Molecular Ecology of Key Organisms in Sulfur and Carbon Cycling in Marine Sediments

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Abstract

The World's oceans host a variety of sulfidic habitats. Yet, microorganisms oxidizing reduced inorganic sulfur compounds have mostly been studied at hydrothermal vents, in anoxic basins, conspicuous microbial mats and symbioses but rarely in coastal sediments.

In this thesis sulfur-oxidizing prokaryotes (SOP) of a eutrophic intertidal sand flat in the German Wadden Sea were investigated by molecular techniques. The diversity, abundance and activity of SOP were analyzed in particular among the *Gammaproteobacteria*. Comparative sequence analysis of the 16S rRNA and three genes involved in sulfur oxidation revealed a high diversity of mainly gammaproteobacterial SOP. Most of them were closely related to thiotrophic symbionts, including those of the tubeworm genus *Oligobrachia*. A group of free-living relatives accounted for up to 4% of all cells (~1.3×10^8 cells ml^{-1}). Consistent with a presumed chemolithoautotrophic utilization of inorganic sulfur compounds, these and numerous other members of the *Gammaproteobacteria* incorporated $^{14}CO_2$ as revealed by microautoradiography (MAR). The findings demonstrate that non-filamentous *Gammaproteobacteria* are important catalysts of sedimentary sulfur oxidation and contribute to CO_2-fixation in coastal surface sediments.

Similarly, *Roseobacter* clade bacteria (RCB) accounted for unexpectedly high abundances of up to 10% of all cells in surface sediments (~2.5×10^8 cells ml^{-1}). A RCB-related genome fragment of 35 kb was recovered from a metagenomic fosmid library. It encoded genes of the SOX multienzyme system including the sulfur dehydogenase SoxCD, but also the complete rDSR pathway, a gene arrangement that is unique among SOP. Gene-targeted FISH confirmed the presence of the gene *dsrA* in sedimentary RCB enriched in anaerobic sulfidic medium. In addition, a novel gene, which encodes a putative dioxygenase, designated as *dsrU*, was identified in the rDSR pathway.

Protocols were developed for application of MAR and nano-scale secondary ion mass spectroscopy (nanoSIMS) to marine sediment samples to follow assimilation of acetate in single cells. Members of the *Gammaproteobacteria* appeared to assimilate slightly more acetate than RCB, whereas sulfate-reducing bacteria showed no significant incorporation. Particularly the combination of flow cytometry and nanoSIMS proved to be powerful for up-scaling of the analysis of substrate uptake by sediment bacteria enabling an efficient, high-resolution profiling of single cells from complex microbial communities.

Zusammenfassung

Die Weltmeere umfassen ein Vielzahl sulfidischer Habitate. Mikroorganismen, welche reduzierte inorganische Schwefelverbindungen oxidieren, wurden bisher intensiv an Hydrothermalquellen, in anoxischen Becken, mikrobiellen Matten und Symbiosen erforscht, wenig aber in küstennahen Sedimenten.

In dieser Studie wurden schwefeloxidierende Prokaryoten (SOP) einer eutrophen Sandwattfläche des deutschen Wattenmeeres mit molekularbiologischen Techniken untersucht. Es wurde deren Artenvielfalt, Anzahl und Aktivität analysiert. Ein Fokus lag auf der Gruppe der *Gammaproteobacteria.* Vergleichende Sequenzanalyse der 16S rRNA und dreier Gene der Schwefeloxidation zeigte eine hohe Diversität vorrangig gammaproteobakterieller SOP. Viele waren nahverwandt zu thiotrophen Symbionten, unter anderem zu jenen der Röhrenwurmgattung *Oligobrachia*. Eine Gruppe freilebender Verwandter umfasste bis zu 4% aller Zellen (~10^8 Zellen ml^{-1}). Wie Mikroautoradiographie (MAR) zeigte, inkorporierten diese und viele andere Gammaproteobakterien $^{14}CO_2$, was auf eine chemolithoautrotrophe Nutzung inorganischer Schwefelverbindungen hindeutet. Die Resultate verdeutlichen, dass auch nicht-filamentöse Gammaproteobakterien wichtige Katalysatoren der sedimentären Schwefeloxidation sind und zur CO_2-Fixierung in küstennahen Sedimenten beitragen.

Bakterien der *Roseobacter* Gruppe (RCB) erreichten ebenso hohe Abundanzen von bis zu 10% aller Zellen im Oberflächensediment (~2.5×10^8 Zellen ml^{-1}). Aus einer Metagenombank konnte ein 35 kb langes, RCB-verwandtes Genomfragment identifiziert werden. Jenes kodierte Gene des SOX Multienzymsystems mitsamt der Schwefeldehydrogenase SoxCD sowie des kompletten rDSR Stoffwechselwegs – eine für SOP einzigartige Genkonstellation. Eine Gen-spezifische FISH wies das Gen *dsrA* in RCB aus Sediment nach, welche anaerob in sulfidischem Medium angereichert wurden. Darüber hinaus wurde eine neues Gen im rDSR Stoffwechselweg entdeckt, das, vorläufig als *dsrU* bezeichnet, vermutlich eine Dioxygenase kodiert.

Es wurden Protokolle zur Anwendung von Mikroautoradiographie und nano-skalierter Sekundärionenmassenspektrometrie (nanoSIMS) auf marine Sedimentproben entwickelt, um die Assimilation von Acetat in einzelnen Zellen zu verfolgen. Gammaproteobakterien assimilierten offenbar mehr Acetat als RCB, während sulfatreduzierende Bakterien keine signifikante Aufnahme zeigten. Die Kombination von Durchflußzytometrie and nanoSIMS erwies sich als besonders zielführend für eine Hochskalierung der Analyse der Substrataufnahme durch Zellen aus Sedimenten, da sie ein effizientes, hochauflösendes Screening von vielen Einzelzellen aus komplexen mikrobiellen Gemeinschaften ermöglicht.

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1. Introduction

Altogether 195 countries are located at the shorelines of our World's oceans and, notably, more than half of the World's population lives within a 100 km distance from the coasts. Some coastal ecosystems are among the most productive on Earth (CIA World Factbook 2010). Germany with a total coast length of 2389 km is located at two seas at once. At the Baltic Sea, it borders to one of the large anoxic marine basins. In addition, the Wadden Sea of the North Sea comprises one of the largest tidal zones. In both ecosystems microorganisms catalyze oxidation and reduction processes that are central to the cycling of inorganic sulfur compounds in the water column and the sediment (Al-Raei *et al*., 2009; Grote *et al*., 2007; Jansen *et al*., 2009; Jost *et al*., 2010). In view of these facts, it is remarkable that the main actors, sustaining ecosystem function in pelagic and benthic habitats along Earth's shorelines are poorly characterized.

Nowadays, eutrophication, which refers to the increased production of organic matter by excess load of mineral nutrients, threatens coastal marine ecosystems all around the world (Selman *et al*., 2008, Fig. 1). Large quantities of nutrients are delivered to coastal seas, where increased population sizes drive production of industry and agriculture. This leads to increased primary production and adds new organic matter to the ecosystem. In coastal areas, the large majority of biomass is not degraded in the water column but is deposited at the sea floor. During stable stratifications of water bodies concentrations of dissolved oxygen decline in particular in deeper water layers. Here, hypoxic to anoxic conditions develop and lead to accumulation of toxic sulfide that arises from the sediment through anaerobic carbon mineralization by sulfate reducing prokaryotes (SRP). Currently many ecosystems are severely stressed by hypoxia, i.e. the condition of low dissolved oxygen beyond the point that sustains most animal life (Fig. 1). The expansion of hypoxic zones can have serious consequences for ecosystems and coastal economies, leading to loss of fisheries, loss of biodiversity and the alteration of food webs (Selman *et al*., 2008). Mass mortality and the ultimate absence of benthic macrofauna follow as consequence of stable anoxia. For example, at Breton beaches the input of anthropogenic nutrients is the main contributor to the annual development of massive algae blooms. Their decay at the sediment is accompanied by drastic sulfide concentrations, which causes tourists to avoid local holiday resorts during spring and summer (Spiegel Online, 20.8.2009, http://www.spiegel.de/reise/aktuell/0,1518,643807,00.htm).

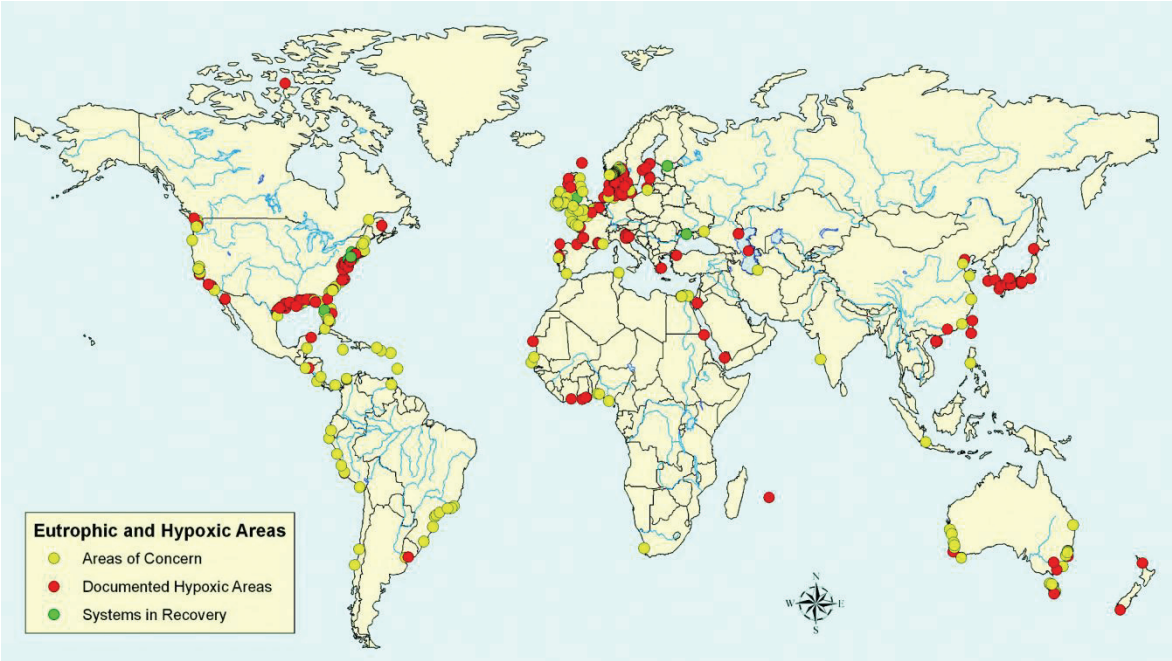

Figure 1 World's eutrophic and hypoxic coastal areas (Selman and colleagues, 2008).

The map illustrates the location of earth's "dead" (hypoxic) zones. These occur downriver of places where human population density is high. The fertilizer applied to crops triggers explosive planktonic algae growth in coastal areas. The algae die and rain down into deep waters. Microbes decompose organic matter, using up oxygen. As a consequence, mass killing of fish and other sea life often results. Currently, the microbial cycling of sulfur and carbon is in focus of research in many systems that experience seasonal or persistent hypoxia, including the Black Sea (Lin *et al*., 2006; Grote *et al*., 2008)), the Baltic Sea (Grote *et al*., 2007; Grote *et al*., 2008), the Cariaco Basin (Lin *et al*., 2006) and the Saanich Inlet (Zaikova *et al*., 2009). Source: http://rs.resalliance.org/wp-content/uploads/2010/07/dead_zones_lrg.jpg

Particularly, coastal marine sediments take a central role in both the production and consumption of hydrogen sulphide (Jørgensen and Nelson, 2004). As declining oxygen concentrations and organic matter accumulation ultimately influence sediment biogeochemistry (Freitag *et al*. 2003; Middelburg and Levin, 2009) it is of fundamental interest to characterize prevailing sediment microbial communities to better understand and predict the effects of changing environmental conditions on cycling of the key elements sulfur and carbon.

The following chapter introduces the current knowledge on the identity and activity of microbes that mediate cycling of both elements in coastal sediment ecosystems. It will provide an overview of the fate of sulfur and carbon and explain how the input of organic matter leads to the formation of hydrogen sulfide. With respect to the focus of the study, it particularly introduces those organisms that mediate the oxidation of reduced inorganic sulfur compounds.

1.1 Role and Fate of Reduced Inorganic Sulfur Compounds in Coastal Sediment

In organic-rich coastal sediments microbial sulfate reduction accounts for a large fraction of organic matter mineralization and results in the formation of substantial amounts of hydrogen sulfide (Jørgensen, 1982). Since sulfide inhibits aerobic respiration of multi-cellular life, its release into the water column can severely affect pelagic organisms. Particularly in recent years, hypoxic events that can be

accompanied by sulfide release frequently occur in coastal waters (Diaz and Rosenberg, 2008). However, the energy-yielding oxidation of inorganic sulfur compounds ("dissimilatory sulfur oxidation", Fig. 2) by sulfur-oxidizing prokaryotes (SOP) can protect marine organisms from sulfide toxification (Lavik *et al*., 2009).

Generally, hydrogen sulfide is very quickly precipitated as iron minerals such as iron monosulfides and pyrite (Howarth, 1984; Heijs *et al*. 1999, Jansen *et al*. 2009). However, in near-shore sediments the rate of accretion of these sulfide minerals is below the rate of sulfate reduction. Between 80 to 99% of the sulfide is re-oxidized to sulfate at sediment surfaces before it reaches the water column (Jørgensen, 1977a; Howarth, 1984). Here, the relative contribution of chemical versus biological oxidation of sulfur/sulfide and the involved organisms are still unknown for most sediments (Jørgensen and Nelson, 2004). Hydrogen sulfide that diffuses to the surface layers of sediment where oxygen, nitrate or light are available can be microbially oxidized. Organisms that rely on oxidation of sulfide with oxygen compete with precipitation of metal sulfides and the spontaneous chemical reaction with oxygen. For the latter process, half-life periodes in the order of 1 to 3 hours have been reported (Cline and Richards, 1969; Chen and Morris, 1972; Almgren and Hagstrom, 1974). However, microbial mediated sulfur oxidation by some SOP can be highly efficient as it has been estimated to be 10.000 –100.000 times faster than the autocatalytic oxidation with oxygen (Jørgensen and Revsbech, 1983).

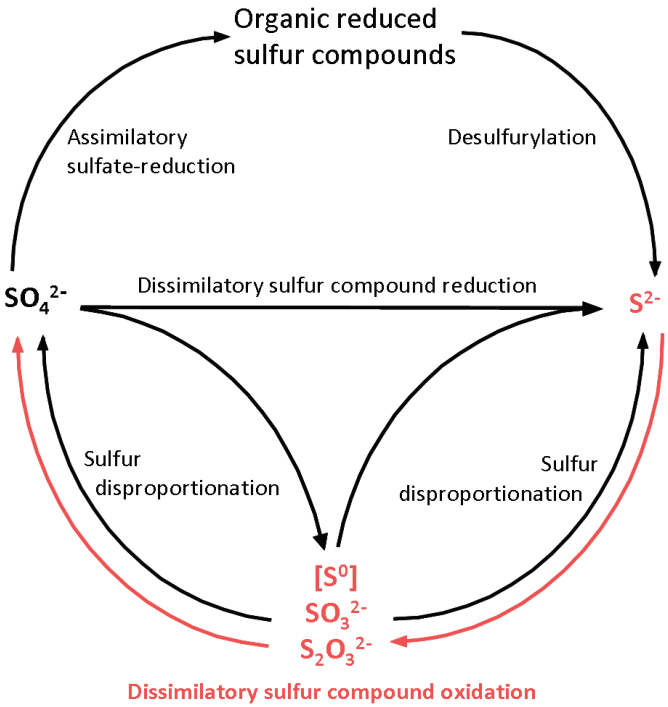

Figure 2 The microbial sulfur cycle.

Hydrogen sulfide is formed by microbial processes such as respiration of sulfate and partially oxidized sulfur species (Dissimilatory sulfur compound reduction) or disproportionation reactions. In addition, hydrogen sulfide is released from sulfur-containing amino acids during decomposition of organic material (desulfurylation). Sulfur-oxidizing prokaryotes re-oxidize sulfide to intermediate sulfur species or oxidize it completely to sulfate (dissimilatory sulfur compound oxidation). In addition, it recycles reduced sulfur compounds back to the oxidized form and thus facilitates their use as electron acceptors by anaerobic microorganisms. In coastal sediments of the Wadden Sea, hydrogen sulfide and intermediate sulfur species such as elemental sulfur and polysulfide occur in high concentrations (Jansen *et al*. 2009; Kamyshny *et al*., 2010). Modified from Brüser *et al*. (2000)

Studies, which investigated benthic SOP in near-shore sediments mostly focused on morphologically conspicuous bacteria that occur at sediment surfaces (Jørgensen, 2010). Those comprise mat-forming filamentous *Beggiatoa* or *Thioploca* (Jørgensen and Nelson, 2004; Jørgensen, 2010) and giant *Thiomargarita* (Schulz *et al*., 1999). These peculiar organisms can account for 3 to 91% of total

sedimentary sulfur oxidation (Otte *et al*., 1999; Ferdelman *et al*., 1997; Brüchert *et al*., 2003; Preisler *et al*., 2007). However, they occur as rather local phenomena at coastal sites and may represent only a small fraction of marine sedimentary SOP. In contrast, non-mat forming, unicellular SOP from the oxic-anoxic transition zone have rarely been investigated, although they may significantly contribute to sulfur oxidation in marine sediments (Brüchert *et al*., 2003).

1.2. Sulfur-Oxidizing Prokaryotes

SOP comprise diverse physiologies (Fig. 3) and are distributed among various phylogenetic lineages (Fig. 4). Phototrophic sulfur bacteria (PSB) oxidize inorganic sulfur compounds for use as electron donors in carbon dioxide fixation during anoxygenic photosynthetic growth (Fig. 3; Frigaard and Dahl, 2009). The most prominent anoxygenic photrophs comprise the purple sulfur bacteria in the class of *Gammaproteobacteria* (*Chromatiaceae* and *Ectothiorhodospiraceae*), the purple non-sulfur bacteria in the classes of *Alpha-* and *Betaproteobacteria*, and the green sulfur bacteria in the family *Chlorobiaceae* and of the genus *Chloroflexus.*

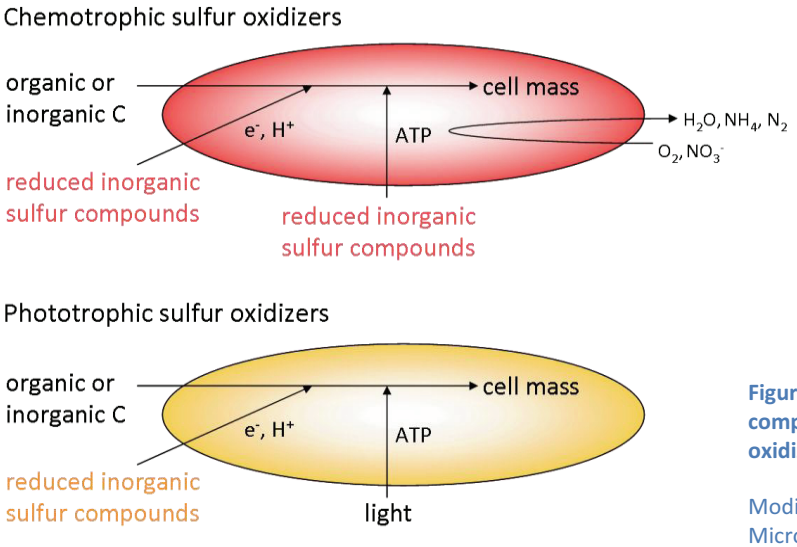

Figure 3 Utilization of reduced inorganic sulfur compounds by physiological distinct sulfur-oxidizing prokaryotes.

Modified from F. Widdel, MarMic lecture series Microbiology (2005).

Chemotrophic sulfur bacteria (Fig. 3) derive energy from the oxidation of reduced inorganic sulfur compounds (Kuenen, 1975; Robertson and Kuenen, 2006). Representatives in the domain *Archaea* belong to the order *Sulfolobales* and mediate sulfur oxidation in extreme environments such as sulfidic hot springs, solfataras and deep-sea hydrothermal vents. These extremophile archaeal SOP comprise the aerobic *Acidianus* and *Sulfolobus* species. They metabolize elemental sulfur as electron donor via the enzyme sulfur oxygenase reductase (SOR) which catalyzes a unique disproportionation reaction. In contrast, most chemotrophic SOP belong to the domain *Bacteria* (Friedrich *et al*., 2005; Sievert *et al*., 2007). Similar to phototrophs, they utilize a variety of sulfur compounds including sulfide, elemental sulfur and thiosulfate in a number of different pathways. The most prominent members that have previously been implicated in marine sulfur cycling belong to the alpha-, gamma- or epsilonproteo-bacterial classes of the *Proteobacteria* (Grote *et al*., 2007; Mussmann *et al*., 2007; Lavik *et al*., 2009; Loy *et al*. 2009; Walsh *et al*. 2009).

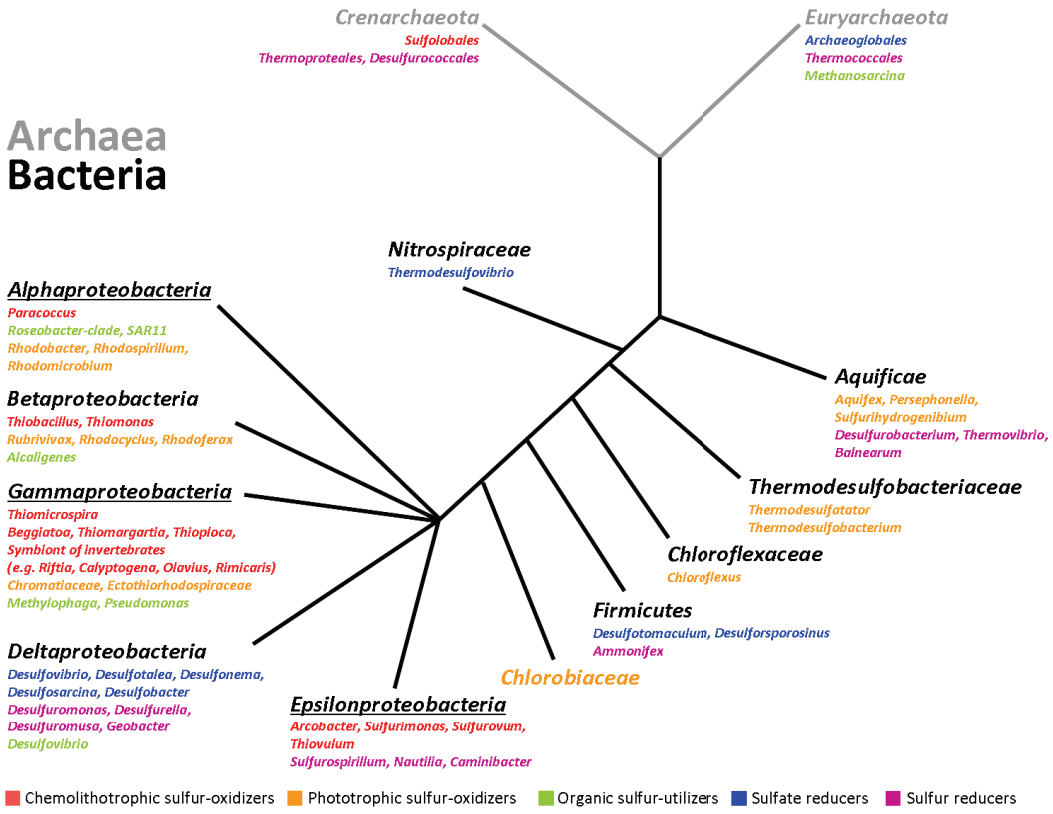

Figure 4 Diversity of sulfur-compound metabolizing prokaryotes.
Lineages of dissimilatory sulfur-compound-oxidizing prokaryotes investigated during this study are underlined. Modified from Sievert *et al.* (2007)

Sulfur-oxidizing members of the Gammaproteobacteria

Sulfur-oxidizing members of the *Gammaproteobacteria* are highly versatile with respect to their physiology. They comprise anoxygenic phototrophic purple sulfur bacteria of the family *Chromatiaceae* (e.g. *Allochromatium vinosum*, *Thiocapsa roseopersicina*) that form intracellular sulfur globules during growth on sulfide, polysulfides, thiosulfate or elemental sulfur. In addition, they comprise phototrophic members of the family *Ectothiorhodospiraceae* (*Ectothiorhodospira shaposhnikovii*, *Halorhodospira halophila*) that accumulate sulfur extracellular. *Alkalilimnicola ehrlichii* and *Thioalkalivibrio sp*. represent non-phototrophic members of the *Ectothiorhodospiraceae*. Large non-phototrophic sulfur bacteria of the genera *Beggiatoa, Thioploca* or *Thiomargarita* store elemental sulfur and nitrate in vacuoles (Fig.5). They probably represent the most conspicuous sulfur-oxidizing *Gammaproteobacteria* (Schulz and Jørgensen, 2001; Jørgensen, 2010). *Beggiatoa* species have repeatedly been detected in temperate coastal sediments of Limfjorden/Denmark (Jørgensen, 1977b), Dangast/Wadden Sea (Mussmann *et al*., 2003) and Eckernförde Bay/Baltic Sea (Preisler *et al*., 2007). In addition, microscopic small, chemolithoautotrophic *Thiomicrospira* species (Fig. 5) have frequently been isolated from coastal intertidal sediments and were suggested to be environmentally relevant (Kuenen and Veldkamp, 1972; Brinkhoff *et al*., 1998; Brinkhoff *et al*., 1999).

