygen and nitrate), their adaptations to different sulfide concentration result in niche partitioning. This either leads to the competitive success of one group in homogeneous environments, or permits co-existence in environments with discrete sulfide gradients.

In most sulfidic marine habitats environmental conditions are highly dynamic and fluctuating. Although divergent and unique metabolic capabilities are advantageous during competition, they can endanger the maintenance of a population if they define a very narrow ecological niche in an unsteady environment. Temporary collapses of individual niches may occur and cause the collapse of a SOP population. An example is the decline of anoxygenic phototrophic communities during holomixis in the usually stable and stratified seawater lake Lake Rogoznica. In such circumstances, metabolic versatility provides a selective advantage. Most likely, it is the combination of metabolic versatility and increased competitiveness for certain substrates and conditions, which forms the base for the wide habitat range and extensive distribution of the SUP05 clade *Gammaproteobacteria*, and *Sulfurimonas*/*Sulfurovum*-group and *Arcobacter*-related *Epsilonproteobacteria* in various sulfidic marine environments.

7.3 Outlook

The new insight on diversity, distribution and function of SOPs in marine systems gathered during my thesis research furthers the current understanding of ecological mechanisms and environmental conditions influencing the composition of sulfur-oxidizing communities. However, the reasons for extensive intra-clade diversity within the two most widely distributed SOP groups, the SUP05 clade and the *Sulfurimonas*/*Sulfurovum* group, are still unclear.

Few isolates of *Sulfurimonas/Sulfurovum*-related *Epsilonproteobacteria* are well described, while only one isolate of a SUP05-related bacterium is available to date. On the other hand, public databases harbor an enormous diversity of sequences from organisms related to these clades. Especially within the *Sulfurimonas*/*Sulfurovum*-group, great diversity within and between samples from similar environments is apparent (López-Garcia *et al.,* 2003; Opatkiewicz *et al*., 2009, personal observations). The entire

sequence group, however, is still interpreted as one coherent ecotype (Campbell *et al*., 2006). For some marine microorganisms (e.g. SAR11 clade *Alphaproteobacteria*, Morris *et al*., 2005; *Prochlorococcus*, Rocap *et al*., 2003; *Vibrio*, Thompson *et al*., 2005) it has been confirmed that individual sequence clusters relate to specific ecological niches (Polz *et al*., 2006). At the same time, genome data from *Sulfurimonas*/*Sulfurovum*-related *Epsilonproteobacteria* revealed that these lack many DNA repair genes, which affects the frequency of mutations, gene loss and horizontal gene transfer (Nakagawa *et al*., 2007). A comprehensive analysis of the publically available sequence data, along with any accompanying metadata, would be a first step towards determining whether sequence diversity and sequence clusters represent environmentally distinct groups and reflect niche differentiation, or if the broad microdiversity is a consequence of genetic shortfalls in the *Sulfurimonas*/*Sulfurovum* group. The same approach could be applied to analyze diversity within the SUP05 cluster. In this case, particular attention should be paid to the effect of oxygen concentrationson sequence clustering.

Furthermore, while we were able to resolve the influence of S^0 and sulfide concentration on the diversity and composition of sulfur-oxidizing communities in the investigated habitats, other factors that were speculated to influence niche differentiation among SOPs like mildly acidic and alkaline pH or availability of multiple sulfur oxidation pathways remain insufficiently characterized. Co-variance of multiple parameters in the environment complicates the detection of significant effect from small-scale variations in environmental conditions. Cultivation-based laboratory studies would allow the evaluation of single parameter effects on sulfur-oxidizing communities. In the future, representative *Sulfurimonas/Sulfurovum* and SUP05 strains should be used to study ecophysiological responses to changing environmental conditions, as well as intra- and intergroup competition. Insights from laboratory studies can be thereafter used to complement environmental observations. The concepts of substrate-specific and sulfide concentration-dependent niche differentiation as well as the potential for organic carbon assimilation under variable environmental conditions have been proposed on the basis of environmental observations. These too need to be further examined in controlled growth and competition experiments with a variety of phylogenetically and physiological different SOPs, to ensure robustness of the suggested diversification mechanisms.

7.4 References

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APENDIX A

Microbial Lipid Remnants in Hydrothermal Structure Interiors: Evidence for Transport from Subseafloor Environments

Nadine Goldenstein, Marcos. Y. Yoshinaga, Eoghan P. Reeves, Petra Pjevac, Wolfgang Bach and Kai-Uwe Hinrichs

Manuscript in preparation

Microbial Lipid Remnants in Hydrothermal Structure Interiors: Evidence for Transport from Subseafloor Environments

Nadine Goldenstein[1,*], Marcos. Y. Yoshinaga[1], Eoghan P. Reeves[1], Petra Pjevac[2], Wolfgang Bach[1] and Kai-Uwe Hinrichs[1]

[1] MARUM Center for Marine Environmental Sciences & Department of Geosciences, University of Bremen
[2] Max Planck Institute for Marine Microbiology, Bremen