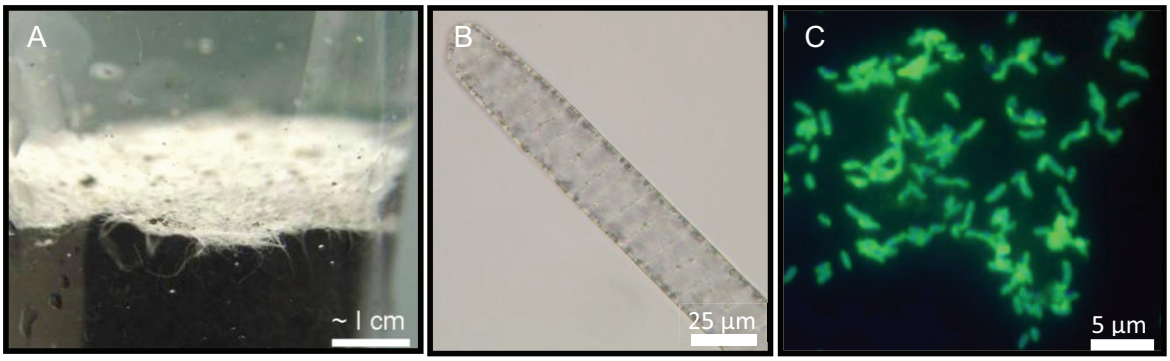

Figure 5 Prominent sedimentary sulfur-oxidizing prokaryotes
Images of sulfur-oxidizing prokaryotes showing (A) a macroscopic mat of (B) large, vacuolated *Beggiatoa sp*. filaments attached to surface sediment and (C) microscopic small, fluorescently stained *Thiomicrospira sp*. isolated from coastal sediments. Images A and B provided by M. Mußmann

Sulfur-oxidizing members of the Epsilonproteobacteria

Members of the *Epsilonproteobacteria* have most often been implicated in sulfur cycling at hydrothermal vent sites (López-García *et al*., 2003; Campbell *et al*., 2006; Nakagawa and Takai, 2008; Sievert *et al*., 2008a). Morevover, they are abundant in anoxic pelagic habitats (Campbell *et al*., 2006; Grote *et al*., 2007; Lavik *et al*., 2009). Prominent chemolithoautotrophic species such as *Arcobacter sulfidicus* (Wirsen *et al*., 2002) and *Sulfurimonas denitrificans* (Timmer-Ten Hoor, 1975) have been isolated from coastal sediments. Using fluorescence in situ hybridization (FISH) Llobet-Brossa and colleagues (1998) found *Arcobacter sp*. related bacteria to account for up to 1.6% of total cells (10^7 cells ml^{-1}) in an intertidal mudflat.

Alphaproteobacteria of the marine Roseobacter clade

Marine *Roseobacter* clade bacteria (RCB) are ubiquitously distributed throughout the oceans. They employ versatile mechanisms for the acquisition of carbon and energy and are among the most abundant heterotrophic pelagic bacteria in coastal ecosystems RCB (Gonzalez and Moran, 1997). In the North Sea they comprise on average 12% of all bacterioplankton cells (Eilers *et al*., 2001). While early findings of Gonzalez and Moran (1997) suggested that RCB account for 3-11% of the total community 16S rRNA gene pool in coastal sediment, further information on the community structure and function of RCB in marine sediment is scarce.

Pelagic RCB are involved in carbon monoxide utilization, aromatic compound degradation and aerobic anoxygenic photosynthesis (Wagner-Doebler and Biebl, 2006; Moran *et al*., 2007; Brinkhoff *et al*., 2008). Owing their global abundance and metabolic versatility, RCB have been termed ‘ecological generalists’. Accordingly, they are important to oceanic biogeochemical cycles. Here, they have long been identified as important catalysts in the degradation of climate relevant organosulfur compounds (Moran *et al*., 2003; Wagner-Doebler and Biebl, 2006; Newton *et al*., 2010). Particularly cultivation based studies revealed a variety of DMS and DMSP degrading strains (Gonzalez *et al*., 1999; Gonzalez *et al*., 2003). Likewise, culture-independent approaches proved their environmental contribution to organosulfur compound oxidation (Zubkov *et al*., 2002; Vila *et al*., 2004; Howard *et al*., 2008).

Cultivation-based studies moreover revealed RCB that oxidize inorganic sulfur compounds like thiosulfate, sulfide, elemental sulfur and sulfite. Representatives include the anaerobic species of *Sulfitobacter*, that couple sulfite oxidation to nitrate reduction (Sorokin, 1995; Ivanova *et al*., 2004) and the aerobic thiosulfate, sulfide and sulfur-oxidizing *Citreicella thiooxidans* (Sorokin *et al*., 2005), all isolated from the chemocline of the Black Sea. *Silicibacter pomeroyi* oxidizes thiosulfate aerobically and was isolated from coastal seawater (Gonzalez *et al*., 2003). Several thiosulfate-oxidizing strains were moreover isolated from deeps sea sediments (Teske *et al*., 2000). However, in contrast to organosulfur-compound degradation, the environmental relevance of sulfur-oxidizing RCB has received little attention.

Genetics and biochemistry of sulfur oxidation

The phylogenetic diversity among SOP is reflected in their versatile sulfur oxidation pathways. The underlying physiology and genetic basis of sulfide, sulfur and thiosulfate oxidation has so far been revealed for the readily cultivable phototrophic *Chlorobi* and *Chromatiales* and the chemotrophic alphaproteobacterium *Paracoccus pantotrophus*. In addition, the utilization of sulfur has been studied in acidophilic species of *Acidithiobacillus* (*Gammaproteobacteria*) and *Acidiphilium* (*Alphaproteobacteria*) and in the archaeal sulfur oxidizer *Acidianus ambivalens*.

The different sulfur oxidation pathways have been extensively reviewed (Kletzin *et al*., 2004; Friedrich *et al*., 2005; Mohapatra *et al*., 2008; Frigaard and Dahl, 2009; Ghosh and Dam, 2009). Table 1 provides an overview of enzymes that are involved in the sulfur-energy metabolism of different SOP. Diverse SOP have different pathways and numerous genes potentially involved in sulfur oxidation have been identified in the genome of a variety of organisms (Meyer *et al*., 2007; Meyer and Kuever, 2007a; Frigaard and Dahl, 2009; Loy *et al*., 2009).

The present study investigated uncultured SOP that employ the reverse dissimilatory sulfite reductase (rDSR) pathway and the Sox multienzyme system for sulfur oxidation.

Table 1 Central enzymes of sulfur energy metabolism in sulfur-oxidizing prokaryotes

Organism/Affiliation	Substrate	Enzymes[a,b,c]	Reference
Archaea			
A. ambivalens/Sulfolobales	S^0	Sulfur oxygenase reductase Sulfite acceptor oxidoreductase Thiosulfate oxidase	Kletzin *et al*., 2004
Bacteria			
Chlorobium species/Chlorobiales	H_2S S^0 $S_2O_3^{2-}$	Sulfide quinone reductase reverse DSR pathway, Sox multienzyme system	Frigaard & Dahl, 2009
Alphaproteobacteria			
A. acidophilum/Rhodospirillales	S^0	Sulfur dioxygenase	Rohwerder & Sand, 2003
P. panthotropus/Rhodobacterales	$S_2O_3^{2-}$	Sox multienzyme system	Friedrich *et al*., 2001
R. sulfidophilum/Rhodobacterales	H_2S, $S_2O_3^{2-}$	Sox multienzyme system	Appia-Ayme *et al*., 2001
Gammaproteobacteria			
A. thiooxidans/Acidithiobacillales	S^0	Sulfur dioxygenase	Rohwerder & Sand, 2003
A. vinosum/Chromatiales	H_2S S^0 $S_2O_3^{2-}$	Sulfide quinone reductase reverse DSR pathway, Sox multienzyme system	Frigaard & Dahl, 2009
*C. okutanii symbionts/*unclassified	H_2S S^0 $S_2O_3^{2-}$	Sulfide quinone reductase reverse DSR pathway, Sox multienzyme system	Harada *et al*., 2009
Epsilonproteobacteria			
Sulfurovum sp.	$S_2O_3^{2}$, S^0	Sox multienzyme system	Yamamoto *et al*., 2010

a. Enzymes involved in sulfide oxidation include sulfide:quinine oxidoreductase (SQR), flavocytochrom c sulfide dehydrogenase (FccAB) and the Sox system; **b.** Enzymes involved in elemental sulfur oxidation include the unique archaeal sulfur oxygenase reductase (SOR), the bacterial sulfur dioxygenase, the reverse dissimilatory sulfite reductase (rDSR); **c.** Enzymes involved in thiosulfate oxidation include the Sox system

The Sox multienzyme system

The Sox pathway (Fig. 6) is well characterized for the chemotrophic alphaproteobacterium *Paracoccus pantotrophus*. Here it mediates the complete oxidation of thiosulfate to sulfate (Friedrich *et al*., 2000; Friedrich *et al*., 2001). It has moreover been demonstrated to oxidize sulfide, sulfite and elemental sulfur *in vitro* (Rother *et al*., 2001). In addition, it is essential for the oxidation of thiosulfate and sulfide in the phototrophic purple non-sulfur bacterium *Rhodovulum sulfidophilum* (Appia Aime 2001). Sox genes are distributed over a wide range of phylogenetic lineages (*Alpha-, Beta-, Gamma-*, *Epsilonproteobacteria, Chlorobi*) which comprise numerous heterothrophic SOP (Meyer *et al*., 2007). In addition 'Sox' genes occur in chemoautotrophic sulfur-oxidizing *Gamma-* and *Epsilonproteobacteria* like *Thiomicrospira crunogena* (Scott *et al*., 2006) and *Arcobacter butzleri* (Miller *et al*., 2007), *Sulfurimonas denitrificans* (Sievert *et al*., 2008b) or *Sulfurovum* sp. (Yamamoto *et al*., 2010). However, they have also been detected in organisms unable to oxidize thiosulfate, like the heterotrophic gammaproteobacterium *Congregibacter litoralis* (Fuchs *et al*., 2007).

Organisms possessing the Sox multienzyme system separate into two groups (Fig. 6): those that form sulfur globules as intermediates from thiosulfate oxidation (for example *Allochromatium vinosum*) and those that do not (for example *Paracoccus pantotrophus*). The respective physiological trait is attended by the presence or absence of the enzyme sulfur dehydrogenase SoxCD. Organisms that form intermediate sulfur deposits lack SoxCD and thus possess an "incomplete" or "truncated" Sox multienzyme comlex. In these organisms reverse dissimilatory sulfite reductase (*dsr*) genes are encoded in addition to the other *sox* genes. Enzymes of the rDSR-pathway compensate for the lack of the sulfur dehydrogenase in SoxCD-deficient organisms as they are responsible for the oxidation of the intermediary formed sulfur deposits (Frigaard and Dahl, 2009).

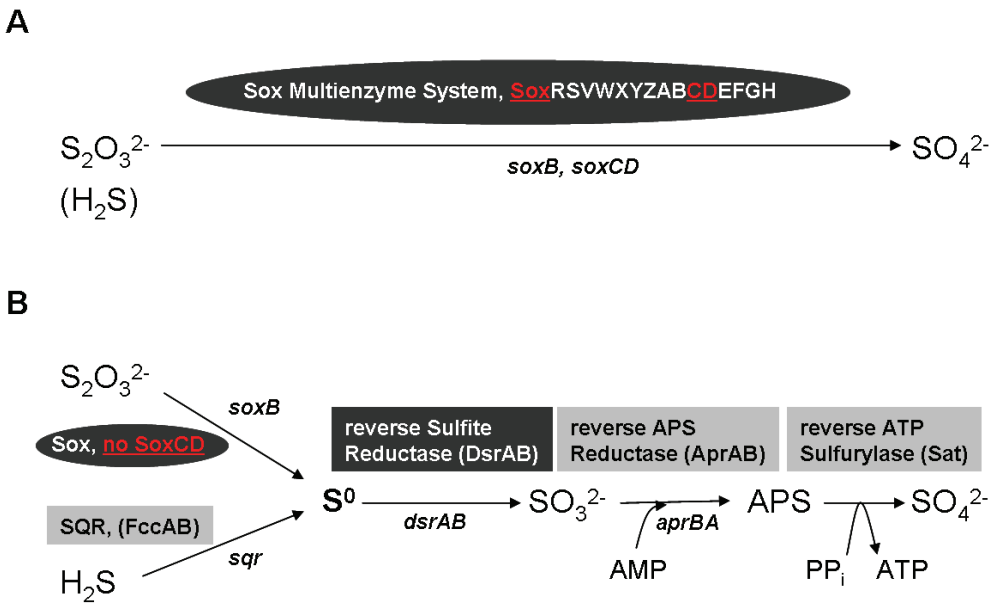

Figure 6 Pathways of sulfur oxidation in *P. pantotrophus* and *A. vinosum*.
(A) In *P. pantotrophus* the Sox multienzyme system mediates the complete oxidation of thiosulfate to sulfate. Similarly sulfide is oxidized to sulfate in *R. sulfidophilum*. (B) In *A. vinosum* a truncated Sox multienzyme system that lacks the sulfur dehydrogenase (SoxCD) oxidizes thiosulfate to elemental sulfur. In contrast, sulfide is oxidized to elemental most likely via SQR. In addition, flavocytochrom c sulfide dehydrogenase (FccAB) activity has been detected. The intermediary sulfur is oxidized to sulfite via the reverse dissimilatory sulfite reductase (rDSR). Sulfite, the product of the rDsr system is further oxidized to sulfite via an indirect sulfite oxidation pathway. Here, the enzyme adenosine-5' phosphosulfate (APS) reductase (adenylylsulfate reductase, AprAB) consumes sulfite and adenosinemonophosphate (AMP) and generates adenosine-5'-phosphosulfate (adenylylsulfate, APS). Subsequently, ATP sulfurylase (Sat) generates sulfate. The genes *sqr*, *soxB*, *dsrAB* and *aprBA* are diagnostic for respective sulfur oxidation pathways. Modified after Frigaard and Dahl (2009)

The rDSR pathway

The rDSR-pathway is a widespread sulfur oxidation pathway (Dahl *et al*., 2008). Its genes are present in diverse autotrophic *Gammaproteobacteria* (including PSB, symbiotic SOP in invertebrates, *Beggiatoa, Thiothrix*) and *Chlorobi* (Frigaard and Dahl, 2009; Loy *et al*., 2009). It is best studied in the phototrophic gammaproteobacterium *Allochromatium vinosum*, in which it is indispensable for the oxidation of transiently formed sulfur (Pott and Dahl, 1998). Of special interest is the formation and subsequent utilization of transiently produced elemental sulfur, as the underlying mechanism and enzymes are not

fully resolved yet. A basic model has been proposed in 1998 by Pott and Dahl (1998). Since then Dahl and colleagues continuously proceeded in elucidating the role of rDSR proteins (Dahl *et al*., 2005). Only recently new functions could be assigned to the DsrC and DsrEFH proteins. Regarding the fate of periplasmic stored sulfur in the cytoplasm and its further oxidation a sulfur transferase activity has been suggested for DsrEFH which, together with DsrC, participate in sulfur transfer reactions that shuttle persulfidic sulfur from sulfur globules to the rDSR (Cort *et al*., 2008).

While the rDSR pathway has been extensively studied in phototrophic organisms detailed studies on the function of the rDSR proteins in aerobic chemotrophic SOP are lacking.

1.3. Molecular Approaches for Characterization of Uncultured SOP

The following section introduces cultivation independent molecular approaches that can be applied towards the phylogenetic and functional characterization of uncultured microorganisms.

16S rRNA approach

So far, the identification of SOP in microbial communities has been mostly based on 16S rRNA phylogeny. Culture-independent studies on coastal sediment have consistently found *Gammaproteobacteria* in high frequencies in 16S rRNA gene libraries (Asami *et al*., 2005; Hong *et al*., 2006; Gillan and Pernet, 2007; Edlund *et al*., 2008; Kim *et al*., 2008; Bi-Wei *et al*., 2009) or in high relative cell numbers (Ravenschlag *et al*., 2001; Ishii *et al*., 2004; Buehring *et al*., 2005; Musat *et al*., 2007). For some of the mostly uncultured groups the capability to oxidize sulfur has been suggested, because of their phylogenetic relationship with known chemoautotrophic SOP, in particular with thiotrophic symbionts of marine invertebrates (Ravenschlag *et al*., 1999; Bowman *et al*., 2003, Meyer and Kuever, 2007b). In one study Ravenschlag and colleagues (2001) detected 10^7 putative, gammaproteobacterial SOP ml^{-1} in arctic surface sediment based on 16S rRNA phylogeny and fluorescence *in situ* hybridization. However, specific populations were not identified and no evidence for *in situ* activity has been provided.

Functional gene approach

Although the 16S rRNA gene is the most common phylogenetic marker, it is widely recognized that the assignment of physiological traits based on 16S rRNA phylogeny is of limited use as close relatives do not necessarily display the same physiology. Instead, related species might significantly differ in physiology and ecology. The diversity and identity of uncultured SOP can be better examined by comparative sequence analysis of 'functional' (or 'diagnostic') marker genes. Table 2 provides an overview of genes that encode key enzymes involved in sulfur oxidation. The following genes have been applied as 'functional' markers:

- the *sqr* gene that encodes sulfide: quinone reductase which is responsible for the oxidation of sulfide (Pham *et al*., 2008),
- the *soxB* gene that encodes the sulfate thiohydrolase, which catalyzes the oxidation of thiosulfate (Petri *et al*., 2001; Meyer *et al*., 2007; Chen *et al*., 2009) and
- the *aprBA* genes that encode the dissimilatory adenosine-5'-phosphosulfate reductase which mediates the oxidation of sulfite (Meyer and Kuever, 2007a; Meyer and Kuever, 2007b).

The variety of sulfur oxidation pathways complicates the choice of a suitable functional marker. Moreover lateral gene transfer can hamper the reliable assignment of unknown sequences to defined taxonomic groups. For example, the SQR is widely distributed but less conserved in both pro- and eukaryotes and might also function in sulfide detoxification without a gain of energy (Grieshaber and Voelkel, 1998; Bronstein *et al*., 2000, Theissen *et al*., 2003). Likewise, SoxB genes of the sulfur-oxidizing multienzyme complex (Sox) also occur in organisms without a confirmed thiosulfate oxidation activity (Meyer *et al*., 2007). Accordingly, the *aprBA* genes are widespread but also occur in some non-sulfide oxidizing organisms (Meyer and Kuever, 2007a; Meyer and Kuever, 2007b).

Table 2 Overview of functional markers diagnostic for sulfur-oxidizing prokaryotes.

Gene	Protein	Compound	Environmental Sample	Reference
sqr	SQR	H_2S	marine & freshwater sediment	Pham *et al.*, 2008
dsrAB	rDSR	S^0	freshwater sediment	Loy *et al.*, 2009
aprA	APR	SO_3^{2-}	marine sediment	Meyer and Kuever, 2007b
aprA	APR	SO_3^{2-}	hydrothermal fluid	Huegler *et al*., 2010
soxB	SoxB	$S_2O_3^{2-}$	freshwater microbial mats	Chen *et al.*, 2009
soxB	SoxB	$S_2O_3^{2-}$	hydrothermal fluid	Huegler *et al*., 2010
soxCD	SoxCD	$S_2O_3^{2-}$	marine surface water	Frigaard, unpublished data

Most recently a molecular assay for the *dsrAB* genes of the reverse operating dissimilatory sulfite reductase has been introduced (Fig. 7, Loy *et al*., 2009). Lateral gene transfer was shown to be widely absent from the rDSR phylogeny. DsrAB genes co-occur with *aprBA* and *soxB* (Fig. 5) in SOP that employ the reverse APS reductase pathway for sulfite oxidation and the truncated SOX multienzyme system for the oxidation of thiosulfate (Meyer and Kuever, 2007a; Meyer *et al*., 2007). Interestingly, *dsrAB* has been detected in ecological relevant sulfur oxidizers such as benthic *Beggiatoa* (Mussmann *et al*., 2007) or the pelagic GSO/SUP05 organisms (Lavik *et al*., 2009; Walsh *et al*., 2009). Thus, they might constitute a valuable functional marker for environmental diversity studies of uncultured sedimentary SOP.

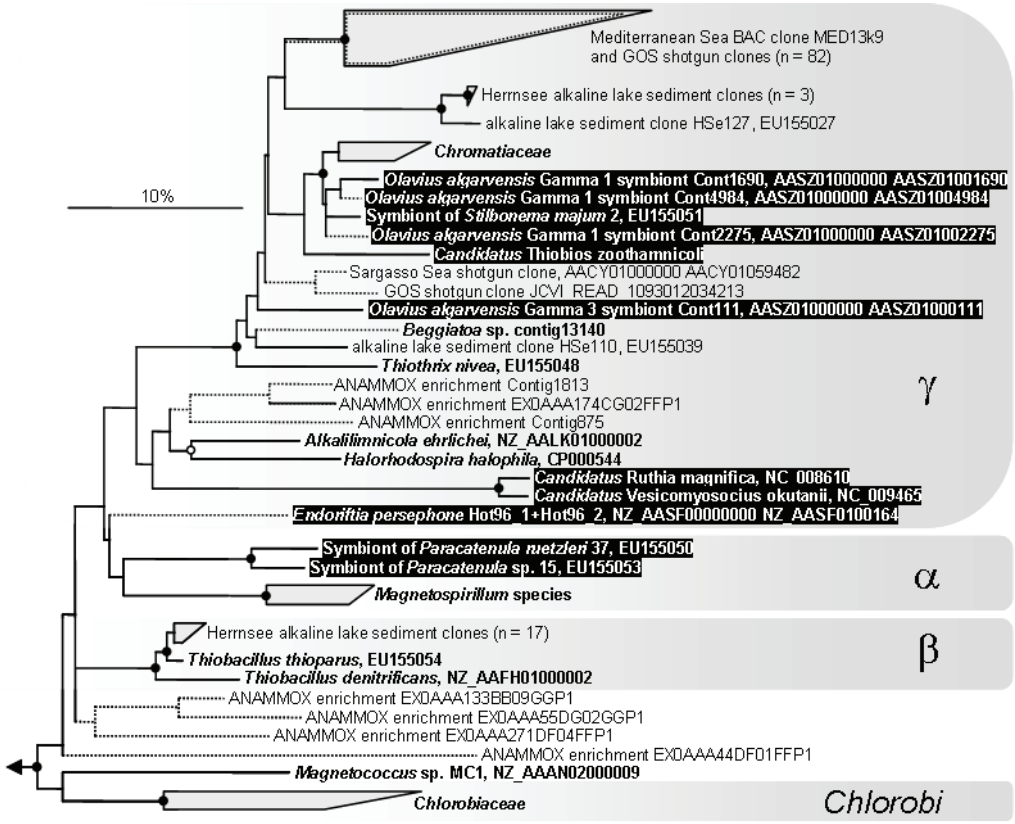

Figure 7 Phylogeny of DsrAB as published by Loy *et al*. (2009)
The tree shows the affiliation of environmental DsrAB sequences with sequences of cultured SOP (bold) and with sequences of known sulfur-oxidizing endosymbionts (shaded).

Metagenomics combined functional gene approach

Metagenomics refers to the genomic analysis of microorganisms by direct extraction and cloning of DNA from an assemblage of microorganisms (Handelsman, 2004). It offers the possibility to study the identity and metabolic potential of SOP by i.e. analyses of long DNA sequences or high throughput sequencing of numerous genomic fragments. Unlike the 16S rRNA and functional gene approaches, which focus on single genes only, the analysis of large fragments allows the linkage of phylogenetic informative genes with genes that encode enzymes of metabolic pathways. One major aim is the discovery of new molecular mechanisms. Ideally, this involves the discovery of new genes, including those that are too diverged from known genes to be amplified with PCR, by analysis of metagenomic data, followed by a formulation of hypotheses, which are then verified by laboratory experiments.

Table 3 Characterization of uncultured gammapoteobacterial SOP by metagenomic approaches.

Affiliation	Habitat	Sulfur oxidation genes	Reference
SUP05	anoxic fjord water	*sqr, fccAB, sox, dsr, apr, sat*	Walsh *et al*., 2009
Endosymbionts	coastal sediment	*sqr, fccAB, sox, dsr, apr, sat*	Woyke *et al*., 2006
unknown	open ocean surface water	*dsr*	Sabehi *et al*., 2005
unknown	hydrothermal vent chimney	*sqr, fccAB, dsr*	Xie *et al*., 2010
Halothiobacillus sp.	hydrothermal vent chimney	*sox*	Xie *et al*., 2010
Thiomicrospira sp.	hydrothermal vent chimney	*sox*	Brazelton *et al*., 2010

Metagenomic approaches have contributed new discoveries on uncultured sulfur-oxidizing *Gammaproteobacteria* from open ocean waters, coastal oxygen minimum zones, hydrothermal vent habitats and eukaryotic host species (Table 3). For example, Sabehi and colleagues (2005) discovered a novel *dsr* gene by analyzing a large insert BAC library generated from surface water of the Mediterranean Sea. A subsequent survey of the global ocean sampling dataset suggested that the respective phylotype belongs to a group of abundant mutually photoheterotrophic *Gammaproteobacteria* that are widespread in open ocean waters (Loy *et al*., 2009). In another study, Walsh and colleagues (2009) investigated the full gene complement of an anoxic fjord inhabiting microbial community and reconstructed the genome sequence of a widespread chemolithoautotrophic gammaproteobacterium. Recent studies on hydrothermal vent associated chemolithoautotrophs revealed the genetic repertoire of candidate SOP related to *Thiomicrospira* (Brazelton and Baross, 2010) and *Halothiobacilli* spp. (Xie *et al*., 2010). In addition, Woyke and colleagues (2006) provided first comprehensive insights into the energy and carbon metabolism of symbiotic sulfur-oxidizing members of the *Gammaproteobacteria* that live associated with oligochaetes in coastal sediment.

Studies on free-living SOP of coastal sediments are so far not available. Previously, Mussmann and colleagues (2005) recovered large genomic fragments from a sulfidic coastal sediment. This effort yielded insights into the phylogeny of genes responsible for dissimilatory sulfate reduction and revealed a group of novel sulfate reducing prokaryotes.

1.4. Thiotrophic Carbon Assimilation and Carbon Mineralization in Coastal Sediments

SOP exhibit diverse metabolic aerobic and anaerobic lifestyles including auto-, hetero- and mixotrophic carbon assimilation (Gray and Head, 1999; Jørgensen and Nelson, 2004; Kelly and Wood, 2006; Robertson and Kuenen, 2006). Thus, they link the cycling of sulfur compounds to the cycling of carbon in diverse oxic and anoxic marine habitats.

Autotrophic sulfur oxidizers use the energy gained from sulfur oxidation to assimilate carbon dioxide into organic biomass. Generally, CO_2 fixation can proceed via six different pathways: the Calvin-Bassham-Benson (CBB) cycle, the reductive citric acid (rTCA) cycle, the 3-hydroxypropionate cycle, the reductive acetyl coenzyme A pathway, the 3-hydroxypropionate/4-hydroxybutyrate cycle and the dicarboxylate/4-hydroxybutyrate cycle (Jones, 2008; Huber *et al*., 2008). In deep-sea vent habitats the rTCA cycle is considered as important CO_2 fixation pathway (Nakagawa and Takai, 2008) as it operates in the abundant

sulfur-oxidizing epsilonproteobacteria. In contrast, sulfur-oxidizing gammaproteobacteria such as the phototrophic *Chromatiales* (Sander and Dahl, 2009), chemotrophic *Thiomicrospira* spp. (Tourova *et al*., 2006) and symbiont species (Woyke *et al*., 2006; Scott and Cavanaugh, 2007) employ the CBB cycle for CO_2 fixation. In addition, both pathways occur in the gammaproteobacterial sulfur-oxidizing symbiont of the deep-sea vent worm *Riftia pachyptila* (Robidart *et al*., 2008).

In permanently dark habitats such as deep sea hydrothermal vents (Campbell *et al*., 2006), cold seeps (Lichtschlag, *et al*., 2010) and anoxic marine basins (Grote *et al*., 2008; Glaubitz *et al*., 2009) chemoautotrophic SOP are important non-photosynthetic primary producers of organic carbon, providing a food source for diverse organisms. Early investigations suggested that microbial autotrophy also constitutes a source of organic carbon in sulfidic coastal sediments (Kepkay *et al*., 1979). Kepkay and Novitsky (1980) hypothesized a contribution of chemotrophic SOP (Fig. 8). However, data on the productivity of SOP in coastal sediment ecosystems are scarce as *in situ* studies that link their abundance and activity have not been conducted.

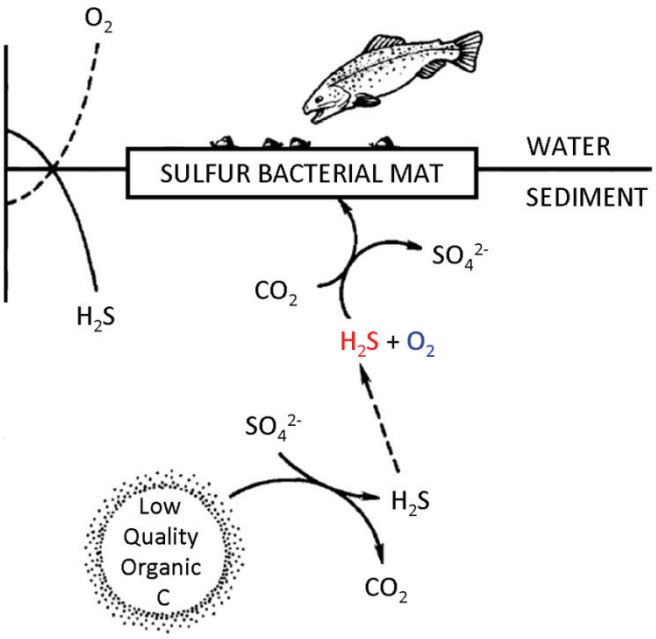

Figure 8 Ecological importance of the sulfur cycle.

SOP concentrate high-quality food near the sediment surface. According to Howarth (1984) a combined cycle of anaerobic decomposition through sulfate reduction and associated fermentation leads to energy conservation as reduced inorganic sulfur compounds. Chemolitho-autotrophic production of new organic carbon serves to take relatively low-quality organic matter, i.e. material, which is only slowly decomposed and distributed throughout the sediments and concentrate the energy as living biomass in a discrete zone near the sediment surface, where it serves as high quality food for animals. The chemolithoautotrophic production represents an input of new organic carbon to sediments from CO_2 fixation. Since the energy came originally from the decomposition of organic matter through sulfate reduction, the production is secondary production and not primary production. Modified after Howarth (1984)

Facultative autotrophic, mixotrophic and heterotrophic bacteria can couple the oxidation of sulfur compounds to mineralization of organic carbon in sulfidic, organic matter rich sediment (Howarth, 1984; Podgorsek and Imhoff, 1999; Teske *et al*., 2000). Particularly, coastal shelf sediments face a high input of organic matter. Here, up to 50% of the pelagic primary production reaches the sediment surface (Wollast, 1991). In addition, benthic photosynthetic microorganisms like cyanobacteria and diatoms contribute to total primary production by sediment autochthonous carbon fixation (Middelburg *et al*., 2000; Gattuso *et al*., 2006; Evrard *et al*., 2010).

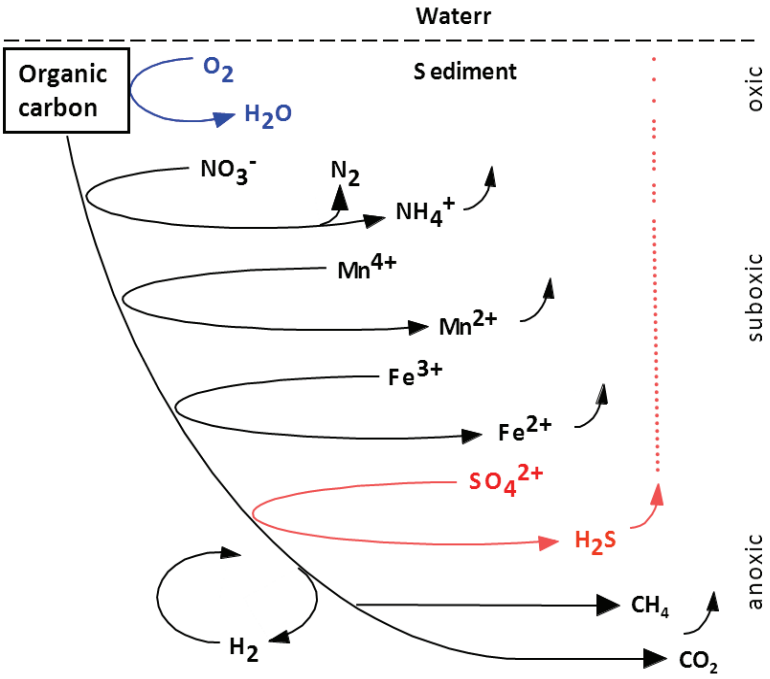

Figure 9 Degradation of organic matter in marine sediment.

The graphic illustrates a schematic view of microbial mineralization processes using different terminal electron acceptors. Aerobic respiration (blue) in the oxic surface layer and sulfate reduction (red) in anoxic sediment layers account for most of the organic carbon decay. The graphic illustrates how the microbial cycling of carbon and sulfur are coupled. In anaerobic sediment layers organic carbon is degraded by microbial sulfate reduction, a reaction that produces hydrogen sulfide. This hydrogen sulfide can be removed from the system by biological oxidation, metal sulfide precipitation, volatilization or chemical oxidation. Sulfur-oxidizing prokaryotes utilize hydrogen sulfide in suboxic to oxic zones of the sediment by coupling its oxidation to nitrate and oxygen respiration. Modified after Jørgensen (2000)

Within the sediment organic matter is rapidly turned over as it serves as energy and/or carbon source for microorganisms (Nealson, 1997). Figure 9 illustrates the microbial mineralization processes, which proceed via a cascade of reactions that consume different terminal electron acceptors (Canfield *et al.*, 1993). The stepwise degradation is catalyzed by microbes that consume oxygen in the uppermost sediment layer followed by nitrate, manganese and iron (oxyhydr) oxides, sulfate, carbon dioxide and acetate. Generally, aerobic respiration and anaerobic sulfate reduction are the dominant terminal pathways of organic matter degradation (Sørensen *et al.*, 1979; Jørgensen, 1982). In aerobic respiration all the free energy resulting from the respired organic matter is available to the metabolizing organisms. In contrast, during anaerobic respiration of organic matter via sulfate reduction a large amount of energy is conserved in reduced inorganic-sulfur compounds (Howarth, 1984). Its oxidation can then further support chemolithoautotrophic, -heterotrophic and -mixotrophic bacterial production or production by anoxygenic photosynthetic SOP. The volatile fatty acid acetate represents a central intermediate of anaerobic organic matter degradation. It is generally considered as important substrate of sulfate reducing prokaryotes. In addition, SOP among the *Gammaproteobacteria* utilize acetate as carbon source (Kuenen and Veldkamp, 1973; Hagen and Nelson, 1996; Otte et al., 1999; Nielsen et al., 2000; Schulz and de Beer, 2002). Similarly, acetate is used as a growth substrate by heterotrophic sulfur-oxidizing members of the marine *Roseobacter* clade (Sorokin, 2005; Sorokin *et al.* 2005).

Recent progress identified permeable sandy sediments, which cover a majority of continental shelf areas of the world (Emery, 1968), as giant biocatalytic filters (Boudreau *et al.*, 2001). They decompose organic matter extraordinary efficiently and ensure that inorganic nutrients are released back into the water column (de Beer *et al.*, 2005; Rusch *et al.* 2006, Werner *et al.*, 2006; Cook *et al.*, 2007; Gao *et al.*, 2009; Joye *et al.*, 2009; Chipman *et al.*, 2010). By now, a number of studies characterized the composition of microbial communities indigenous to sandy sediment sites (Hunter *et al.*, 2006; Musat *et al.*, 2006; Boer

et al., 2009). Evrard and colleagues (2008) used stable isotopes and microbial biomarker techniques to follow the fate of inorganic carbon through the benthic microbial compartment. Analysis of phospholipid-derived fatty acids (PLFA) and hydrolysable amino acids (HAA) demonstrated that a major portion of inorganic carbon fixed by microphytobenthos is excreted as extracellular polymeric substances, which are assimilated by heterotrophic bacteria residing at the sediment surface. Additional inorganic carbon incorporation in deeper sediment layers revealed the presence of chemoautotrophic bacteria (Evrard *et al*., 2008). While chemoautotrophic and chemoheterotrophic bacterial activity has previously investigated in muddy sediment (Kepkay and Novitsky, 1980) little is known about the importance and interactions of these processes in sandy sites (Evrard *et al*., 2008). To further elucidate key microorganisms involved in production and mineralization processes, investigations that link the activity of uncultured microbes to their *in situ* identification and quantification are desirable.

1.5. Molecular Approaches to Study Activities of Uncultured Microorganisms

The most powerful methods that enable an identification of uncultured microorganisms actively involved in carbon turnover are nucleic acid based stable-isotope probing (Radajewski *et al*., 2000), microautoradiography combined FISH (Lee *et al*., 1999; Ouverney and Fuhrman, 1999) and nanoSIMS combined HISH (Behrens *et al*., 2008; Musat *et al*., 2008). These technologies have been comprehensively reviewed (Friedrich, 2006; Wagner *et al*., 2006; Neufeld *et al*., 2007, Wagner et. al. 2009). The following section briefly addresses their application to marine sediments.

Stable isotope probing

Stable isotope probing (SIP) of nucleic acids has most frequently been used to track the flow of simple organic carbon compounds into sediment microbial communities. Using DNA-SIP Webster and colleagues (Webster *et al*., 2006; Webster *et al*., 2010) followed the utilization of acetate, glucose and CO_2 by the microbial community of the Severn Estuary, UK. Members of the *Gammaproteobacteria*, *Epsilonproteobacteria* and *Archaea* were identified as major consumers of respective substrates under aerobic and anaerobic conditions in slurry microcosms. In another study, MacGregor and colleagues (2006) combined stable isotope probing with paramagnetic bead capture of the 16S rRNA to track the utilization of acetate, propionate, amino acids, and glucose by bacteria and eukaryotes inhabiting Wadden Sea sediment. Later, Miyatake and colleagues (2009) adapted the method to more specifically trace the uptake of glucose, propionate and acetate by sulfate reducing *Deltaproteobacteria* of the *Desulfobacteraceae* in intact sediment cores. In addition, Kittelmann and Friedrich (2008) identified perchloroethene degrading *Chloroflexi* in Wadden Sea sediment. All these studies successfully applied SIP, however, two major drawbacks of the method are that substrate incorporation cannot be traced quantitatively on the single cell level and that SIP experiments usually require extended incubation times that introduce biases with respect to community composition and primary substrate consumers (Wagner *et al*., 2006).

Microautoradiography combined with fluorescence in situ hybridization (MAR-FISH)

Microautoradiography (MAR) combined with fluorescence in situ hybridization (FISH) or catalyzed reporter deposition FISH (CARD-FISH) was the first technology that could link phylogenetic identity and metabolic function of single cells in mixed microbial communities. In contrast to DNA or RNA based stable isotope probing substrate incorporation is related to individual cells but not mere sequences

(Wagner *et al*., 2006). It relies on incubation of the sample with a radioactively labeled substrate, subsequent FISH on glass slides or membrane filters, followed by exposure to an autoradiographic emulsion, which leads to a precipitation of silver grains around substrate-incorporating (substrate-active) cells. Fluorescently labeled target cells and substrate-active cells can be visualized by epifluorescence microscopy. Thus MAR-FISH offers the unique opportunity to directly observe and enumerate substrate incorporating cells of uncultured microorganisms in their natural habitat and to quantify the metabolic activity to a limited extent (i.e. determination of cell-specific uptake rates). Since its first application to environmental samples (Lee *et al*., 1999; Ouverney and Fuhrman, 1999) MAR-FISH has been extensively applied for carbon incorporation studies of natural bacterioplankton communities. Alonso and Pernthaler (2005 and 2006) combined MAR with CARD-FISH to trace the uptake of glucose and leucine into bacterioplankton populations of the North Sea. In its only application to sediment samples Gray and colleagues (2000) analyzed acetate and bicarbonate utilization by a freshwater population of sulfur-oxidizing *Achromatium oxaliferum* cells. However, the cell size of these comparatively large organisms allowed for a physical separation of target cells prior to MAR analysis. To date, the method has not been applied to study substrate uptake by different phylogenetic groups inhabiting marine sediments.

Mass spectrometry of single cells (nanoSIMS)

The latest technology to study phylogenetic identity and metabolic activity of individual cells in complex microbial communities combines RNA-based *in situ* hybridization coupled catalyzed reported deposition of halogen labeled tyramids (Halogen In Situ Hybridization, HISH) with stable isotope imaging based on nanometer-scale secondary-ion mass spectrometry (nanoSIMS). It represents a promising method to identify and quantify isotope labeled microorganisms and to trace substrate uptake by individual cells quantitatively (Musat *et al*., 2008; Halm *et al*., 2009; Ploug *et al*., 2010). Like MAR-FISH, nanoSIMS has so far not been used to image metabolically active cells in complex sediment microbial communities.

1.6 The Wadden Sea – An UNESCO World Heritage Site

The Wadden Sea has become an UNESCO World heritage site in 2009 (World Heritage Convention, 2009). With respect to its uniqueness, key features and values the United Nations Environment Programme and World Conservation Monitoring Centre state:

"The Wadden Sea is the largest unbroken contiguous tidal flat area in the world. As such it is also among the last remaining intertidal ecosystems. Here, dynamic natural processes create a variety of different coastal and sedimentary features including barrier islands, channels, flats and salt marshes. The multitude of transitional zones between land, sea and freshwater provides the basis for its species richness. The productivity of biomass is one of the highest in the world, most significantly demonstrated in the numbers of fish, shellfish and birds supported by the site. The salt marshes host around 2,300 species of flora and fauna, and the marine and brackish areas a further 2,700 species, including 30 species of breeding birds. Up to 6 million birds can be present at the same time, and up to 12 million pass through each year."

Given a total area of 10,000 km^2 this makes 1.2 birds a square meter. Most excitingly beyond this square meter reside > 1,000,000,000 bacteria per milliliter of Wadden Sea sediment that help to maintain the ecosystem.

Figure 10 The intertidal sandflat Janssand within the coastal ecosystem Wadden Sea.
Images taken from DFG Research Group 'Watt', MPI Bremen/ICBM Oldenburg

The Janssand intertidal sand flat – biogeochemistry of coastal elemental cycling

The original ecosystem of the Wadden Sea consisted of salt marshes, mudflats, and sea grass beds that developed after the last ice age. Nowadays, sandflats dominate in the area of the barrier islands and the large tidal channels (Lotze *et al*., 2005).

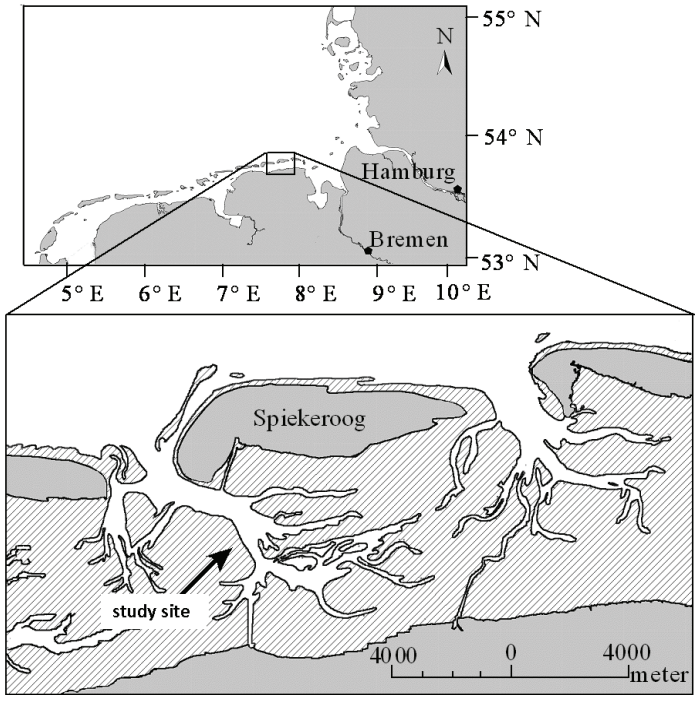

Figure 11 Geographic location of the Janssand intertidal sand flat.

Image taken from Billerbeck *et al*. (2009)

The sampling site *Janssand* (Fig. 10) is a characteristic intertidal sand flat. It is about 11 km^2 in size and located in the back-barrier area of the East Frisian island Spiekeroog, within the German Wadden Sea (Fig. 11). Its biogeochemistry has been extensively investigated by the DFG founded "Forschergruppe Watt" during the research program "Biogeochemistry of tidal flats". The dynamics of key chemical parameters, key chemical elements and key biogeochemical processes have been in focus of research (Boettcher *et al*., 2004; Polerecky *et al*., 2005; Billerbeck *et al*., 2006a; Billerbeck *et al*., 2006b; Billerbeck *et al*., 2007; Roy *et al*., 2008; Al-Raei *et al*., 2009; Beck *et al*., 2009; Jansen *et al*., 2009; Kamyshny *et al*.,

2010). Furhter studies focused on the characterization of the sediment microbial community prevailing at Janssand and nearby sites (Ishii *et al*., 2004; Koepke *et al*., 2005; Mussmann *et al.* 2005; Wilms *et al*., 2006a; Wilms *et al*., 2006b; Webster *et al*., 2007; Gittel *et al*., 2008; Bischof, unpublished data)

Sulfur cycling at the Janssand intertidal sand flat

The study site represents a habitat of extensive sulfur transformations. Already upon arrival in the field a strong smell of hydrogen sulfide can be experienced. Sulfidic channels and pools with whitish sulfur precipitates are apparent phenomena of intensive sulfur cycling within the flats sediments. As illustrated in Figure 12, hydrogen sulfide is formed by the activity of heterotrophic sulfate reducing bacteria in deeper sediment layers (Al-Raei *et al*., 2009). Additional sulfur compounds such as elemental sulfur and polysulfides accumulate in sulfidic pore waters, channels and pools (Jansen *et al*., 2009; Kamyshny *et al*., 2010). Sulfide is usually re-oxidized within the upper 3 cm of the surface sediment in the transition zone of oxygen, nitrate and sulfide (Gao *et al*., 2009; Jansen *et al*., 2009). Although Jansen and colleagues (2009) expected motile sulfide oxidizing organisms to be present at sites with high sulfidic outflow macroscopic SOP such as filamentous *Thiothrix*-like bacteria have only been occasionally observed. Likewise, large vacuolated sulfur oxidizers like *Beggiatoa sp*. are absent from the flat (Ishii *et al*., 2004; Arnds J., 2006; Lenk S., 2006; Jansen *et al*., 2009). Instead the re-oxidation of sulfides was hypothesized to be catalyzed by metal oxides (Jansen *et al*., 2009; Al-Raei *et al*., 2009).

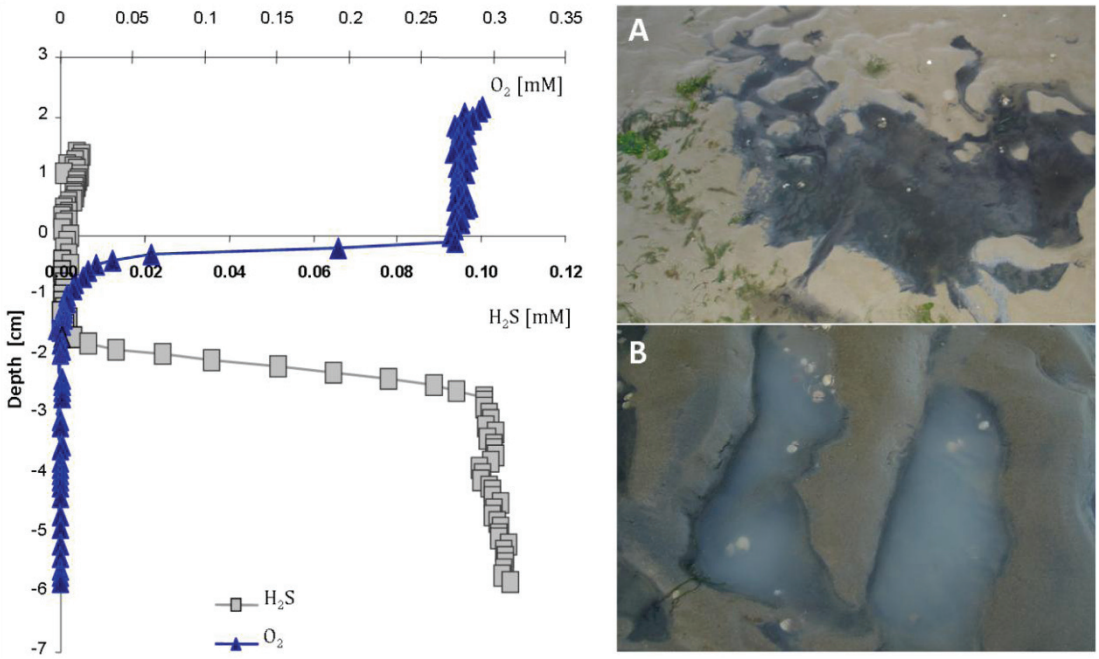

Figure 12 Intense sulfur transformations at Janssand site.
The graph shows a characteristic microsensor profile of the concentrations of oxygen and sulfide assessed during a field measurement. The images show "black spots" and sulfidic pools encountered at the sand flat. (A) Black spots are iron sulfide (FeS) precipitates. Here, they occur next to buried macroalgae. The high input of organic mater leads to local oxygen depletion. As a consequence the sediment surface turns anoxic. Large-scale occurrence of black spots can indicate increased eutrophication (Freitag *et al*. 2003). (B) Sulfidic pools form where hydrogen sulfide seeps into local water accumulations that remain on the flat during low tide. Over time elemental sulfur and polysulfide precipitates accumulate. This leads to a whitish coloration of the pools, called 'sulfur milk'. Microsensor profiles were provided by S. Jansen and previously published by Jansen and colleagues (2009).