*Corresponding author: nadine.goldenstein@uni-bremen.de

Several microbiological investigations have detected enigmatic evidence for microbes in high temperature (> 200°C) interiors of hydrothermal vent 'chimney' structures (e.g. [1]) well above the known temperature limit for life. It is unclear whether these findings reflect flooding during collection [1], cross-contamination with exterior microbial biomass, or genuine natural phenomena. While microbial populations on vent exteriors have been investigated with DNA- and lipid-based methods, the interior of these structures have rarely been characterized and the origin of this microbial material is poorly understood. In this study, we used a novel coring approach to carefully sample the microbial organic matter present within the interiors of a moderate temperature 'diffuser' structure and an inactive 'black smoker' chimney for lipid-based characterization. SEM-based mineralogy indicates estimated vent fluid temperatures of 170-200°C for the diffuser and >200°C during former venting of the inactive black smoker, and no DNA was extractable from interior samples. However, archaeal core tetraethers (GDGT) were present in the interior of both structures and the intact polar lipid (IPL) diglycosyl-archaeol (2Gly-AR), was widely distributed inside the diffuser (Table 1). Both core GDGT and IPL recovered from the diffuser interior samples were highly distinct relative to exterior microbial lipids, which strongly suggests flooding and/or exterior cross-contamination did not occur. Given temperature estimates indicating uninhabitable conditions, a lack of DNA, fatty acids and the presence of exclusively 2Gly-AR only, the possibility of an active microbial community in the diffuser interior is highly unlikely. This would imply that 2Gly-AR is not representative of living cells in the structure, in contrast to traditional assumptions regarding IPL degradation. Hence, IPL in these chimney interiors do not reflect living community compositions.

Table 1: Distribution of various lipid types in different chimney samples

	'Active black smokers'		'Diffuser'		'Inacitve	
	interior	exterior	interior	exterior	interior	exterior
Bacterial IPL	-	yes	no	yes	no	yes
Bacterial FA	-	yes	no	yes	yes	yes
Archaeal IPL	-	yes	yes	no	no	yes
Archaeal core lipids	-	yes	yes	yes	yes	yes

To further explore causes of lipid variations in both structure interiors, core GDGTs present in interior and exterior samples, were assessed using principal component analysis (Figure 1).

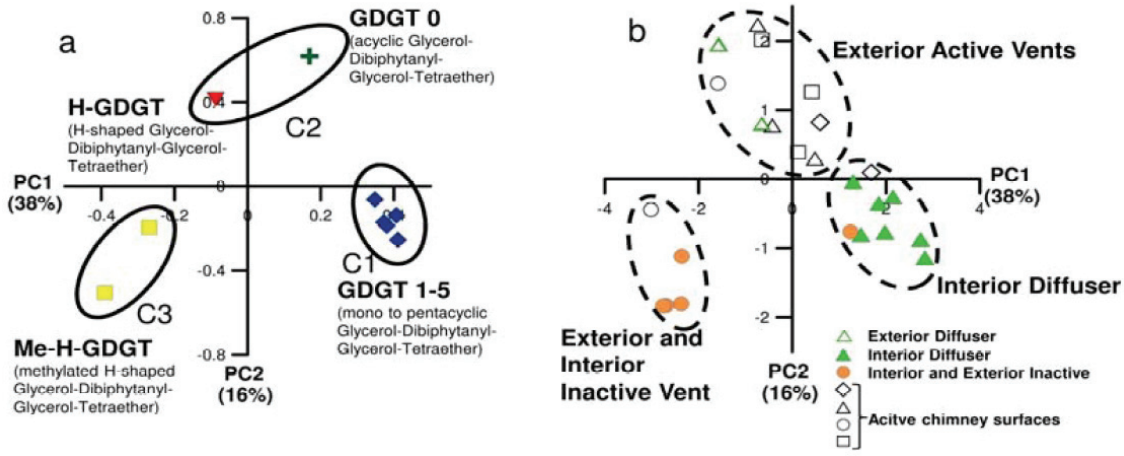

Figure 1: Results of Principal Component Analysis with archaeal core GDGT as loadings (a) and samples as scores (b).

Three distinct clusters (C) were evident - C1 formed by GDGT-1 to -5, C2 containing HGDGT and GDGT-0 and Me-H-GDGT representing C3. Clustering of the active chimney exteriors between C1 and C2 is likely related to the presence of Deep-sea Hydrothermal Vent Euryarchaeota 2 (DHVE-2) and Marine Group I crenarchaeota (MG I) detected in these samples. DHVE-2, rarely found in low-temperature sedimentary environments, are predominantly associated with GDGT-0 and H-GDGT (C2) [2]. Therefore, samples plotting with C2 appear more related to thermophilic archaea (i.e. DHVE-2). However, clustering of diffuser interior samples with C1 indicates a distinctly different source of lipid material compared to exterior samples clustering with C2. In MG-I-dominated sediments, GDGT-0 and -5 were reported to be predominant [4], similar to typical marine sediments. A lack of sediments and evidence for any water column

organic matter contributions to hydrothermal fluids in this system [3] renders these potential sources of microbial lipids unreasonable. We hypothesize they may instead derive from the purported deep biosphere inhabiting the crustal aquifer mixing zones, and reflect cell pyrolysis by and transport with upflowing vent fluid/sea-water mixtures. In contrast to the diffuser, Me-H-GDGT (C3) were found along with bacterial fatty acids in the interior and exterior of the inactive black smoker. These compounds are not common in normal marine subseafloor but are indicative of hyperthermophilic microorganisms and may have been transported into the structure by sea-water entrainment during or after cessation of hydrothermal venting.

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