Molecular studies started to characterize microorganisms involved in sulfur cycling. Earlier investigations focused on sulfate reducing prokaryotes (Ishii *et al*., 2004; Gittel *et al*., 2008, Mussmann et al. 2005). By comparative analysis of 16S rRNA genes and FISH different deltaproteobacterial populations were identified. Ishii and colleagues (2004) detected a maxmimum of 4.6×10^8 *Desulfosarcinales*-related cells ml^{-1} and 1.9×10^8 *Desulfobulbaceae*- related cells ml^{-1} in the surface sediment (corresponding to ~ 9 and 4% of total cells, respectively). Gittel and colleagues (2008) found sulfate reducing bacteria to account for 2–5% of the microbial community in the surface sediment. Analysis of the *dsrAB* gene diversity revealed novel, deep-branching populations (Mussmann *et al*., 2005). The 16S rRNA based identity and abundance of these novel SRP has not been resolved yet.

Studies that aimed on identification of SOP were initially conducted by Arnds (2006). While characterizing the diversity of the prevailing sediment microbial community she detected many sequences related to *Gammaproteobacteria* in 16S rRNA gene libraries. Subsequently, FISH revealed that polyphylethic organisms targeted by probe GAM660 accounted for up to 15% of all cells in the surface sediment. As this probe covers many known SOP (Ravenschlag *et al*., 2001) its sedimentary target organisms were hypothesized to participate in sulfur oxidation (Arnds, 2006). However the probe is highly polyphyletic and covers a wide range of uncultured *Gammaproteobacteria*. A novel *dsrAB*-targeting, functional gene approach confirmed the presence of various sulfur-oxidizing populations that affiliate with *Alpha*- and *Gammaproteobacteria* (Lenk, S., 2006). Results from preliminary, quantitative PCR hint at SOP abundances of 10^7 cells ml^{-1} (Lenk, S., 2006). To date, specific gammaproteobacterial populations have not been detected in the sediment of Janssand site nor have they been quantified. Likewise, candidate SOP were not identified, so far. Their identity, diversity and abundance are still largely unknown.

Dynamics of elemental cycling at the Janssand intertidal sand flat

The intertidal sand flat Janssand constitutes a natural, large-scale bioreactor, where key processes such as photosynthesis, mineralization and transport sustain the sediment ecosystem. Two different transport phenomena control mineralization and nutrient release and shape the chemical and microbial architecture of the permeable sand flat (Fig. 13, Billerbeck *et al*., 2006b; Jansen *et al*., 2009). During high tide advective flushing of the surface is driven by currents over ripples. This ‘skin circulation’ infiltrates organic matter and electron acceptors into the top centimeters of the sediment. The infiltration of organic and inorganic carbon promotes mineralization and primary production. It ensures removal of degradation products and replenishment of electron acceptors. The skin circulation moreover controls oxygen dynamics. During high tide, oxygen penetrates several centimeters into the permeable sediment surface where organic matter and oxygen rapidly exchange with each other (Jansen *et al*., 2009). In contrast, penetration depths reach only 5–10 mm during low tide (Werner *et al*., 2006, Jansen *et al*. 2009). The ‘body circulation’ infiltrates the entire sand flat and represents the flow of porewater towards the low water line. Accordingly, a fraction of organic matter arrives in several meters depth where it is degraded by sulfate reducers and methanogens of a deep-subsurface microbial community (Wilms *et al*., 2006b; Gittel *et al*., 2008). The degradation products of these anaerobic processes - predominantly sulfide and methane - seep out at the low water line. Here, they are partially re-oxidized by microbial and chemical processes (Jansen *et al*., 2009). The patterns of porewater flow controll the distribution of

primary electron donors and acceptors and divide the sand flat into different compartments. To understand the microbiology, microbial ecology and biogeochemistry of the sand flat, physical and geochemical data need to be interlinked with studies on microbial diversity and activity.

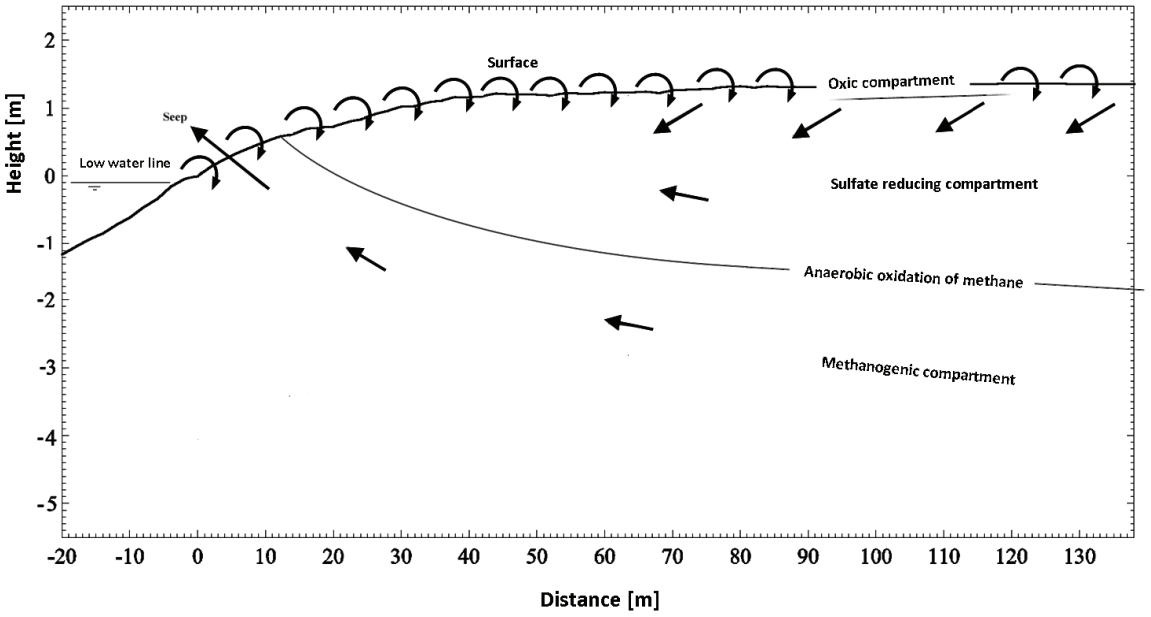

Figure 13 Hypothetical distribution of different functional compartments in Janssand.
The dominant mineralization processes are aerobic respiration, nitrate reduction, sulfate reduction and methanogenesis. Curved arrows indicate advective flushing of the sediment surface. Straight arrows indicate deep porewater flow. Taken from from WATT II research report (2006)

Previous studies on the microbial community composition detected members of the *Alphaproteobacteria*, *Gammaproteobacteria*, *Bacteroidetes* and *Planctomycetes* that comprise typical aerobic but also anaerobic heterotrophic bacteria and comprise distinct physiological groups such as hydrolyzers, fermenters, methanotrophs, nitrate respirers and sulfur oxidizers in high abundances in the surface sediment (Ishii *et al*., 2004; Arnds, 2006). Likewise, members of the *Deltaproteobacteria*, typical anaerobic, heterotrophic bacteria, were found in high numbers (Ishii *et al*., 2004; Gittel *et al*., 2008). However, despite their potential contribution to carbon mineralization little is known about the *in situ* activity of abundant phylogenetic groups.

1.7 Objectives of This Thesis

The present study aimed to identify, quantify and functionally characterize microorganisms that are involved in the oxidation of reduced inorganic sulfur compounds, fixation of inorganic carbon and the turnover of simple organic carbon compounds such as acetate in the surface sediment of the Janssand intertidal sand flat. Using a whole set of molecular tools I aimed to answer the questions: “Who are they?”, “How many are they?” and “What are they doing? “. As the majority of marine microbes still resist cultivation, culture-independent techniques were applied. In addition, targeted enrichment approaches complemented the study. The main objectives were:

The diversity of potential sulfur-oxidizing organisms should be characterized using the 16S rRNA gene approach. Candidate SOP were identified based on their relatedness to known SOP. Chapter 2

The candidate SOP should be detected and quantified *in situ*. In particular, the community composition of the highly abundant and diverse *Gammaproteobacteria* was analysed. Chapter 2

The identity and diversity of SOP should be characterized using the functional gene approach. By investigating genes diagnostic for sulfur oxidation the community structure of SOP was resolved. Chapter 2 and 3

The genomic inventory of uncultured SOP should be investigated using a metagenomic approach. Novel insights into the metabolic capabilities and putative identity of uncultured SOP were gained by the analysis of large genome fragments. Chapter 3

The MAR-FISH method should be adapted for its application in sediments. Using this technique the assimilation of inorganic and organic carbon by single cells of the sediment microbial community was investigated. Chapter 2 and 4

A protocol was developed that allows the nanoSIMS-based analysis of substrate incorporation patterns of single cells in particle-rich sediment samples. A workflow is provided that enables an efficient analysis of target populations. Chapter 4

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2. Sulfur-Oxidizing *Gammaproteobacteria* in Coastal Sediments

Contributions to the study:

Sabine Lenk: performed 16S rRNA gene clone library construction of the March 2007 sample, performed *dsrAB* clone library construction, designed and tested probes for the application in FISH and CARD-FISH, quantified total *Gammaproteobacteria* along the sediment profile of August 2007, quantified distinct populations along the sediment profiles of the April 2005 and August 2007 sample, performed MAR-FISH analysis of the June 2009 and October 2009 sample, performed data analysis and processing, developed the concept of the manuscript, wrote the manuscript

Julia Arnds: performed 16S rRNA gene clone library construction of the Novemeber 2004 and April 2005 sample; quantified total *Gammaproteobacteria* along the sediment profile of April 2005

Katrice Zerjatke: assisted in 16S rRNA gene clone library construction of the March 2007 sample, performed MAR-FISH analysis of the October 2006 sample, assisted in MAR-FISH analysis of the June 2009and October 2009 sample

Niculina Musat: developed experimental set up and performed bulk dark CO_2 fixation measurements of the October 2006 sample

Marc Mußmann: performed *aprA* clone library construction

Sabine Lenk, Rudolf Amann and Marc Mussmann: designed research, discussed data, conceived the manuscript and edited the manuscript

Novel Groups of *Gammaproteobacteria* Catalyze Sulfur Oxidation and Carbon Fixation in a Coastal, Intertidal Sediment

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Key words: reverse dissimilatory sulfite reductase, sulfur-oxidizing prokaryotes, *Gammaproteobacteria*, microautoradiography, carbon fixation, marine sediment

Summary

The oxidation of hydrogen sulfide is essential to sulfur cycling in marine habitats. However, the role of microbial sulfur oxidation in marine sediments and the microorganisms involved are largely unknown, except for the filamentous, mat-forming bacteria. In this study we explored the diversity, abundance and activity of sulfur-oxidizing prokaryotes (SOP) in sulfidic intertidal sediments using 16S rRNA and functional gene sequence analyses, fluorescence in situ hybridization (FISH) and microautoradiography. The 16S rRNA gene analysis revealed that distinct clades of uncultured *Gammaproteobacteria* are important SOP in the tidal sediments. This was supported by the dominance of gammaproteobacterial sequences in clone libraries of genes encoding the reverse dissimilatory sulfite reductase (rDSR) and the adenosine phosphosulfate reductase (APR). Numerous sequences of all three genes grouped with uncultured autotrophic SOP. Accordingly, *Gammaproteobacteria* accounted for 40–70% of all $^{14}CO_2$-incorporating cells in surface sediments as shown by microautoradiography. Furthermore, phylogenetic analysis of all three genes consistently suggested a discrete population of SOP that was most closely related to the sulfur-oxidizing endosymbionts of the tubeworm *Oligobrachia* spp. FISH showed that members of this population (WS-Gam209 group) were abundant, reaching up to 1.3×10^8 cells ml^{-1} (4.6% of all cells). Approximately 25% of this population incorporated CO_2, consistent with a chemolithoautotrophic metabolism most likely based on sulfur oxidation. Thus, we hypothesize that novel, gammaproteobacterial SOP attached to sediment particles may play a more important role for sulfide removal and primary production in marine sediments than previously assumed.

Introduction

Large amounts of hydrogen sulfide are produced by sulfate-reducing bacteria in organic-rich coastal sediments (Jørgensen, 1982). Most of this toxic metabolite is already re-oxidized at sediment surface. Here, the relative contribution of chemical versus biological sulfide oxidation and the majority of involved organisms are still unknown (Jørgensen and Nelson, 2004). Studies on benthic sulfur-oxidizing prokaryotes (SOP) to date mostly focused on 'large sulfur bacteria' such as *Beggiatoa* or *Thioploca* that often form conspicuous mats on sediment surfaces (Jørgensen and Nelson, 2004). However, they occur as local phenomena and represent only a small fraction of marine SOP. In contrast, non-mat-forming, unicellular SOP from the oxic–anoxic transition zone have rarely been investigated, although they may significantly contribute to sulfur oxidation in marine sediments (Bruechert *et al*., 2003).

Most studies of benthic SOP relied on cultivation, where estimated numbers range from 10^5 cells ml^{-1} to 10^9 cells ml^{-1} based on MPN counts (Schaub and Gemerden, 1996; Brinkhoff *et al*., 1998; Heijs *et al*., 1999; Podgorsek and Imhoff, 1999). Chemolithoautotrophic sulfur-oxidizing *Gammaproteobacteria* and *Epsilonproteobacteria* such as *Thiomicrospira*, *Arcobacter sulfidicus* and *Sulfurimonas denitrificans* have frequently been isolated from marine sediments and were suggested to be environmentally important (Kuenen and Veldkamp, 1972; Timmer-Ten Hoor, 1975; Brinkhoff *et al*., 1999; Wirsen *et al*., 2002). Detailed studies on the abundance of SOP avoiding the cultivation bias are scarce. Culture-independent studies on coastal sediment have consistently found *Gammaproteobacteria* in high frequencies in 16S rRNA gene libraries or in high relative cell numbers (Ishii *et al*., 2004; Asami *et al*., 2005; Buehring *et al*., 2005; Hong *et al*., 2006; Musat *et al*., 2006; Gillan and Pernet, 2007; Feng *et al*., 2009). For some of the mostly uncultured groups the capability to oxidize sulfur has been suggested because of their phylogenetic relationship with chemoautotrophic sulfur-oxidizing SOP, in particular with sulfur-oxidizing symbionts of marine invertebrates (Ravenschlag *et al*., 1999; Bowman *et al*., 2003; Meyer and Kuever, 2007a). In one study Ravenschlag and colleagues (2001) detected 10^7 cells ml^{-1} of putative, gammaproteobacterial SOP in arctic surface sediments based on 16S rRNA phylogeny and fluorescence *in situ* hybridization. However, specific populations were not identified and there is no evidence for *in situ* activity.

It is widely recognized that the assignment of physiological traits based on 16S rRNA phylogeny is of limited use. To explore the environmental diversity of SOP in more detail, genes coding for key enzymes of different sulfur oxidation pathways have been applied as phylogenetic and functional markers (Petri *et al*., 2001; Meyer *et al*., 2007; Pham *et al*., 2008). For example, the *aprBA* genes encoding the APS reductase are widespread but also occur in some non-sulfide-oxidizing organisms (Meyer and Kuever, 2007a,b). Recently, the reverse dissimilatory sulfite reductase (rDSR) has been introduced as a suitable molecular marker for SOP as 16S rRNA and rDSR phylogeny are largely consistent (Loy *et al*., 2009). It is involved in the oxidation of transiently formed elemental sulfur/polysulfide to sulfite (Pott and Dahl, 1998; Dahl *et al*., 2005). Genes encoding the alpha and beta subunits of the rDSR (*dsrAB*) have been found in SOP such as free-living and many symbiotic bacteria that are key players in sulfur cycling in marine habitats (Mussmann *et al*., 2007; Lavik *et al*., 2009; Loy *et al*., 2009; Walsh *et al*., 2009).

In this study we investigated the diversity, abundance and activity of potential SOP in tidal surface sediments by cultivation independent methods. We focused on the intertidal sand flat 'Janssand' that is

located in the German Wadden Sea and is characterized by extensive sulfur transformations. Here, sulfide is usually re-oxidized within the upper 3 cm of the surface sediment in the transition zone of oxygen, nitrate and sulfide (Gao *et al*., 2009; Jansen *et al*., 2009). This results in turbid suspensions of elemental sulfur and polysulfides at the sediment surface (Kamyshny and Ferdelman, 2007). The diversity of SOP was studied by combining the full 16S rRNA cycle with comparative phylogenetic analysis of *dsrAB* and of *aprA* genes. The *dsrAB* genes were amplified with novel and published primers to cover a higher diversity. Fluorescence *in situ* hybridization (FISH) was applied with novel and published probes to identify candidate SOP in the sediment. Since many recognized SOP are autotrophic, we combined FISH with microautoradiography (MAR) to identify actively CO_2-incorporating cells.

Results

Diversity of 16S rRNA genes and candidate SOP

A comprehensive 16S rRNA gene analysis was performed to identify candidate SOP in Janssand sediments. Three 16S rRNA gene libraries were constructed from DNA that was extracted from surface sediment sampled in November 2004, April 2005 and March 2007. In total 790 clone sequences were retrieved and were screened for candidate SOP among all bacterial phyla. None of the retrieved sequences were closely related to SOP among the *Chlorobi* or *Epsilonproteobacteria*. Although *Gammaproteobacteria* accounted for 40% of all sequences (Fig. S1), no sequences related to known gammaproteobacterial SOP such as phototrophic *Chromatiales*, filamentous sulfur oxidizers as *Beggiatoa* or previously isolated *Thiobacillus* or *Thiomicrospira* species were detected. To take a closer look at other potential SOP among the *Gammaproteobacteria* 169 full-length 16S rRNA gene sequences were phylogenetically analysed in more detail. These were classified into 69 operational taxonomic units (OTUs) based on a 97% sequence identity cut-off. All sequences were phylogenetically analysed and representative sequences of each OTU were selected for illustration in the phylogenetic tree (Fig. 1). The majority (54 OTUs) grouped with sequences originating from coastal and deep-sea sediments.

Overall, 21 OTUs displayed stable branching patterns with both applied maximum-likelihood methods and displayed relationships with sulfur-oxidizing bacteria (Fig. 1, Table S1). The next known relatives of most of these OTUs were symbionts of marine invertebrates (89–98% sequence identity, Table S1). A set of 15 sequences grouped with endosymbionts of the siboglinid polychaetes *Oligobrachia haakonmosbiensis* and O. *mashikoi* (5 OTUs, e.g. clone WS202, 89–92%) and with the cultured strain *Leucothrix mucor* (89%), which is a chemolithoheterotrophic sulfur oxidizer (Grabovich *et al*., 1999). One OTU grouped with the ciliate symbiont *Candidatus* Thiobios zoothamnicoli (e.g. clone 321, 94–95%). We also recovered sequences that were related to the sulfur-oxidizing Gamma 1 and Gamma 3 symbionts of the gutless oligochaete *Olavius algarvensis* (e.g. clone 147, 95% and clone WS114 98%, respectively). Several sequences branched off more deeply and were most closely related to the free-living, sulfur-oxidizing *Thioalkalivibrio thiocyanodenitrificans* (6 OTUs, e.g. clone WS299, WS524, 90–92%). Twelve OTUs grouped within the NOR5/OM60 clade (Fig. 1). Although several isolates contain genes involved in thiosulfate oxidation, members of NOR5/OM60 have not yet been shown to oxidize thiosulfate. Their role in sulfur oxidation is therefore still debated (Fuchs *et al*., 2007; Yan, 2009). Few alphaproteobacterial sequences (1% of all clones) were recovered, of which four OTUs grouped within the planktonic *Roseobacter* lineage. These organisms are mainly known for transformation of organo-sulfur compounds.

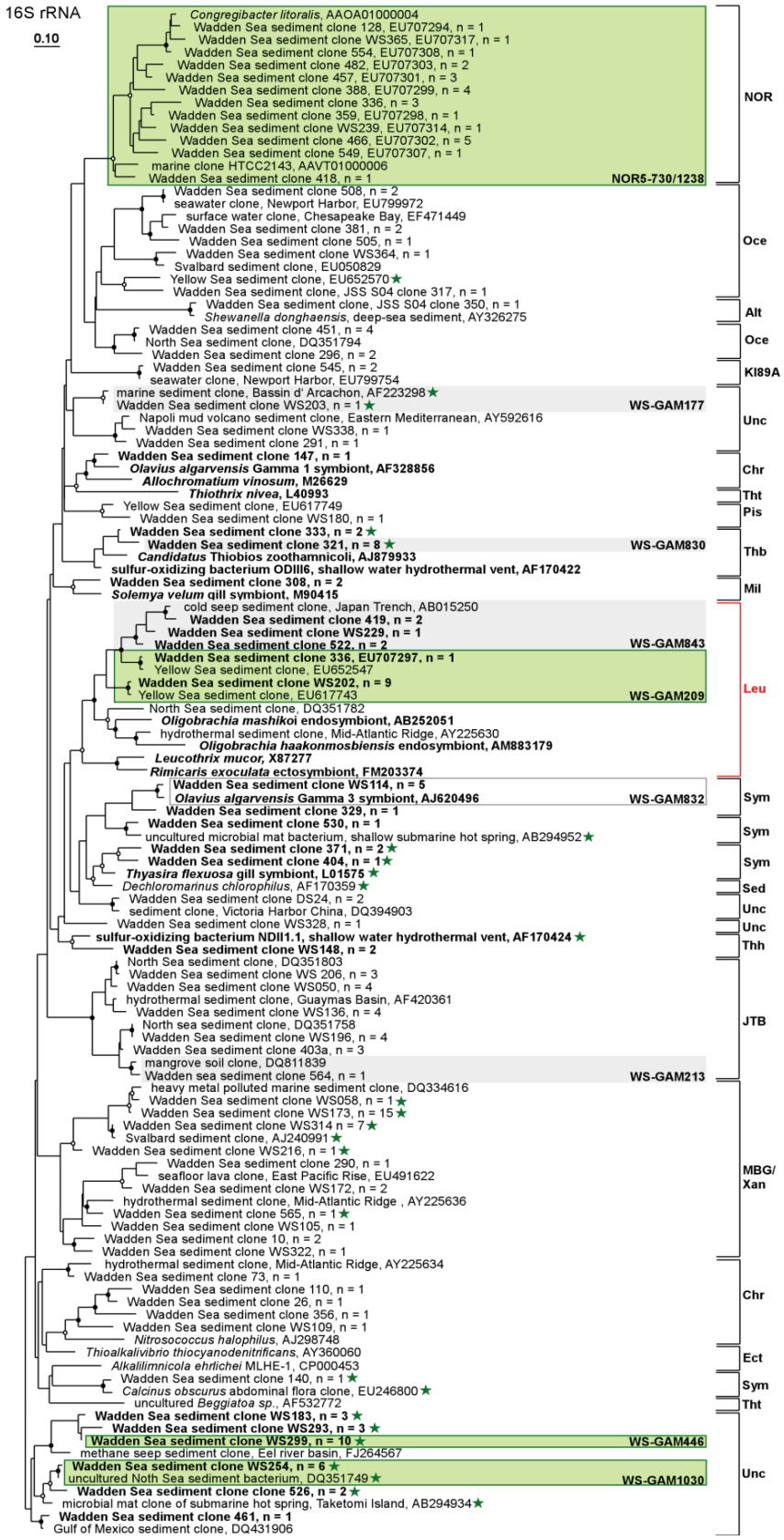

Figure 1 Maximum-likelihood phylogeny (RAxML) of gammaproteobacterial 16S rRNA gene sequences from intertidal sediments of the Janssand.

Single OTUs are represented by selected clones ('Wadden Sea sediment clone'), 'n' equals numbers of sequences per OTU. Known sulfur-oxidizing bacteria and related OTUs are shown in bold. Candidate SOP targeted by probe GAM660 are indicated by an asterisk (★). OTUs that were targeted by population-specific oligonucleotide probes applied in this study are shaded. Grey shading indicates groups that were clearly detected in sediment samples by FISH. Green shading indicates groups that were quantified by FISH (Table 1, Fig. 3). RAxML bootstrap values are indicated for lineages with > 70% (filled circles) or > 50% (open circles) support. The bar indicates 10% sequence divergence. Taxonomic assignment is given according to the guide tree of the SILVA SSU Reference database release 100. NOR, NOR5/ OM60 clade; Oce, *Oceanospirillales*; Pse, *Pseudoalteromonadales*; Alt, *Alteromonadales*; KI89A, KI89A clade; Unc, uncultured organisms; Chr, *Chromatiaceae*; Tht, *Thiotrichaceae*; Pis, *Piscirickettsiaceae*; Thb, *Candi-datus* Thiobios (uncultured sym-bionts); Mil, Milano-WF1B-44 clade; Leu *Leucothrix/ Thiothrichaceae*; Sym, uncultured symbionts; Sed, *Sedimen-ticola*; Thh, *Thiohalophilus*; JTB, JTP148 clade; MBG/Xan, marine benthic group of *Xanthomonadales*; Ect, *Ectothio-rhodospiraceae*.

dsrAB and aprA diversity in intertidal sediments

The diversity and identity of uncultured SOP was further examined by comparative sequence analysis of the two key genes *dsrAB* and *aprA* that are diagnostic for sulfur oxidation. The dominance of candidate sulfur-oxidizing *Gammaproteobacteria* in the 16S rRNA gene libraries was reflected in a high diversity of mostly gammaproteobacterial sequences in the functional gene libraries.

Using both novel and previously published primers (Table S2) in total 115 *dsrAB* sequences were retrieved from different sediment layers. Based on a 90% sequence identity cut-off (Loy *et al*., 2009) 44 distinct OTUs were identified. All sequences were phylogenetically analysed and representative sequences of each OTU were selected for illustration in the phylogenetic tree (Fig. 2). Rarefaction analysis suggested that the *dsrAB* diversity was nearly covered after pooling data sets recovered with both primer pairs (Fig. S2). The phylogenetic analysis of deduced DsrAB amino acid sequences grouped the majority (88 sequences) into 33 OTUs among the *Gammaproteobacteria* (Fig. 2). A broad diversity was covered as indicated by 67–98% overall sequence identity. Consistent with the 16S rRNA phylogeny also several DsrAB grouped with sequences from sulfur-oxidizing symbionts. For example, several OTUs formed stable monophyletic branches with *Candidatus* T. zoothamnicoli (7 OTUs, 88–89% sequence identity), with the Gamma 3 symbiont of the oligochaete *O*. *algarvensis* (9 OTUs, 93–96%) and with the symbiont of *O*. *haakonmosbiensis* (3 OTUs, 85–86%). In addition, two OTUs grouped with *Endoriftia persephone*, the symbiont of the tubeworm *Riftia* (2 OTUs, 87–88%). Five OTUs affiliated with the free-living sulfur oxidizer *Thiothrix nivea* as closest cultured relative (83–85%). Moreover, eleven OTUs formed stable phylogenetic groups with uncultured *Alphaproteobacteria*. They were most closely related to DsrAB sequences of uncultured organisms from sulfidic waters of the Namibian upwelling region. The phototrophic, purple non-sulfur bacterium *Rhodomicrobium vannielii* was the next cultured relative (77–80%). Again, none of the recovered sequences was closely related with betaproteobacterial SOP or with phototrophic SOP of the *Chlorobi* or *Chromatiales*.

To further investigate the diversity of SOP in the oxygen–sulfide transition zone on the rDSR level, we set up an enrichment culture inoculated with Janssand sediment with opposing gradients of oxygen and sulfide and cloned and sequenced *dsrAB* from these enrichments. From the layer with visible precipitations of elemental sulfur (Fig. S3) 32 *dsrA* sequences were recovered. All of these except one formed a single OTU (Fig. 2) that was most closely related to the DsrAB of the *O*. *haakonmosbiensis* endosymbiont (93% sequence identity) and to the Wadden Sea clone d1H5 (96%).

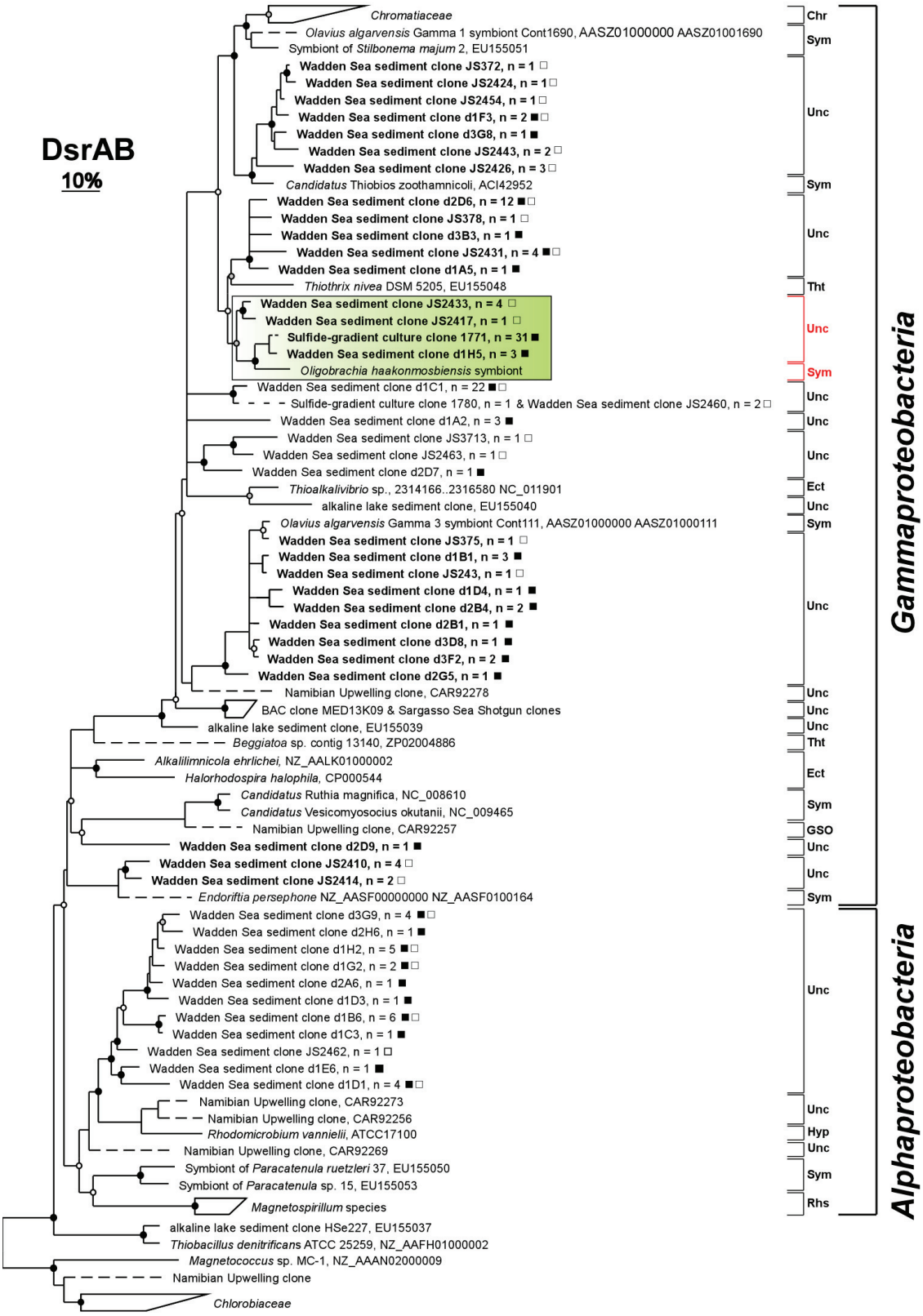
DsrAB
10%
Chromatiaceae
Olavius algarvensis Gamma 1 symbiont Cont1690, AASZ01000000 AASZ01001690
Symbiont of Stilbonema majum 2, EU155051
Wadden Sea sediment clone JS372, n = 1
Wadden Sea sediment clone JS2424, n = 1
Wadden Sea sediment clone JS2454, n = 1
Wadden Sea sediment clone d1F3, n = 2
Wadden Sea sediment clone d3G8, n = 1
Wadden Sea sediment clone JS2443, n = 2
Wadden Sea sediment clone JS2426, n = 3
Candidatus Thiobios zoothamnicoli, ACI42952
Wadden Sea sediment clone d2D6, n = 12
Wadden Sea sediment clone JS378, n = 1
Wadden Sea sediment clone d3B3, n = 1
Wadden Sea sediment clone JS2431, n = 4
Wadden Sea sediment clone d1A5, n = 1
Thiothrix nivea DSM 5205, EU155048
Wadden Sea sediment clone JS2433, n = 4
Wadden Sea sediment clone JS2417, n = 1
Sulfide-gradient culture clone 1771, n = 31
Wadden Sea sediment clone d1H5, n = 3
Oligobrachia haakonmosbiensis symbiont
Wadden Sea sediment clone d1C1, n = 22
Sulfide-gradient culture clone 1780, n = 1 & Wadden Sea sediment clone JS2460, n = 2
Wadden Sea sediment clone d1A2, n = 3
Wadden Sea sediment clone JS3713, n = 1
Wadden Sea sediment clone JS2463, n = 1
Wadden Sea sediment clone d2D7, n = 1
Thioalkalivibrio sp., 2314166..2316580 NC_011901
alkaline lake sediment clone, EU155040
Olavius algarvensis Gamma 3 symbiont Cont111, AASZ01000000 AASZ01000111
Wadden Sea sediment clone JS375, n = 1
Wadden Sea sediment clone d1B1, n = 3
Wadden Sea sediment clone JS243, n = 1
Wadden Sea sediment clone d1D4, n = 1
Wadden Sea sediment clone d2B4, n = 2
Wadden Sea sediment clone d2B1, n = 1
Wadden Sea sediment clone d3D8, n = 1
Wadden Sea sediment clone d3F2, n = 2
Wadden Sea sediment clone d2G5, n = 1
Namibian Upwelling clone, CAR92278
BAC clone MED13K09 & Sargasso Sea Shotgun clones
alkaline lake sediment clone, EU155039
Beggiatoa sp. contig 13140, ZP02004886
Alkalilimnicola ehrlichei, NZ_AALK01000002
Halorhodospira halophila, CP000544
Candidatus Ruthia magnifica, NC_008610
Candidatus Vesicomyosocius okutanii, NC_009465
Namibian Upwelling clone, CAR92257
Wadden Sea sediment clone d2D9, n = 1
Wadden Sea sediment clone JS2410, n = 4
Wadden Sea sediment clone JS2414, n = 2
Endoriftia persephone NZ_AASF00000000 NZ_AASF0100164
Wadden Sea sediment clone d3G9, n = 4
Wadden Sea sediment clone d2H6, n = 1
Wadden Sea sediment clone d1H2, n = 5
Wadden Sea sediment clone d1G2, n = 2
Wadden Sea sediment clone d2A6, n = 1
Wadden Sea sediment clone d1D3, n = 1
Wadden Sea sediment clone d1B6, n = 6
Wadden Sea sediment clone d1C3, n = 1
Wadden Sea sediment clone JS2462, n = 1
Wadden Sea sediment clone d1E6, n = 1
Wadden Sea sediment clone d1D1, n = 4
Namibian Upwelling clone, CAR92273
Namibian Upwelling clone, CAR92256
Rhodomicrobium vannielii, ATCC17100
Namibian Upwelling clone, CAR92269
Symbiont of Paracatenula ruetzleri 37, EU155050
Symbiont of Paracatenula sp. 15, EU155053
Magnetospirillum species
alkaline lake sediment clone HSe227, EU155037
Thiobacillus denitrificans ATCC 25259, NZ_AAFH01000002
Magnetococcus sp. MC-1, NZ_AAAN02000009
Namibian Upwelling clone
Chlorobiaceae
Chr
Sym
Unc
Sym
Unc
Tht
Unc
Sym
Unc
Unc
Unc
Ect
Unc
Sym
Unc
Unc
Unc
Tht
Ect
Sym
GSO
Unc
Unc
Sym
Unc
Unc
Hyp
Unc
Sym
Rhs
Gammaproteobacteria
Alphaproteobacteria

Figure 2 Phylogenetic reconstruction of DsrAB amino acid sequences retrieved from intertidal sediments of the Janssand. A consensus tree based on maximum-likelihood (RAxML) and Bayesian analysis was generated. Single OTUs are represented by selected clones ('Wadden Sea sediment clone'), 'n' equals numbers of sequences per OTU. Gammaproteobacterial OTUs that formed stable groups with sulfur-oxidizing bacteria are shown in bold. The sequence cluster related to endosymbionts of *O. haakonmosbiensis* cluster is highlighted. Squares indicate OTUs comprising sequences that were retrieved with (■) the novel primer set designed in this study, (□) primers designed by Loy and colleagues (2009), (■□) both primer sets. Nine OTUs were shared by both data sets. Sequences that were amplified with the novel primer set published in this study originate from different sediment depths: 0–3 cm ('d1'), 8–19 ('d2') or > 19 cm ('d3'). The 'JS' clone sequences were amplified with primers published by Loy and colleagues (2009) and originate from 0–3 cm depth. Bootstrapping was performed including all public full-length sequences. Sequences shorter than 550 amino acids (indicated by dashed line) were added to the tree without changing the overall tree topology. Filled circles indicate lineages that have both > 70% RAxML bootstrap support and > 90% MRBAYES posterior probability values. RaxML bootstrap values are given for lineages with > 70% (grey circles) and > 50% (open circles) support. The bar indicates 10% sequence divergence. Chr, *Chromatiaceae*; Sym, symbionts; Unc, uncultured organisms; Tht, *Thiotrichaceae*, Ect, *Ectothiorhodospiraceae*; GSO, GSO447/SUP05 clade; Hyp, *Hyphomicrobiaceae/Rhizobiales*; Rhd, *Rhodospirillaceae*.

In addition, an *aprA* gene library was generated from the intertidal sediment sampled in 2003. The library comprised 65 sequences that did not fully cover the *aprA* diversity as shown by the rarefaction analysis (Fig. S2). The majority of the recovered sequences also grouped with *Gammaproteobacteria* (45 sequences, 22 OTUs), of which many affiliated with symbiotic SOP (Fig. S4). Similar to 16S rRNA and DsrAB phylogenies four sequences were most closely related to the endosymbiont of *O. haakonmosbiensis* (3 OTUs, e.g. clone A82, 94% sequence identity). Furthermore, sequences grouped with the free-living *Thioalkalivibrio* sp. HL-EbGR7 and with the sulfur-oxidizing Gamma 3 symbiont of *O. algarvensis* (1 OTU, clone 092, 95%). One cluster of sequences formed a monophylethic group with the Gamma 1 symbiont of *O. algarvensis* (e.g. clone A073, 97%) and with the endosymbiont of *Inandrilus leukodermatus* (e.g. clone 096, 97%). Five OTUs clustered with the free-living, sulfur-oxidizing strain DIII5 (e.g. clone A81, 96%). In addition, sequences were found that were not related to SOP, but affiliated with the alphaproteobacterium *Pelagibacter ubique* (10 sequences) and with sulfate-reducing *Deltaproteobacteria* (10 sequences).

Abundance of candidate SOP in intertidal sediments

To quantify abundant populations of candidate SOP fluorescence *in situ* hybridization combined with catalysed reporter deposition (CARD-FISH) of the 16S rRNA was performed at different depths (Fig. 3). The applied probes targeted cultured and uncultured, proteobacterial SOP (Table 1). In agreement with the 16S rRNA gene libraries, *Epsilonproteobacteria* were not detected in numbers above the detection limit of 0.5% in repeated FISH experiments. Since we consistently found relatives of uncultured gammaproteobacterial SOP in clone libraries of all the three genes (16S rRNA, *dsrAB*, *aprA*), we focused our study on the quantification of gammaproteobacterial populations. Cells of *Gammaproteobacteria* as detected by probe GAM42a accounted for 25% (April 2005) and 30% (August 2007) of all cells, which equalled absolute abundances of up to 8.1×10^8 (April) and 7.6×10^8 cells ml^{-1} (August 2007, Fig. 3). For a more detailed resolution of the gammaproteobacterial community we applied published and novel probes (Table 1). The probe TMS849 (Brinkhoff and Muyzer, 1997) that is specific for *Thiomicrospira* spp. was optimized for FISH. Individual cells were stained, but relative abundances of detected cells were below the detection limit of 0.5%. Eight novel probes were developed in this study that targeted 10 OTUs (46 sequences) from the Janssand intertidal sand flat related to sequences of symbiotic SOP or sequences originating from other sulfidic sediments (Fig. 1, Table 1). The target range of these probes

comprised not only sequences from this study but also related sequences from other sulfidic marine sediments.

Three of the novel probes (WS-Gam209, WS-Gam446 and WS-Gam1030, Table 1) revealed relative cell abundances above the detection limit (Fig. 3). Double hybridization with the more general probes GAM660 or GAM42a confirmed that the cells identified by our specific probes belonged to *Gammaproteobacteria* (Fig. 4). Probe WS-Gam209 that targeted the population related to the endosymbionts of *Oligobrachia* spp. and to the strain *L*. *mucor* (Fig. 1) displayed the highest counts throughout the study period (Fig. 3). Maximum relative abundances accounted for up to 4.6% of all cells in April 2005 (1.3×10^8 cells ml^{-1}). The cells appeared as rods or cocci and often formed aggregates (Fig. 4). Two of the OTUs most closely related to *T*. *thiocyanodenitrificans* were targeted by probes WS-Gam446 and WS-Gam1030 (Fig. 1). The detected populations accounted for up to 1.4% (WS-Gam446, 5.6×10^7 cells ml^{-1}) and 1.9% (WS-Gam1030, 6.5×10^7 cells ml^{-1}) of all cells (Fig. 3). Analysis of the public sequence database and additional FISH surveys indicated that WS-Gam209, WS-Gam1030 and the WS-Gam446 populations occur in sulfidic sediments worldwide (Fig. S5). Probes WS-Gam177, WS-Gam213, WS-Gam830 and WS-Gam843 clearly hybridized with cells; however, their abundances were too low for a confident quantification. The NOR5/OM60 clade (Figs 1 and 4) constituted up to 3.8% (1.5×10^8 cells ml^{-1}) of all cells (Fig. 3). Overall, the mean relative abundances of the populations, which were targeted by probes WS-Gam209, -1030, -446 and NOR5, added up to 8% of all cells and represented approximately one third of all *Gammaproteobacteria* in the upper sediment layers.

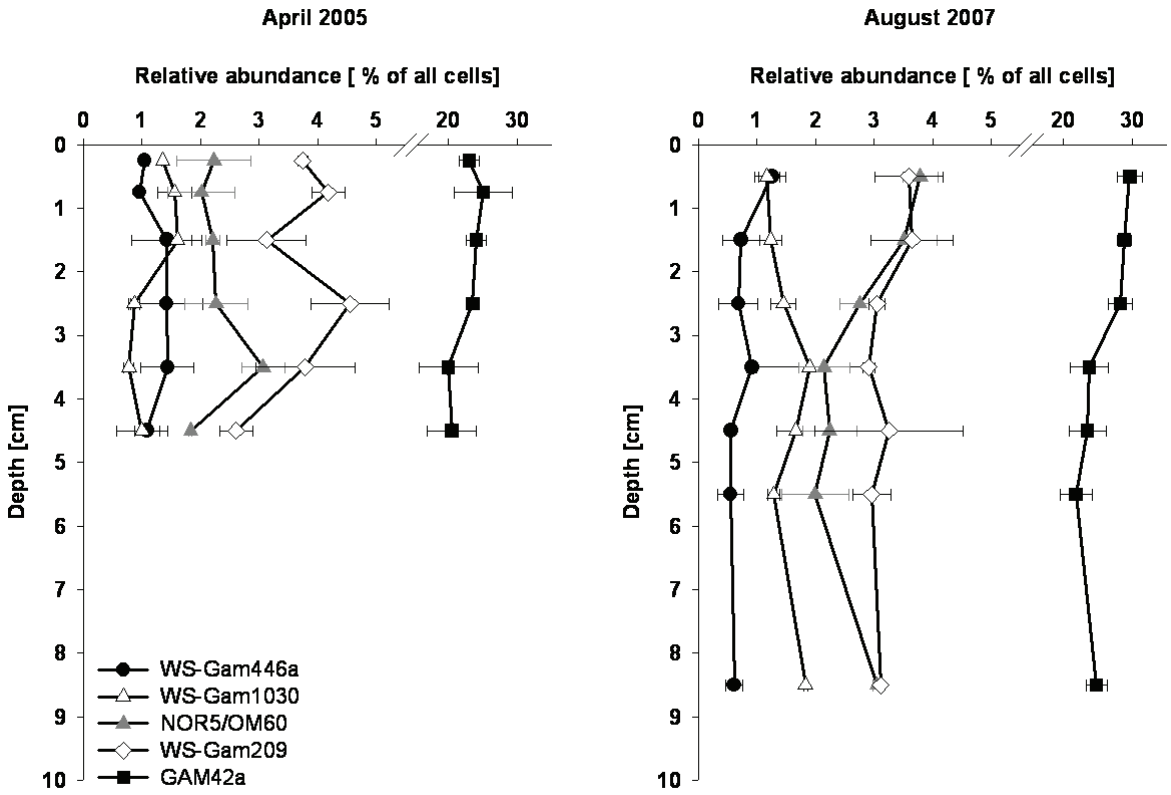

Figure 3 Relative abundances of distinct gammaproteobacterial populations and total *Gammaproteobacteria* in two vertical sediment profiles in April 2005 and August 2007.

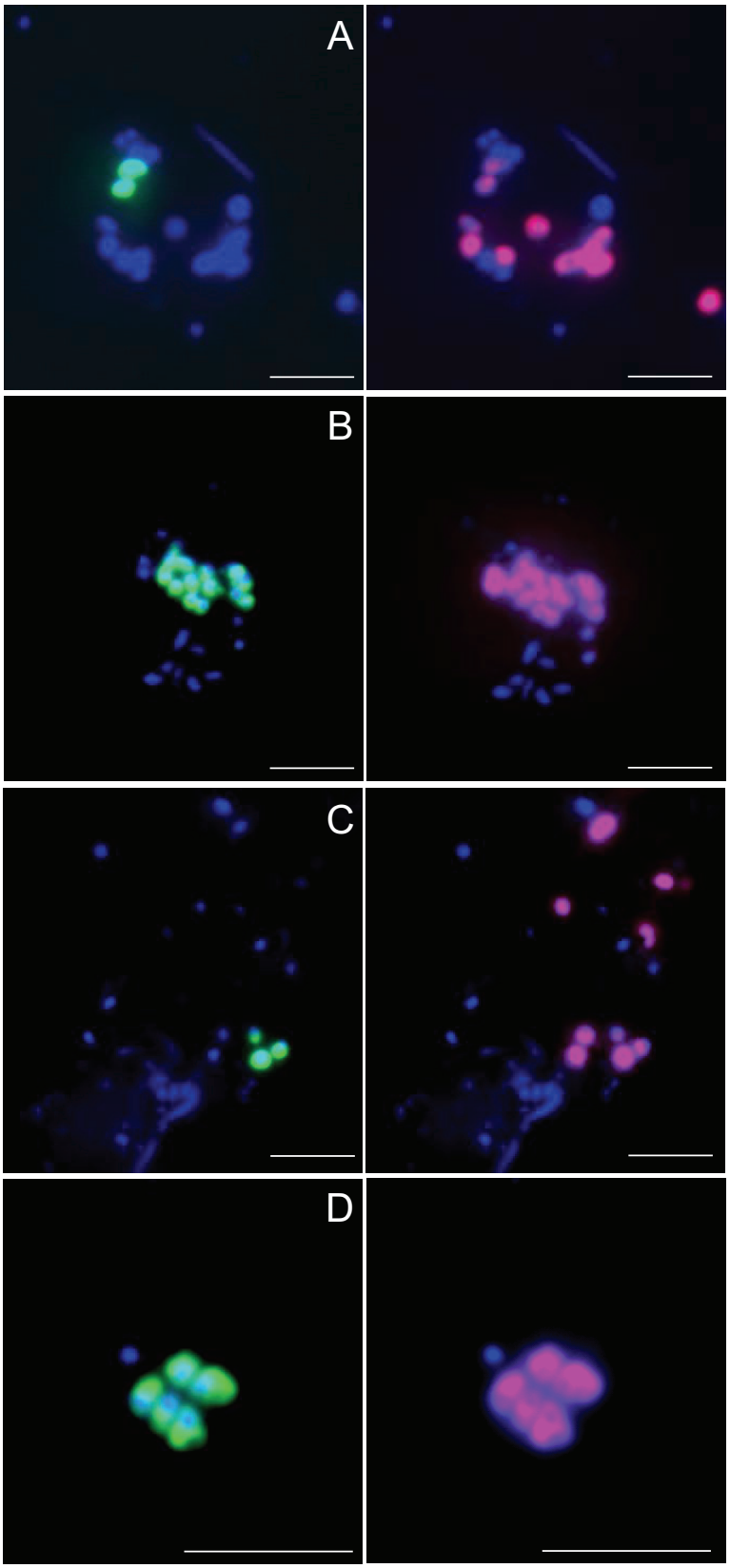

Figure 4 Epifluorescence microscopy images of distinct gammaproteobacterial populations identified in the intertidal sediments of Janssand.

For all images, green (Alexa 488): cells that were identified by population-specific probes (left panel); red (Alexa 594): *Gamma-proteobacteria* that hybridized with the general probe GAM42a (right panel); blue: DAPI-stained DNA. The scale bar corresponds to 5 µm. **A.** NOR5/OM60 target cells of probe NOR5-730/1238.; **B.** Probe WS-Gam209-targeted cells related to *Oligobrachia* spp. endosymbionts.; **C.** Probe WS-Gam446-targeted cells related to *T. thiocyanodenitrificans*.; **D.** Probe WS-Gam1030-targeted cells related to *T. thiocyanodenitrificans.*

Table 1 Oligonucleotide probes applied for the detection of distinct populations in FISH

Probe name	Specificity	Sequence (5' - 3')	Target position (*E. coli*)	FA[a]	Signal[b]	Reference
GAM42a [d,f] Δ	*Gammaproteobacteria*	GCC TTC CCA CAT CGT TT	1027 – 1043[c]	35	++	Manz *et al*., 1992
GAM660 [f]	*Gammaproteobacteria*	TCC ACT TCC CTC TAC	660 – 674	35	++	Ravenschlag *et al*., 2001
NOR5-730 [e,f]	NOR5/OM60 clade	TCG AGC CAG GAG GCC GCC	730 – 747	50	++	Eilers *et al*., 2001
NOR5-1238 [e,f]	NOR5/OM60 clade	CCC TCT GTG CGT TCC ATT	1238 – 1255	50	++	Yan *et al*., 2009
WS-Gam209 [d, f]	OTU WS202	CTA CTA GTG CCA GGT CCG	209 – 227	25	++	This study
WS-Gam446 [f]	OTU WS299	ACC CGC AAC TGT TTC CTC	446 – 462	20	++	This study
WS-Gam1030 [d,f]	OTU WS254	CCT GTC AAC CAG TTC CCG	1030 – 1048	25	++	This study
WS-Gam177	OTU WS203	TCC CTC GTA AGG ATT ATG	177 – 195	10	+	This study
WS-Gam213	OTU 403a	TTA TCT CAA AGC GCG AGC	213 – 231	10	+	This study
WS-Gam830	OTU 321	TTA AAT GAG CCC GAC GGC	830 – 846	10	+	This study
WS-Gam843	OTU WS229, 336, 419, 522	CTG CAC CAC TGA GAC CTC	843 – 861	20	+	This study
WS-Gam832 [f]	OTU WS114	GGT ACT AAG ACC CCC AAC	832 – 848	10	-	Ruehland, C., unpublished
TMS849 [f]	*Thiomicrospira sp*.	CTT TTT AAT AAG RCC AAC AG	830 – 849	10	-	Brinkhoff *et al*, 1997
GSO477 [f]	GSO cluster	CTA AAG TTA ACG TCA AGG	477 – 469	15	-	Lavik *et al*., 2009
Nscoc128	*Nitrosococcus halphilus*.	CCC CTC TAG AGG CCA GAT	128 – 146	10	+	Juretschko S., 2000
EP404 [f]	*Epsilonproteobacteria*	AAA KGY GTC ATC CTC CA	404 – 420	30	-	Macalady *et al*., 2006
EPSY549 [f]	*Epsilonproteobacteria*	CAG TGA TTC CGA GTA ACG	549 – 566	35	-	Lin *et al*., 2006
ARC1430 [f]	*Arcobacter sp*.	TTA GCA TCC CCG CTT CGA	1430 – 1444	20	+	Snaidr *et al*., 1997
EUBI-III [f]	*Bacteria*	GCW GCC WCC CGT AGG WGT	338-255	10	+	Amann *et al*. 1990, Daims *et al*. 1999
NON338 [f]	control probe	ACT CCT ACG GGA GGC AGC	338 – 355	10	-	Wallner *et al*., 1993

a. Formamide concentration (v/v) in hybridization buffer (46°C)
b. Cell detection in the sediment: ++, many signals; +, few signals (relative abundance < 0.5% of all cells); –, no signals
c. 23S rRNA
d. Probe was applied with competitor
e. Probe was applied with helpers
f. Probe labeled with horseradish peroxidase (HRP)

Dark CO_2 fixation revealed by MAR combined with CARD-FISH

Since many SOP fix inorganic carbon during obligate or facultative autotrophy, the detection of CO_2 uptake in single cells can indirectly provide support for lithoautotrophic sulfur oxidation (Sandrin *et al*., 2008). To test whether *Gammaproteobacteria*, in particular the candidate SOP, were able to incorporate inorganic carbon, we combined CARD-FISH with MAR (MAR–FISH). Intact sediment cores were percolated with [^{14}C]-labeled bicarbonate. In addition, surface sediment was incubated with [^{14}C]-labeled bicarbonate in glass vials. Bulk carbon uptake rates and the relative abundance of cells that have incorporated $^{14}CO_2$ were determined. Within the cores the CO_2 uptake rate and relative abundance of $^{14}CO_2$-incorporating cells were maximal in the top 2 cm and decreased with depth (Fig. 5A). In the upper 2 cm the mean uptake rates ranged from 51.2 to 57.1 µg carbon l^{-1} h^{-1}, whereas in the anoxic sediment layer in 7–8 cm depth 18.0 ± 0.2 µg carbon l^{-1} h^{-1} were incorporated. The uptake rate declined to 4.3 ± 1.5 µg carbon l^{-1} h^{-1} in 10–11 cm depth.

We identified $^{14}CO_2$-incorporating *Bacteria* and *Gammaproteobacteria* in the top 3 cm and in 7–8 cm depth by MAR–FISH (Fig. 5A). In 1–2 cm depth the relative abundance of $^{14}CO_2$-incorporating *Bacteria* was highest (13.9%). Here, *Gammaproteobacteria* accounted for nearly half (45%) of all $^{14}CO_2$-incorporating cells. In contrast, in 7–8 cm depth only 1.5% of all *Bacteria* were $^{14}CO_2$-positive. Of these, *Gammaproteobacteria* accounted for less than 25%.

For the vial incubation only sediment from the oxic–anoxic transition zone in 1–2 cm depth was sampled in June and October 2009. The vials were mildly agitated in oxic seawater to facilitate oxygen penetration into the sediment. Bulk CO_2 uptake rates amounted to 106.2 ± 23.2 (July) and 96.7 ± 4.1 µg (October) carbon l^{-1} h^{-1}. Of all cells 22.4% ± 3.8% (July) and 18.1% ± 2.3% (October) incorporated $^{14}CO_2$ (Fig. 5B). *Gammaproteobacteria* displayed relative abundances of 50% (July) and 70% (October) of all $^{14}CO_2$-positive *Bacteria* and were thus overrepresented compared with their relative abundance of up to 30% of the total microbial community (Fig. 3).

Based on counts of $^{14}CO_2$-positive cells and bulk CO_2 uptake we calculated the mean cell-specific CO_2 uptake rates. In the upper 2 cm of the sediment core, on average, 13.1 fg carbon day^{-1}, which corresponds to 45 amol carbon h^{-1}, was incorporated per cell. In the vial incubations the CO_2 uptake rate were similar and ranged from 12.5 ± 2.7 (June) and 11.4 ± 2.8 (October) fg carbon $cell^{-1}$ day^{-1}. To check whether our candidate SOP such as the WS-Gam209 group also incorporated $^{14}CO_2$, we applied specific probes for MAR–FISH. Exclusively populations related to symbionts of the *Oligobrachia* spp. (probe WS-Gam209) and to *T. thiocyanodenitrificans* (probe Gam1030) showed significant uptake of $^{14}CO_2$ (Fig. S6A–D). Here, approximately 25% of all probe WS-Gam209 hybridized cells were $^{14}CO_2$-positive. Generally, silver grain precipitates around individual cells varied in number and size, which likely reflected different uptake efficiencies.

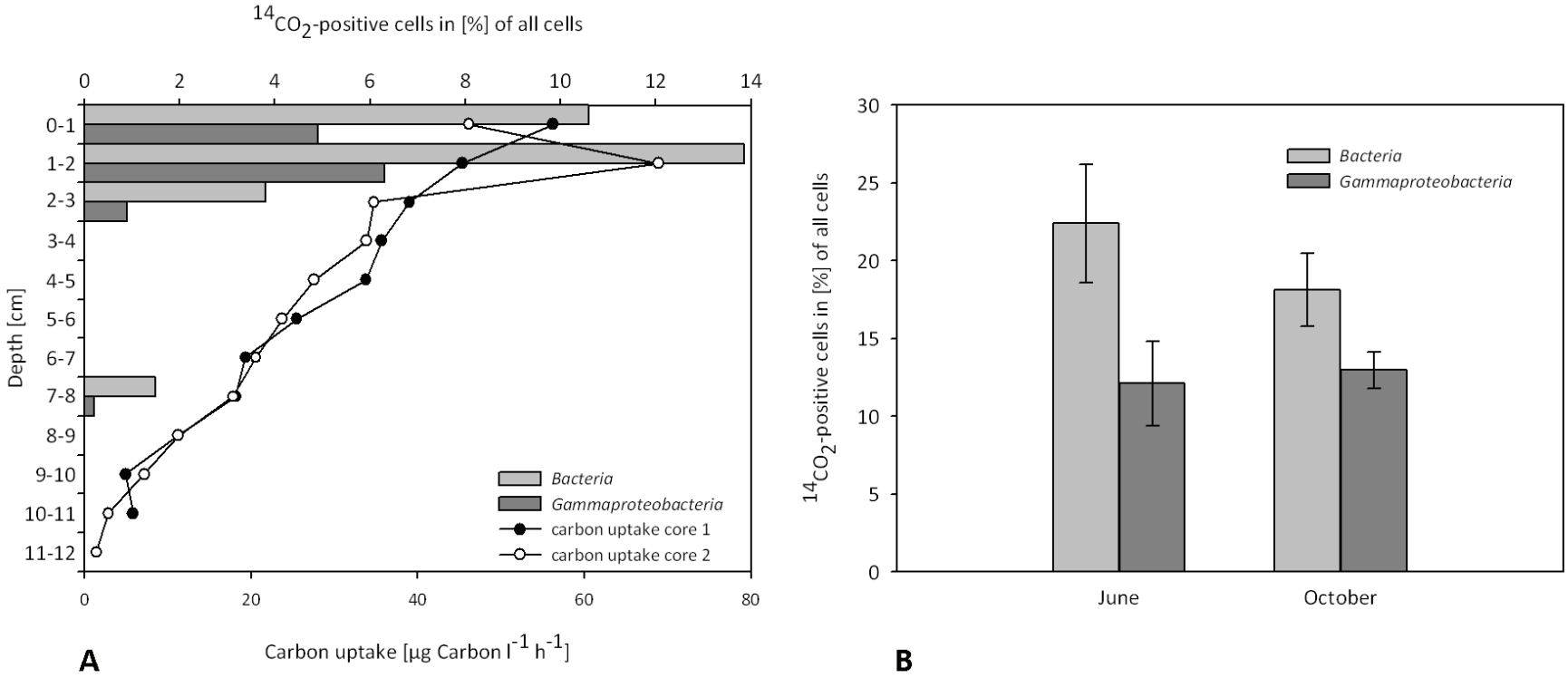

Figure 5

A. Dark CO_2 fixation rates and relative abundances of $^{14}CO_2$-incorporating *Bacteria* and *Gammaproteobacteria* in core incubations of October 2006. The relative abundances of $^{14}CO_2$-incorporating cells in 0–1, 1–2, 2–3 and 7–8 cm depth were determined only for core 2.

B. Relative abundances of $^{14}CO_2$-incorporating *Bacteria* and *Gammaproteobacteria* in vial incubations of June 2009 (duplicates) and October 2009 (triplicates).

Discussion

Our 16S rRNA approach suggested only a minor role for well-characterized SOP such as *Arcobacter*, *Sulfurimonas* or other *Epsilonproteobacteria* in the sediments, although they are important SOP in other habitats such as hydrothermal vent systems and sulfidic water columns in oxygen minimum zones (Campbell *et al*., 2006; Grote *et al*., 2007). Likewise, there was little evidence that known gammaproteobacterial SOP, e.g. *Beggiatoa*, phototrophic *Chromatiales, Thiobacillus* and *Thiomicrospira* spp., exhibited a significant role in sulfur oxidation in Janssand sediments, even though they have previously been isolated from other tidal sites (Kuenen and Veldkamp, 1972; Brinkhoff *et al*., 1999; Mussmann *et al*., 2003). Primer and probe bias cannot be completely ruled out, but our findings suggest, for example, that *Thiomicrospira* species are rare in the investigated intertidal flat. Accordingly, previous MPN counts revealed low abundances of 10^3–10^4 *Thiomicrospira* cells per gram of tidal sediment (Brinkhoff *et al*., 1998).

Uncultured *Gammaproteobacteria* that are not closely related to the above mentioned organisms have been hypothesized to oxidize sulfur in marine sediments based on their 16S rRNA phylogenetic relationship to other uncultured SOP (Ravenschlag *et al*., 2001; Bowman *et al*., 2003). In our study, we recovered many gammaproteobacterial 16S rRNA gene clones (Fig. S1), which were reflected in a high *in situ* abundance of *Gammaproteobacteria* of up to 30% (Fig. 3). In addition, even though the applied primers have a broad target range, the *dsrAB* and *aprA* gene libraries indicated a highly diverse community of uncultured, mainly gammaproteobacterial SOP. In particular, the phylogenies of 16S rRNA, DsrAB and AprA were congruent for several sequences that were related to symbiotic SOP such as *Candidatus* Thiobios zoothamnicoli, to symbionts of *O*. *algarvensis* and to symbionts of *O*. *haakonmosbiensis* and *O*. *mashikoi*. Most of the related symbiotic SOP were presumed or were shown to assimilate CO_2 autotrophically (Rinke *et al*., 2006; Woyke *et al*., 2006; Scott and Cavanaugh, 2007; Loesekann *et al*., 2008; Robidart *et al*., 2008). Therefore, we hypothesized that the gammaproteobacterial SOP in the tidal sediments may also incorporate CO_2.

To test this hypothesis that was solely based on phylogenetic affiliation we studied CO_2 incorporation into single cells by MAR–FISH. This provided evidence for the lithoautotrophic metabolism, which could be powered by sulfur oxidation in *Gammaproteobacteria*. We percolated sediment columns with artificial seawater that was spiked with ^{14}C-labeled bicarbonate but free of alternative electron donors. Although not very likely, we cannot completely exclude that yet unidentified autotrophic groups such as hydrogen, ammonia or metal oxidizers contributed to CO_2 fixation. However, sequences related to gammaproteobacterial autotrophs other than SOP like ammonia-oxidizing *Nitrosococcus* were rarely found. Accordingly, these organisms were not detectable by FISH (Table 1). Therefore, most likely sulfide produced by sulfate reduction was the major available electron donor in the experiments. In addition, heterotrophic CO_2 fixation was also not likely to account for the majority of the observed carbon uptake, since the cell-specific uptake rates of up to 45 amol cell^{-1} h^{-1} resembled those of other uncultured autotrophic SOP. For comparison, the gammaproteobacterial symbionts of *O*. *algarvensis* assimilated 1–100 amol carbon cell^{-1} h^{-1} (C. Bergin, pers. comm.). Freshwater phototrophic SOP were shown to incorporate 5–20 amol carbon cell^{-1} h^{-1} (Musat *et al*., 2008) and SOP in pelagic redox clines of the Baltic Sea assimilated 35–78 amol carbon cell^{-1} h^{-1} (Jost *et al*., 2008). Moreover, we tested by MAR–FISH,

whether cells of typical heterotrophic bacteria were $^{14}CO_2$ positive. Members of *Planctomycetales* and the *Roseobacter* lineage did not incorporate $^{14}CO_2$. This observation further indicated that the incorporation by the microbial community is autotrophic rather than a heterotrophic, anapleurotic CO_2 incorporation.

In summary, the consistent phylogenetic affiliation of the recovered sequences with gammaproteobacterial, autotrophic SOP was reflected in a high proportion of CO_2-incorporating *Gammaproteobacteria* in surface sediments. Therefore, it is highly suggestive that the observed CO_2 incorporation among *Gammaproteobacteria* was coupled to chemolithotrophic sulfur oxidation.

Novel sulfur-oxidizing populations

We collected strong evidence for a chemolithoautotrophic sulfur-oxidizing potential of the WS-Gam209 group, which is highly abundant in the Janssand intertidal sediments. The congruent phylogenies of DsrAB, AprA and 16S rRNA suggested their presence in the same group of organisms including symbionts of *Oligobrachia* spp. and provided cumulative evidence for a sulfur oxidation potential, although the functional and 16S rRNA genes could not be directly linked. Moreover, the WS-Gam209 cells incorporated $^{14}CO_2$, which indicated an autotrophic physiology as it was suggested for many uncultured chemolithotrophic, symbiotic SOP (Rinke *et al*., 2006; Woyke *et al*., 2006; Loesekann *et al*., 2008). Additional support for the chemolithoautotrophic sulfur-oxidizing potential of free-living relatives of *Oligobrachia* spp. was obtained from the sulfur precipitate layer in the oxygen–sulfide gradient culture (Fig. S3). Since the DsrAB gene library was dominated by a single OTU that was related to the symbionts of *O. haakonmosbiensis*, the sulfide apparently stimulated growth of this population over other rDSR-carrying SOP present in the inoculum. In the surface sediments this population reached abundances of up to 10^8 cells ml^{-1}, exceeding previous MPN-based counts of SOP in tidal sediment estimates by factor 1000 (Brinkhoff *et al*., 1998). A preliminary biogeography survey (Fig. S5) indicated a widespread occurrence of relatives of the WS-Gam209 group in other sediments. Accordingly, free-living forms of the *O. mashikoi* endosymbiont A-related phylotype accounted for up to 9% of bacterial rRNA gene copies in the worm-inhabited and adjacent coastal sites (Aida *et al*., 2008). Despite the grouping of many sequences with symbiotic SOP we do not claim any origin of these populations from such invertebrate hosts, as the public sequence databases are biased to well-characterized, symbiotic SOP. The recovered sequences rather originate from members of a larger phylogenetic group that comprises free-living and symbiotic SOP. In addition, no symbiont-bearing invertebrates have been found in our tidal sediments.

The WS-Gam1030 population also incorporated $^{14}CO_2$. For this group, which was distantly related to the free-living *T. thiocyanodenitrificans* no clearly related DsrAB sequences were found in our data set. Also for the population targeted by probe WS-Gam446 we currently lack further indication for thiotrophy, besides the fact that it is widespread in sulfidic habitats (Fig. S5). Cells of the NOR5/OM60 clade did not significantly incorporate $^{14}CO_2$ and the sulfur oxidation potential of members of this clade is not clear (Fuchs *et al*., 2007; Yan *et al*., 2009). Most $^{14}CO_2$-positive *Gammaproteobacteria* (Fig. S6I–N) could not be identified by the applied probes.

Interestingly, we also retrieved DsrAB sequences that are related to unknown, alphaproteobacterial organisms from the Namibian upwelling zone (Lavik *et al*., 2009). Currently, a more precise identification of these phylotypes is hampered by the limited resolution of the phylogenetic backbone of known rDSR-carrying SOP. In our libraries no 16S rRNA gene sequences of typically cultured, alphaproteobacterial SOP were recovered. However, sequences affiliated with marine, mostly planktonic *Roseobacter*-lineage representatives were sporadically found in the 16S rRNA gene libraries. Marine *Roseobacter* are predominantly known for degradation of organo-sulfur compounds (Wagner-Doebler and Biebl, 2006) and have not yet been shown to carry rDSR genes. Since inorganic sulfur-oxidizing members of the *Roseobacter* lineage have been isolated from pelagic habitats (Sorokin *et al*., 2005), further studies on this group should be performed.

Biological sulfur oxidation potential

Al-Raei and colleagues (2009) proposed that re-oxidation of sulfides is at least partially catalysed by metal oxides in the Janssand sediments. However, the high abundance of candidate SOP and CO_2-incorporating *Gammaproteobacteria* found in this study may point to a more vital role of biological sulfur oxidation. To estimate this biological sulfur oxidation capacity we compared the *in situ* sulfide flux with the abundance of candidate gammaproteobacterial SOP. Based on microsensor profiles obtained during a joint sampling campaign in April 2005 (Jansen *et al*., 2009) we calculated a sulfide flux of 1.9 mmol m^{-2} day^{-1}, which is comparable to fluxes reported for other marine sediments (Bruechert *et al*., 2003; Preisler *et al*., 2007; Nielsen *et al*., 2010). To calculate the potential cell-specific sulfide oxidation rates we conservatively estimated a mixed population of CO_2-incorporating *Gammaproteobacteria* accounting for 10% of all cells (Fig. 5). These were considered as candidate SOP for our calculation. Based on these assumptions their abundance of 3.3×10^8 SOP cells ml^{-1} in the sulfide oxidation zone of the sediment (Fig. 3) would correspond to a mean cell-specific sulfide oxidation rate of 3.7 fmol $cell^{-1}$ day^{-1}. This is comparable to *in situ* sulfur oxidation rates of 1.1 fmol $cell^{-1}$ day^{-1} of a community of *Gamma*- and *Epsilonproteobacteria* inferred from Lavik and colleagues (2009) and rates of 7 fmol $cell^{-1}$ day^{-1} calculated for a pure culture (Gevertz *et al*., 2000). In turn, a single population of approximately 10^8 cells ml^{-1} such as the WS-Gam209 group would explain approximately 10% of sulfide removal assuming an oxidation rate of 1.1 fmol sulfide $cell^{-1}$ day^{-1} (inferred from Lavik *et al*., 2009). Indeed, recent findings of Nielsen and colleagues (2010) illustrated that biological sulfur oxidation potential in marine sediments appeared to be underestimated so far. They proposed that SOP may use conductive solids as primary electron acceptor instead of oxygen to thrive in oxygen-free sediment layers. In addition, nitrogen oxides were present in the upper layers of the sediment (Gao *et al*., 2009), which might have served as alternative electron acceptors. It is intriguing to speculate whether the detected autotrophic SOP in our study could be able to compete with metal-catalysed sulfide oxidation in order to reach such high abundances.

Conclusion

Here, we provide the first evidence that non-filamentous *Gammaproteobacteria* are possibly important catalysts of microbial sulfur oxidation and inorganic carbon fixation in marine sediments. Jørgensen and Nelson (2004) proposed that sulfur-oxidizing bacteria, in particular autotrophs, are of low importance in

marine sediments. In contrast, we found that CO_2-incorporating candidate SOP were highly abundant and might be more important in general sulfur oxidation than previously assumed.

In summary, our findings provide a basis for more detailed studies on the chemolithoautotrophic potential of SOP in marine surface sediments, which are – as the majority of the seafloor – devoid of mat-forming, filamentous sulfur bacteria. Despite a generally high diversity of SOP distinct clades such as WS-Gam209 and WS-Gam1030 may be main catalysts of biological sulfur oxidation, not only in tidal but also in other marine sediments. Future studies should investigate how these organisms are able to compete with chemical oxidation and if they have a similar ecological significance in sulfide detoxification as chemolithotrophic bacteria in pelagic oxygen minimum zones (Lavik *et al*., 2009; Walsh *et al*., 2009). Finally, it should be considered to what extent SOP contribute to primary production in marine sediments. Here, their role as carbon sink deserves further attention.

Experimental Procedures

Field site and sampling

The investigated intertidal sand flat is situated in the backbarrier tidal area of the German Wadden Sea (53°43'N, 07°41'E). The study site and sediment characteristics are described in detail by Billerbeck and colleagues (2006) and Jansen and colleagues (2009). Sampling focused on the transition zone of O_2, NO_3^- and H_2S in the upper 0–3 cm, in which sulfide was oxidized. Samples for different experiments such as gene libraries, FISH and CO_2 incorporation experiments were collected at low tide in November 2004 and 2005, April 2005, October 2006, March and August 2007, October 2008 and June 2009. For DNA extraction sediment was frozen immediately or upon arrival in the laboratory. For fluorescence *in situ* hybridization (FISH) two adjacent cores were taken in April 2005 (0–6 cm, after a storm), March 2007 and August 2007 (0–12 cm). The replicate cores were sliced into 1 cm sections, fixed in 1.8% formaldehyde at 4°C overnight, washed twice with PBS and stored in PBS: ethanol (2:3) at −20°C. To detach cells from sediment particles ultrasonic treatment was performed on ice for 7 × 30 s (amplitude 30%, pulse 20) including 6 × 30 s break intervals using a SonoPlus ultrasonic probe (Bandelin Electronic, Berlin, Germany). The profiles of oxygen and hydrogen sulfide concentrations next to the sampled sediment were measured during the sampling campaign in March/April 2005 and published by Jansen and colleagues (2009).

16S rRNA gene libraries and phylogenetic analysis

As spatial and seasonal fluctuations of bacterial diversity can bias gene library composition the sediments were sampled three times, including the oxygen–sulfide transition zone close to the sediment surface. To assess the diversity of the bacterial community the sediment was sampled in November 2004 (0–2 cm sediment depth), April 2005 (0–3 cm) and in March 2007 (0–12 cm). DNA was extracted from surface sediment samples following the protocol of Zhou and colleagues (1996). Clone libraries were generated as described by Mussmann and colleagues (2005a) using universal bacterial primers GM3F/GM4R (Muyzer *et al*., 1995). Amplified PCR products were cloned with the TOPO TA Cloning Kit for sequencing (pCR4-TOPO, Invitrogen, Karlsruhe, Germany) and sequenced with the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California) according to the manufacturers' instructions. Initially, partial sequences (> 600 bp) were analysed with the ARB software (Ludwig *et al*., 2004) using the

SILVA 16S rRNA SSU Reference database release 89 (Pruesse *et al*., 2007). Representative gammaproteobacterial clones were chosen for nearly full-length sequencing (> 1400 bp). Sequences were checked for chimera formation using the Bellerophon server (Huber *et al*., 2004). For tree reconstruction 650 nearly full-length gammaproteobacterial sequences, including 161 sequences originating from the three different clone libraries of this study, were included. Some sequences that have been recovered from a clone library of silty tidal sediment were included in the analysis (Mussmann *et al*., 2005a). Sequences were classified into OTUs based on 97% sequence identity using the ARB similarity matrix and dotur (Schloss and Handelsman, 2005). The final phylogenetic reconstructions were calculated with the ARB software and were based on the SILVA SSU Reference database release 100 (Pruesse *et al*., 2007). Trees were constructed using the maximum-likelihood methods ARB PhyML and RAxML (JTT substitution matrix) (Stamatakis *et al*., 2008) employing 50% nucleotide conservation filters for *Bacteria* and *Gammaproteobacteria*.

dsrAB libraries

Clone libraries of the rDSR gene were established from oxic and anoxic sediment layers of April 2005 (0–3 cm) and November 2004 (8–9 and > 19 cm). The newly designed primers rDSRA240F and rDSRB808R amplified a 2 kb *dsrAB* fragment including the alpha and beta subunits of rDSR (Table S2). PCR reactions contained 50 pmol of each primer, 6.25 nmol of each dNTP, 1× Master *Taq* Buffer and 1 U of *Taq* DNA Polymerase (Eppendorf, Hamburg, Germany) and were adjusted to a final volume of 25 µl. Thermocycling included an initial denaturation for 4 min at 95°C, followed by 30 cycles of 30 s at 95°C, annealing for 30 s at 54°C and elongation for 3 min at 72°C. A final elongation for 10 min at 72°C was included. To cover a maximum diversity of *dsrAB* sequences an additional clone library was established with a previously published primer set (Loy *et al*., 2009) using DNA of the April 2005 surface sample (0–3 cm). For a better resolution of the DsrAB phylogeny the *dsrAB* gene was amplified, cloned and sequenced from the endosymbiont of the siboglenoid tubeworm *O*. *haakonmosbiensis* (Loesekann *et al*., 2008). Sequences were imported in a *dsrAB*/DsrAB-ARB database (http://www.microbial-ecology.net). Phylogenetic trees for the deduced amino acid sequences were constructed with the maximum-likelihood RAxML (JTT substitution matrix) (Stamatakis *et al*., 2008) and the Bayesian inference method (mixed amino acid and gamma-distributed rate model) employing MRBAYES (Huelsenbeck and Ronquist, 2001) with an insertion/deletion filter. In total 190 full-length DsrAB amino acid sequences (comprising 550 amino acid positions) representing all currently published and sequences recovered in this study were included. Both methods resulted in nearly identical tree topologies. The consensus tree was constructed where branching patterns could not be unambiguously resolved. OTUs for DsrAB sequences were defined based on tree topology and a 90% sequence identity cut-off (Loy *et al*., 2009).

aprA library

A clone library for the alpha subunit (*aprA*) of the adensosine-5'-phosphosulfate (APS) reductase gene was established according to Meyer and Kuever (2007a) using DNA extracted from 5–12 cm sediment collected in 2003 (Mussmann *et al*., 2005b). A RAxML analysis of 151 full-length AprBA database sequences (comprising 214 amino acid positions) was performed using an insertion/deletion filter. Altogether 66 partial AprA sequences (119 amino acid positions) from this study were added to the tree

by applying the ARB parsimony quick add criterion, without changes in the overall tree topology. OTUs for AprA were defined based on tree topology and a 90% sequence identity cut-off.

Probe design, optimization and quantification of bacterial cell numbers and population sizes

Oligonucleotide probes were designed using ARB and the SILVA 16S rRNA SSU Reference database release 89. The specificity of the novel probes was checked against the SSU Parc database release 89 and repeated against the SSU Reference database release 100 (see http://www.arb-silva.de) using the 'probe match' tool of ARB. In addition to the targeted Janssand OTUs closely related sequences from a variety of sites, particularly sulfidic sediments were also covered (see also the NCBI 16S rRNA gene database http://www.ncbi.nlm.nih.gov/entrez/).

Initial hybridizations with newly designed probes (Table 1) were performed with Cy3-labeled oligonucleotides in order to check for positive signals of the targeted gammaproteobacterial populations in the environment. Hybridizations were performed as described by Llobet-Brossa and colleagues (1998) for 3 h and overnight using a formamide concentration of 0–10%. We used extended hybridization times in order to increase hybridization efficiency (Yilmaz and Noguera, 2004). Samples from different seasons and depths were checked for hybridized cells. Signals were considered to be significant when cells were detected repeatedly and uniformly over the filter and exceeded counts with the negative control NON338. Subsequently, the hybridization stringency of selected probes WS-Gam209, WS-Gam446 and WS-Gam1030 was optimized by Clone-FISH (Schramm *et al*., 2002) using full match and mismatch clones (Table S3). Formamide concentrations were defined as stringent when discrimination against the mismatch clone was ensured. In addition, CARD-FISH with increasing formamide concentrations (0–70%) was performed on sediment samples to determine signal cut-off. CARD-FISH was performed as described by Ishii and colleagues (2004) including a standard hybridization time of 3 h for all hybridizations. Counts were corrected by subtracting values obtained with negative control NON338. Probes, competitors and helpers and the corresponding formamide concentrations applied in this study are listed in Table 1.

Dark CO_2 fixation and MAR–FISH

For measurement of dark CO_2 fixation intact sediment cores were sampled in the field and kept at *in situ* temperature (October 2006). Incubations were started in the laboratory within 3 h after sampling. No external sulfur compound was added to the incubations as sulfide arising from the activity of sulfate reduction in anoxic sediment layers and stored elemental sulfur/polysulfides were intended to fuel dark CO_2 fixation by SOP in the incubations. Two intact sediment cores were percolated (Gao *et al*., 2009) with 50 ml of oxic artificial sea water (ASW, pH 8, without ammonium) containing 1 mM [^{14}C] bicarbonate (specific activity, 47.8 mCi mmol^{-1}; Amersham, USA) to displace natural pore water. After homogenous perfusion of the ASW–tracer mix throughout the core percolation was stopped. In addition, the ASW–tracer mix overlaid the sediment surface by 1 cm. Cores were incubated at an *in situ* temperature of 10°C for 36 h in the dark. Subsequently the [^{14}C] bicarbonate was eliminated from the cores by percolating 2 × 50 ml of ASW, respectively, through the sediment, followed by slicing of the core into 1 cm layers. From each layer 0.5 ml was fixed for FISH as described above. For bulk CO_2 fixation 1.5 ml of each layer of core 1 was mixed immediately with the scintillation cocktail (Ultima *Gold* XR, Packard). For core 2, 1.5 ml per layer was incubated for 36 h in 5% trichloracetic acid to remove residual, unincorporated [^{14}C]

bicarbonate prior to addition of scintillation cocktail. The incorporated radioactivity of the individual sediment layers was assessed by a liquid scintillation counter (Tri-Carb 2900 Packard, Perkin Elmer, USA). Dark CO_2 fixation rates were calculated based on the total inorganic carbon content added to the sample, the amount of [^{14}C] bicarbonate added and the amount of [^{14}C]-labeled dissolved inorganic carbon (DIC, ΣCO_2) fixed into biomass during the incubation time.

In June and October 2009, 1 ml portions of sediment from the oxic–anoxic transition zone of 1–2 cm depth were transferred to glass vials and incubated with 1 ml of sterile filtered oxic seawater containing 1 mM [^{14}C] bicarbonate (specific activity, 44.7 mCi mmol^{-1}; Amersham, USA) for 24 h in the dark under mild agitation to facilitate oxygen penetration into the sediment. Incubations were performed in duplicates (June 2009) or triplicates (October 2009). From the resuspended sediment 0.5 ml was fixed for FISH. The sediment samples were then sonicated as described above to detach cells from the grains. The amount of incorporated ^{14}C carbon was quantified in 10 µl of supernatant after sonication by liquid scintillation counting and was corrected by the scintillation counts of the prefixed, dead controls (negative controls). The abundance of [^{14}C] carbon/DIC-incorporating *Bacteria*, *Gammaproteobacteria* and specific populations with visible substrate uptake were determined following the MAR–FISH protocol of Alonso and Pernthaler (2005). Subsamples were diluted to ensure an even distribution of maximum 20–30 cells per microscopic field on the filter. To determine the fraction of $^{14}CO_2$-incorporating cells of the total microbial community cells were stained with the DNA stain SYBR Green I (Molecular Probes, USA). Exposure times of 7 (core) and 4 (vial) days were considered as optimal. In sediment that was fixed with formaldehyde prior to incubation (negative control), no 'false positive' cell-associated silver grain precipitation could be detected. To assess the proportion of $^{14}CO_2$-positive cells relative to the total microbial community at least 1000 SYBR Green stained cells and the number of corresponding MAR–FISH signals were counted. Approximately 300 MAR signals and the corresponding probe-specific signals were counted to assess the fraction of substrate assimilating *Gammaproteobacteria*. For the October 2009 incubations dual colour hybridizations were performed with probes EUBI-III/GAM42a and EUBI-III/WS-Gam209 as described by Pernthaler and Amann (2004). All preparations were inspected manually under an Axioplan epifluorescence microscope (Zeiss, Jena, Germany) at 1000× magnification.

Oxygen–sulfide gradient cultures

Oxygen–sulfide gradient cultures were established according to the protocol of Nelson and Jannasch (1983) and Kamp and colleagues (2006) with the following specifications: 8 ml top agar containing artificial seawater supplemented with 100 µM $NaNO_3$ and 2 mM $NaHCO_3$ overlaid 4 ml of sulfidic bottom agar containing 16 mM Na_2S. The calculated flux of sulfide from the bottom agar established a concentration of 1 mM sulfide in the oxic transition zone of the upper cm of top agar. In June 2009, 0.5 ml of sediment from the oxic–anoxic transition zone of 2–3 cm depth was sampled and diluted with 200 µl of sterile seawater. After mild sonication on ice (amplitude 10% for 30 s, pulse 20) 10 µl of sediment slurry was inoculated into the top agar and incubated in the dark at room temperature. After 2 weeks the zone of visible bacterial biomass accumulation and sulfur precipitation was sampled for subsequent molecular analysis. Clone library construction for the rDSR gene was conducted as described above. Amplification of *dsrA* was performed with the primer pair rDSRA240F and rDSRB403.

Thermocycling included an initial denaturation for 4 min 95°C, followed by 30 cycles of 30 s at 95°C, annealing for 60 s at 55.5°C and elongation for 3 min at 72°C.

Flux calculation

The average sulfide flux within the sediment was calculated based on HS^- and S_2^- profiles recovered during a joint sampling campaign in March/April 2005 during low tide (Jansen *et al*., 2009, fig. 10). The flux (J) was calculated according to Fick's law J = −DdC/dz. D represents the molecular diffusion coefficient of sulfide in the sediment at a temperature of 6°C, 32‰ salinity and a sediment porosity of 0.45 according to $D = 1.2996 \times 10^{-5}\ cm^{-2}\ s^{-1} \times 0.75 \times 0.45$. The factor dC/dz represents the vertical concentration gradient of dissolved sulfide, corresponding to 0.68 mol m^{-3} over a vertical distance of 1.5 cm. The mean cell-specific sulfide oxidation rate (R) was calculated according to the equation $R = J/A \times \text{cell abundance}^{-1}\ cm^{-3}$. Here, A equals the thickness of the active sulfur-oxidizing layer of 4 mm based on the given sulfide profile. The cell abundance of SOP in this zone was conservatively estimated based on the relative abundances of $^{14}CO_2$-incorporating *Gammaproteobacteria* (Fig. 5) and their mean total abundance in the top 3 cm in April 2005 (Fig. 3).

Nucleotide accession numbers

Nucleotide accession numbers are available at GenBank under HQ190958–HQ191103 for 16S rRNA gene sequences, HQ191171–HQ191216 for dsrAB sequences and HQ191104–HQ191170 for aprA sequences.

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Supporting Information

Figure S1 Summarized taxonomic composition of three 16S rRNA gene libraries.

Figure S2 Rarefaction curves of *dsrAB*, *aprA* and gammaproteobacterial 16S rRNA gene sequences recovered from Janssand sediment.
A coverage of 79% for the 16S rRNA, 78% for *dsrAB* and 67% for the *aprA* gene library was calculated according to C = 1 − n/N (Good, 1953) indicating the extent to which the actual species richness is reflected in the libraries.

Figure S3 Agar enrichment tubes with opposing gradients of oxygen and sulfide.
A–C. Tubes inoculated with surface sediment after 2 weeks of incubation in the dark; note the layer of sulfur precipitates few millimetres below the agar surface. D. Control tube, not inoculated.

Figure S4 Consensus tree based on maximum-likelihood (RAxML) of AprA amino acid sequences recovered from the intertidal sediments of Janssand.
Single OTUs are represented by selected clones ('Wadden Sea sediment clone'), 'n' equals numbers of sequences per OTU. Gammaproteobacterial OTUs that affiliated with known sulfur oxidizers are shown in bold. The sequence cluster related to endosymbionts of Oligobrachia spp. is highlighted. Circles indicate lineages with > 70% (closed) and > 50% (open) RAxML bootstrap support. The bar indicates 10% sequence divergence. Unc, uncultured organisms; Sym, symbionts; Tht, *Thiotrichaceae*; Chr, *Chromatiaceae*; Ect, *Ectothiorhodospiraceae*; SAR, SAR11 clade.

Figure S5 Geographic locations where sequences or organisms targeted by probes WS-Gam209 (▲), WS-Gam446 (●) and WS-Gam1030 (■) have been detected in the SILVA SSU Reference database release 100 (green) and by CARD-FISH (red) respectively. In silty intertidal sediments of a second site in the Wadden Sea (Sylt 55°1'31.584"N, 8°25'54.1194"E) single cells were detected by probes WS-Gam1030 and WS-Gam446. Similarly, populations WS-Gam446, WS-Gam1030 and WS-Gam209 were in detected in abundances comparable to those at Janssand site in non-tidal, sandy sediment from the German North Sea (Helgoland Island, 54.182917°N, 7.902617°E). Here, counts averaged 1.2%, 2.3% and 2.4% of all cells respectively. Moreover, the groups were present in sediment from Hydrate Ridge, Cascadia Margin (Knittel *et al*., 2003). In addition, probe WS-Gam446 hybridized to cells from the Logatchev hydrothermal vent field (site Anyas Garden, 14°45'174"N, 44°58'768" W) and arctic coastal sediment from Svalbard (Ravenschlag *et al*., 2001). **B**. Epifluorescence microscopy images of the widely distributed WS-Gam446 group at site Logatchev Hydrothermal vent field (I), Hydrate Ridge (II) and Svalbard (II). Green: Alexa 488-conferred probe signal; blue: DAPI-stained DNA.

Figure S6 Epifluorescence microscopy images of $^{14}CO_2$-incorporating cells revealed by microautoradiography combined with FISH.
Green (Alexa 488): fluorescence-conferred signal of probes targeting different gammaproteobacterial populations; red (Alexa 594): Bacteria (probe EUBI-III); blue, DAPI-stained DNA; black, precipitation of silver grains indicating substrate incorporation. Arrows indicate $^{14}CO_2$-positive/incorporating cells. (A–C) Probe WS-Gam209-targeted cells related to symbionts of *Oligobrachia* spp.; (D) probe WS-Gam1030-targeted cells related to *Thioalkalivibrio thiocyanodenitrificans*; (E and F) probe GAM42a-targeted *Gammaproteobacteria* displaying no significant $^{14}CO_2$ uptake; (G and H) dead control without silver grain precipitation; (I–M) probes GAM42a (*Gammaproteobacteria*) and EUBI-III (Bacteria). The scale bars correspond to 5 µm.

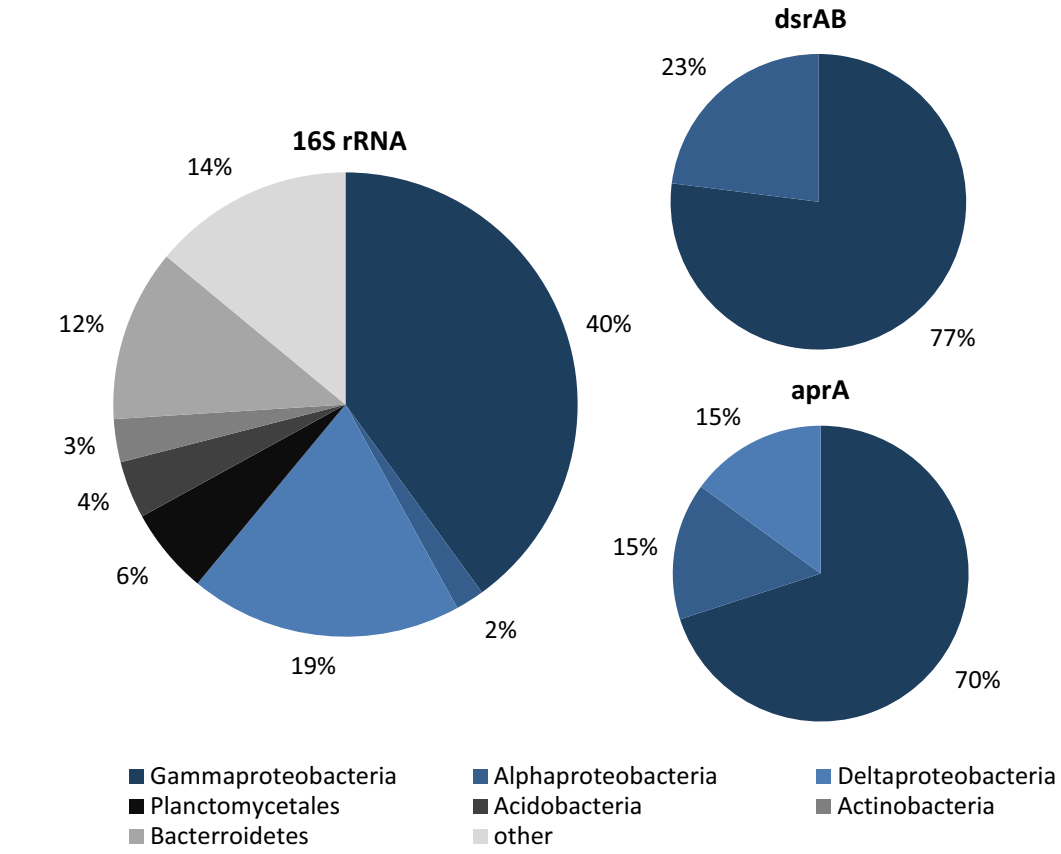


Figure S1

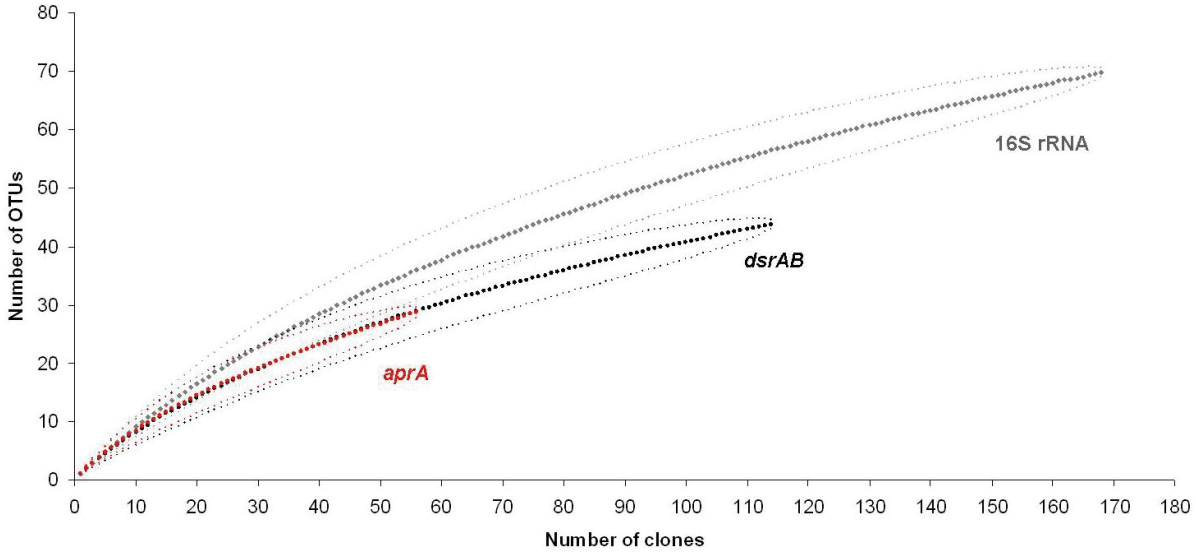


Figure S2

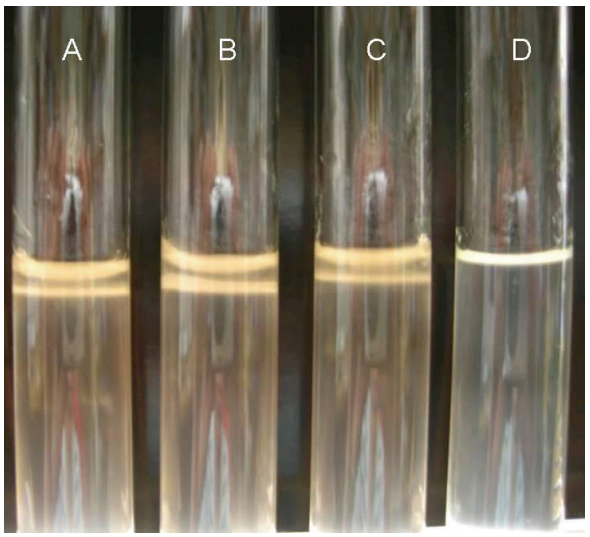


Figure S3

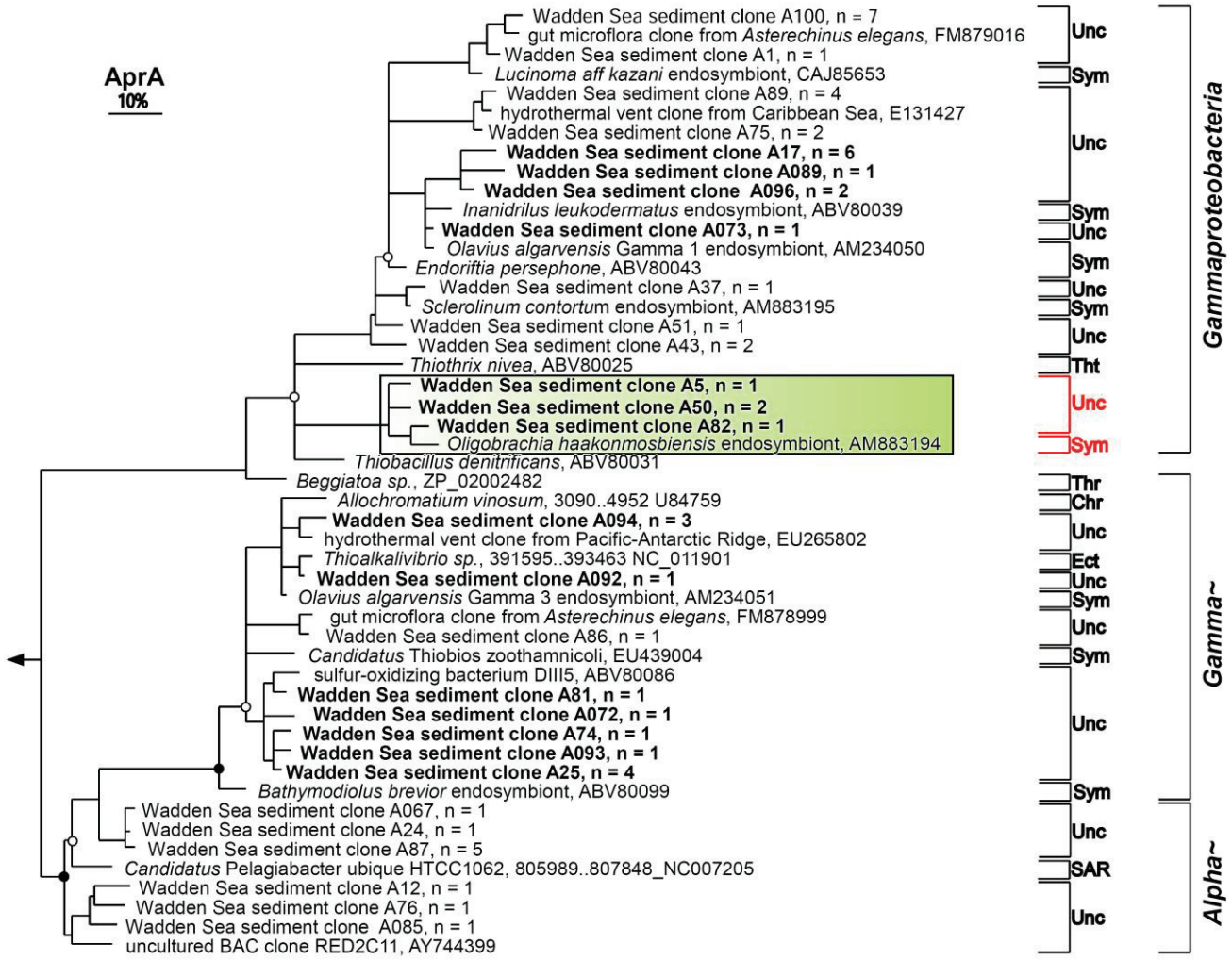
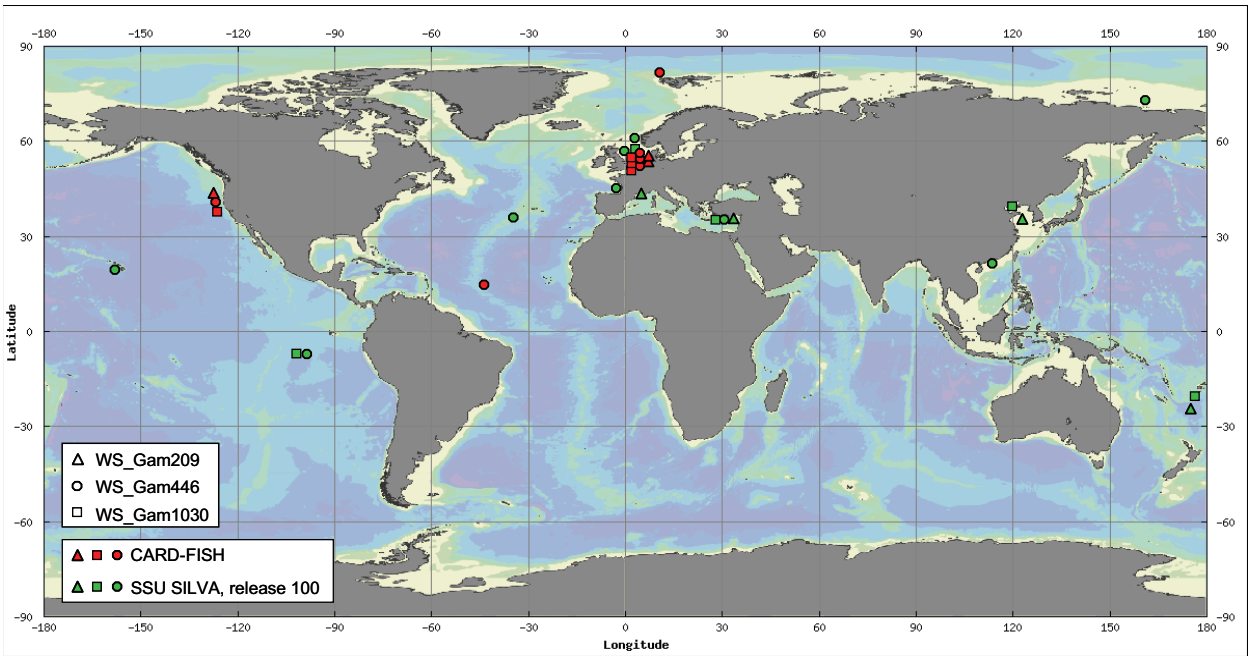


Figure S4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WS_Gam446
WS_Gam1030
CARD-FISH
SSU SILVA, release 100

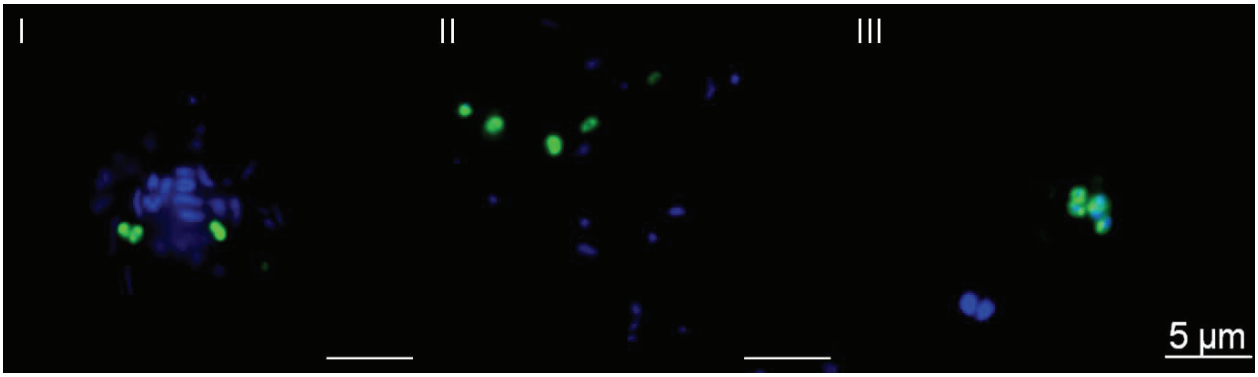
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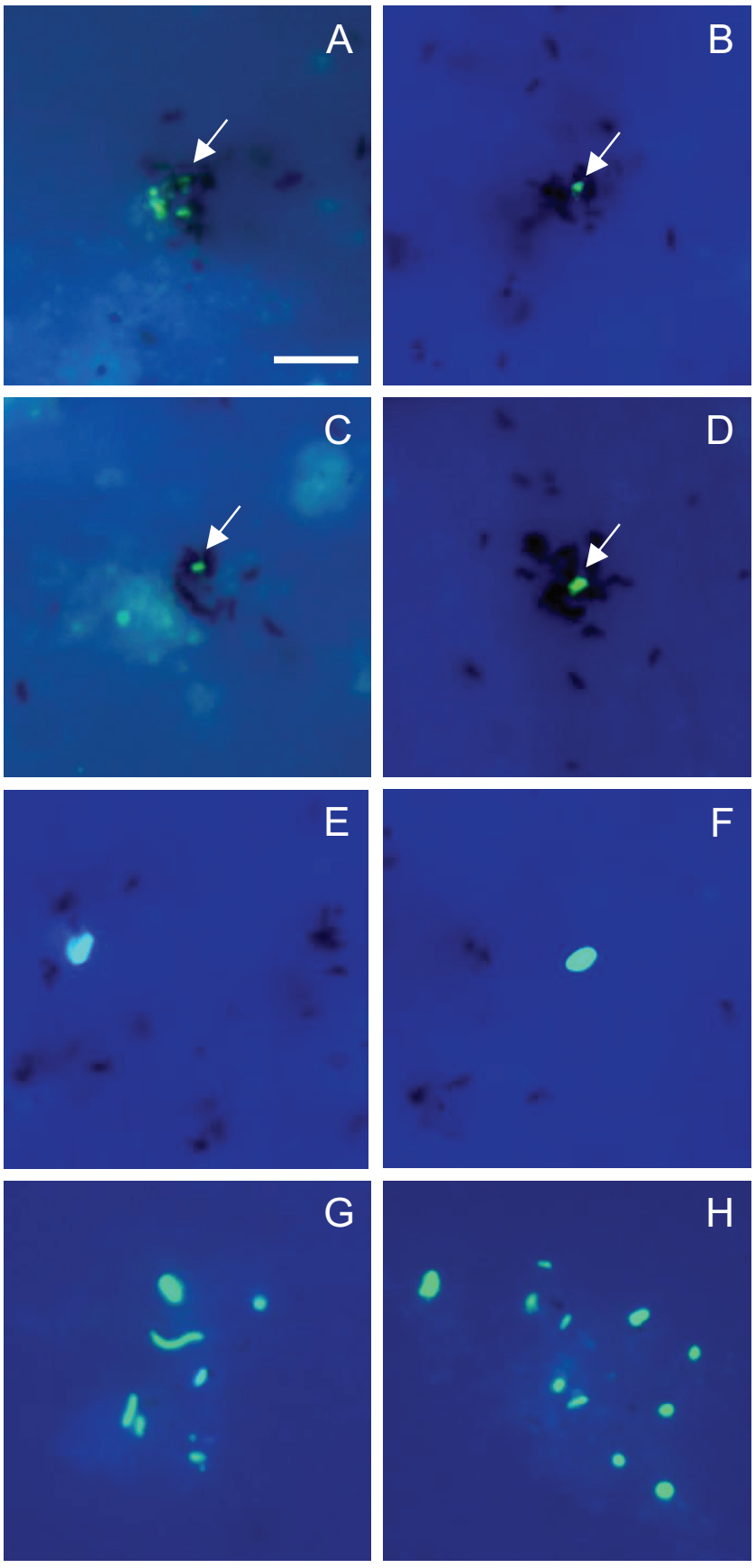
Figure S5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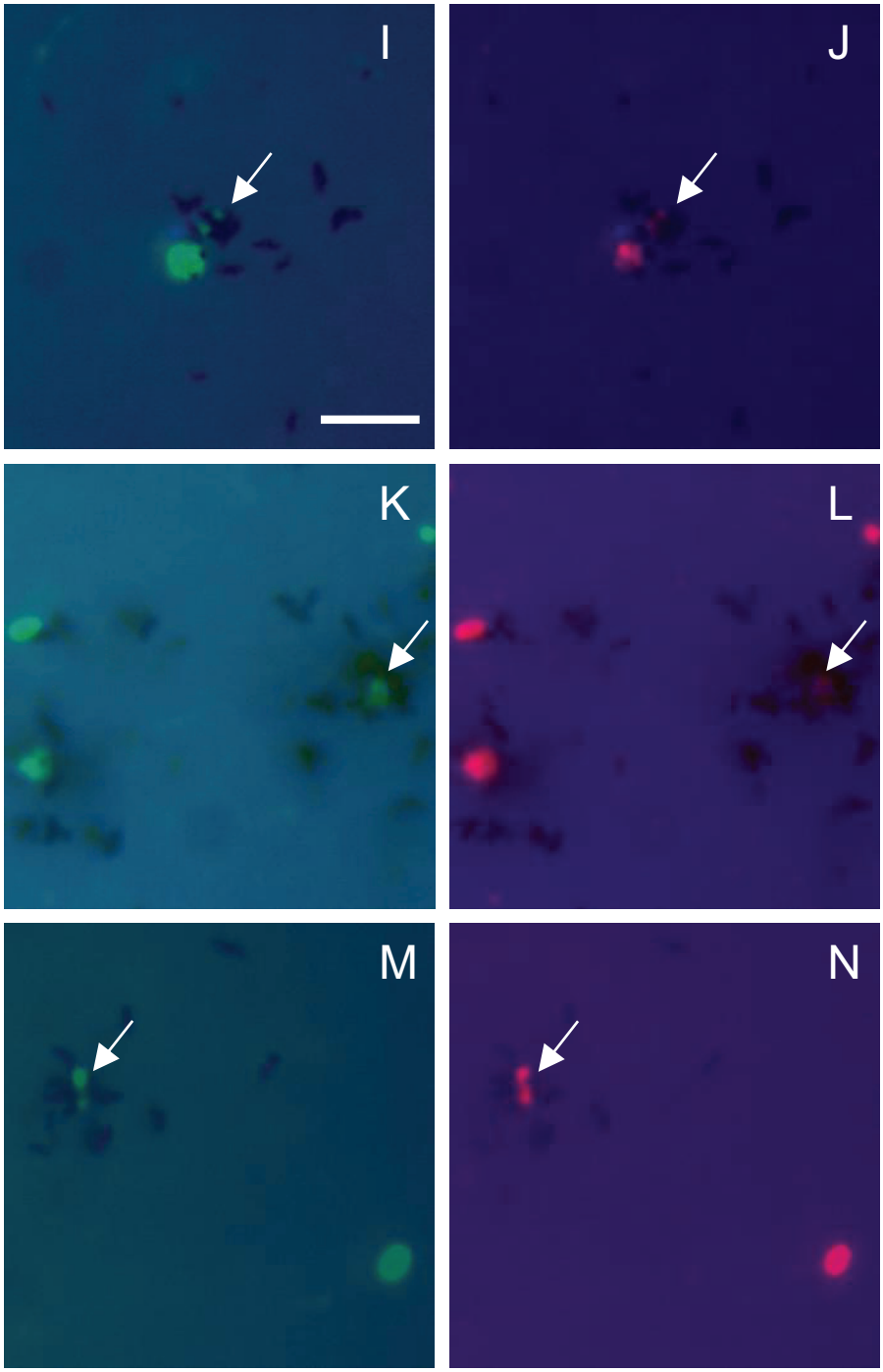

Figure S6

Table S1 Taxonomic affiliation of 16S rRNA sequences related to known sulfur-oxidizing bacteria

Representative sequence for OTU	Sequences[a]	Closest related sulfur-oxidizing bacterium[b]	SI (%)[c]	Probe[d]
Wadden Sea sediment clone 147	1	*Olavius algarvensis* Gamma 1 symbiont, AF328856	95	
Wadden Sea sediment clone 308	2	*Solemya velum gill symbiont, M90415*	93	
Wadden Sea sediment clone 321	8	*Candidatus* Thiobios zoothamnicoli, AJ879933*	94	WS-Gam830
Wadden Sea sediment clone 333	2	*Candidatus* Thiobios zoothamnicoli, AJ879933*	95	
Wadden Sea sediment clone WS202	9	*Oligobrachia haakonmosbiensis* endosymbiont, AM883179*	90	WS-Gam209
Wadden Sea sediment clone 419	2	*Oligobrachia haakonmosbiensis* endosymbiont, AM883179*	90	WS-Gam843
Wadden Sea sediment clone WS229	1	*Oligobrachia haakonmosbiensis* endosymbiont, AM883179*	91	WS-Gam843
Wadden Sea sediment clone 522	2	*Oligobrachia haakonmosbiensis* endosymbiont, AM883179*	91	WS-Gam843
Wadden Sea sediment clone 336	1	*Oligobrachia haakonmosbiensis* endosymbiont, AM883179*	90	WS-Gam843
Wadden Sea sediment clone WS114	5	*Olavius algarvensis* Gamma 3 symbiont, AJ620496*	98	WS-Gam832
Wadden Sea sediment clone WS329	1	*Olavius algarvensis* Gamma 3 symbiont, AJ620496*	93	
Wadden Sea sediment clone 530	1	*Olavius algarvensis* Gamma 3 symbiont, AJ620496*	91	
Wadden Sea sediment clone 371	2	*Thyasira flexuosa* gill symbiont, L01575	93	
Wadden Sea sediment clone 404	1	*Thyasira flexuosa* gill symbiont, L01575	94	
Wadden Sea sediment clone WS148	2	hydrothermal vent strain NDII1.1, AF170424	93	
Wadden Sea sediment clone WS183	3	*Thioalkalivibrio thiocyanodenitrificans*, AY360060	90	
Wadden Sea sediment clone WS293	3	*Thioalkalivibrio thiocyanodenitrificans*, AY360060	89	
Wadden Sea sediment clone WS299	10	*Thioalkalivibrio thiocyanodenitrificans*, AY360060	90	WS-Gam446
Wadden Sea sediment clone WS254	6	*Thioalkalivibrio thiocyanodenitrificans*, AY360060	89	WS-Gam1030
Wadden Sea sediment clone 526	2	*Thioalkalivibrio thiocyanodenitrificans*, AY360060	89	
Wadden Sea sediment clone 461	1	*Thioalkalivibrio thiocyanodenitrificans*, AY360060	89	

a. Operational taxonomic unit (OTU): sequences displaying 16S rRNA gene sequence identity of ≥97 %.
b. Based on Maximum Likelihood phylogenetic reconstruction and sequence identity (Fig. 1).
c. Sequence identity of 16S rRNA in %
d. Oligonucleotide probe applied for detection of the target populations
* Symbiotic sulfur-oxidizing bacteria of which related sequences were also detected in the *dsrAB* libraries.

Table S2 Primer sets used for PCR amplification of the *dsrAB* gene

Primer	Sequence[a] (5' - 3')	Target position[2]	T [°C] [c]	Reference
rDSRA240F[b]	GG**N**TA**Y**TGGAA**R**GG**N**GG	229 – 245	54	This study
rDSRB808R[b]	CC**D**CC**N**ACCCA**D**AT**N**GC	2121 – 2137	54	This study
rDSR1Fa	AA**R**GG**N**TA**Y**TGGAA**R**G	226 – 241	48	Loy *et al*., 2009
rDSR1Fb	TT**Y**GG**N**TA**Y**TGGAA**R**G	226 – 241	48	Loy *et al*., 2009
rDSR1Fc	ATGGG**N**TA**Y**TGGAA**R**G	226 – 241	48	Loy *et al*., 2009
rDSR4Ra	CC**R**AA**R**CA**I**GC**N**CC**R**CA	2058 – 2074	48	Loy *et al*., 2009
rDSR4Rb	GG**R**W**A**R**C**A**I**GC**N**CC**R**CA	2058 – 2074	48	Loy *et al*., 2009
for sequencing:				
rDSRB403F[b]	CA**Y**AC**N**CA**R**GG**N**TGG**Y**T	1725 – 1741	54	This study
rDSRB403R[b]	A**R**CCA**N**CC**Y**TG**N**GT**R**TG	1725 – 1741	54	This study
rDSR874F	TG**Y**ATGCA**Y**TG**YY**T**V**AA**Y**G	913 - 932	48	Loy *et al*., 2009

a. Primer sequences are indicated in UPAC nomenclature with degenerated positions highlighted in bold. Primer combinations used to amplify a *dsrAB* fragment include rDSRA240F – rDSRB808R (2000 bp), rDSRA240F – rDSRB403R (1600 bp), rDSRB403F – rDSRB403R (400 bp) and rDSR1Fa, b, c – rDSR4Ra, b (1900 bp)
b. Target site and numbering according to the nucleotide alignment position of *Chlorobium limicola* DSM245 (AAHJ01000040).
c. Annealing temperature

Table S3 Oligonucleotides and clones applied for probe optimization

Probe/Competitor[a]	Clone considered for probe optimization[b]	Sequence (5' - 3')	Mm[c]	Wm[c]	Reference
Gam209		**CTACTAGTGCCAGGTCCG**			
	Wadden Sea sediment clone 202*	GAUGAUCACGGUCCAGGC			This study
	Wadden Sea sediment clone S04 505	•••U••••••••••••••	1	1.3	This study
	Wadden Sea sediment clone 229*	•••U•••g••••••••••	2	1.6	This study
	Methylomonas sp. LW 15, AF150794	•••A•••g••••••••••	2	1.6	GenBank
cGam209_1		**CTAATAGTGCCAGGTCCG**			
cGam209_2		**CTAWTAGCGCCAGGTCCG**			
Gam446		**ACCCGCAACTGTTTCCTC**			
	Wadden Sea sediment clone WS299*	UGGGCGUUGACAAAGGAG			This study
	Hydrate Ridge sediment clone, AJ535226	•••••A••••••••••••	1	1.7	GenBank
	Wadden Sea sediment clone WS183	C••••AA••••••••••	3	3.4	This study
cGam446		**ACCCGTAACTGTTTCCTC**			
Gam1030		**CCTGTCAACCAGTTCCCG**			
	Wadden Sea sediment clone WS254*	GGACAGTTGGTCAAGGGC			This study
	Wadden Sea sediment clone WS202*	••••••••A•••••••••	1	1.9	This study
cGam1030		**CCTGTCAATCAGTTCCCG**			

a. Unlabeled competitor oligonucleotides applied for discrimination against non-target sequences
b. Full match and mismatch clones used for Clone-FISH and/or competitor design; full match clones are given in bold
c. Number of mismatches (mm) and indication of weighted mismatches (wm) according to ARB (Ludwig *et al*., 2004)
* Clones applied for optimization of probe stringency in Clone-FISH

3. Sulfur-Oxidizing *Roseobacter* Clade Bacteria in Coastal Sediments

Contributions to the study:

Sabine Lenk: screened metagenome library, performed in situ quantification of RCB, conducted 16S rRNA and soxB diversity studies, performed molecular characterization of the sulfidic enrichment culture, tested substrate spectrum of enriched RCB, assisted in the geneFISH experiment, performed data analysis and processing, developed the concept of the manuscript, wrote the manuscript

Cristina Moraru: designed, generated and tested *dsrA*-targeted polynucleotide probes, performed geneFISH experiment

Michael Richter: performed bioinformatic processing of metagenomic data

Michael Kube*:* performed full length sequencing of fosmids

Jens Harder: enriched RCB from sediment

Julia Arnds: quantified RCB and *Alphaproteobacteria* in the April 2005 sample

Sarah Hahnke: performed DGGE analysis of the enrichment culture

Marc Mussmann: assisted in data analysis

Sabine Lenk, Rudolf Amann and Marc Mussmann: designed research, discussed data, conceived the manuscript, edited the manuscript

Uncultured *Roseobacter* Clade Bacteria are Abundant in Intertidal Sediments and Employ Different Sulfur Oxidation Pathways

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Running title: Sulfur-oxidizing *Roseobacter* clade bacteria in coastal sediments

Key words: *Roseobacter*, sulfur oxidation, reverse dissimilatory sulfite reductase (*dsrAB*), sulfate thiohydolase (*soxB*)

Summary

Bacteria of the marine *Roseobacter* clade (RCB) have frequently been implicated in pelagic sulfur cycling. Yet their function and abundance in marine sediments have rarely been investigated. We recovered a 35 kb genome fragment from coastal sediments that could be assigned to RCB. It harbored an unusually clustered set of sulfur oxidation genes as it encoded the reverse dissimilatory sulfite reductase (rDSR) pathway in addition to 14 genes of the Sox multienzyme system (SOX) including subunits SoxCD of the sulfur dehydrogenase. Previously, SoxCD and rDSR were thought to be mutually exclusive in sulfur-oxidizing prokaryotes (SOP). This novel gene arrangement would allow a metabolic flexibility in sulfur oxidation unique among SOP. Between the *dsr* and *sox* operons genes encoding a DMSO reductase (*dmsABC)* were localized, which may hint at sulfur oxidation coupled to DMSO respiration. By comparative analysis we identified a novel gene, designated as *dsrU*, which encodes a putative dioxygenase that is located upstream of *dsrAB* in the RCB assigned fosmid and in aerobic chemotrophic SOP. The derived protein might be involved in an O_2-dependent release of sulfite from an organic donor. To confirm the presence of rDSR in marine *Roseobacter* we performed *dsrA* gene-targeted fluorescence *in situ* hybridization (geneFISH) of closely related RCB that were detected in a sulfur-oxidizing enrichment from anoxic sediments. In addition, phylogenetic analysis of sedimentary 16S rRNA and sulfate thiohydrolase (SoxB) genes provided further evidence for the presence of diverse sulfur-oxidizing RCB in sulfidic, intertidal sediments. Unexpectedly, fluorescence *in situ* hybridization (FISH) revealed dense populations of up to 2.5×10^8 cells ml^{-1} (9.6 ± 0.5% of all cells) in oxic and anoxic layers. Our findings extend the habitat range of marine *Roseobacter* and point at an important, so far unrecognized contribution to sulfur cycling in coastal sediments.

Introduction

Marine *Roseobacter* clade bacteria (RCB) are ubiquitously distributed throughout the world's oceans, where they are engage in a variety of biogeochemical processes (Buchan *et al*., 2005; Wagner-Doebler and Biebl, 2006; Moran *et al*., 2007, Brinkhoff *et al*., 2008). They comprise one of the most abundant heterotrophic groups particularly in coastal bacterioplankton communities (Buchan *et al*., 2005; Wagner-Doebler and Biebl, 2006; Brinkhoff *et al*., 2008). Earlier cultivation-independent findings suggested that they account for 3-11% of the total community 16S rRNA gene pool in coastal sediment (Gonzalez *et al*., 1999). However, knowledge on the activity of sedimentary RCB is scarce. Generally, they are considered as important degraders of climate relevant organosulfur compounds in the water column (Gonzalez *et al*., 1997; Moran *et al*., 2003; Howard *et al*., 2006). In contrast, their contribution to the oxidation of inorganic sulfur compounds ('sulfur oxidation') has received less attention although cultivation based studies repeatedly recovered RCB that utilize sulfur, sulfite, sulfide and thiosulfate (Shiba, 1991; Sorokin, 1995; Gonzalez *et al*., 1999; Teske *et al*., 2000; Sorokin *et al*., 2005; Sass *et al*., 2009). Some of these sulfur-oxidizing isolates were obtained from sulfidic coastal (Sass *et al*., 2009) or deep sea sediments (Teske *et al*., 2000). The presence of genes encoding the Sox multienzmye system ('*sox'* genes) in environmental PCR-based libraries and genome sequences confirmed the implication of diverse RCB in sulfur oxidation (Meyer *et al*., 2007; Moran *et al*., 2007; Swingley *et al*., 2007; Wagner-Doebler *et al*., 2009; www.roseobase.org). The Sox multienzyme system is well characterized for the chemotrophic alphaproteobacterium *Paracoccus pantotrophus,* where it mediates the complete oxidation of thiosulfate to sulfate (Friedrich *et al*., 2000; Friedrich *et al*., 2005). *Sox* genes occur in various phylogenetic clades (Meyer *et al*., 2007) including autotrophic *Gamma*- and *Epsilonproteobacteria* and *Chlorobi*. However, most autotrophic SOP among the *Gammaproteobacteria* and *Chlorobi* contain a truncated Sox system that lacks genes encoding the sulfur dehydrogenase SoxCD (Friedrich *et al*., 2005; Frigaard and Dahl, 2009). These organisms oxidize thiosulfate or sulfide and transiently form sulfur globules, which are further oxidized to sulfite via the reverse-operating dissimilatory sulfite reductase (rDSR) pathway (Pott and Dahl, 1998; Hensen *et al*., 2006; Meyer *et al*., 2007; Grimm *et al*., 2008). This pathway has been found in few *Alphaproteobacteria* but not yet in RCB.

In a previous study we identified novel *Alpha*- and *Gammaproteobacteria* that employ the rDSR for sulfur oxidation in a highly sulfidic, intertidal sand flat of the German Wadden Sea (Lenk *et al*., 2011). In the present study we aimed to further elucidate the metabolic potential and identity of prevailing sulfur oxidizers. Accordingly, we screened a large insert fosmid library for the *dsrAB* and *soxB* diagnostic marker genes as metagenomic approaches have proven a powerful tool to probe deeper into the genome content of environmentally relevant, yet uncultured prokaryotes (Beja *et al*., 2000; Sabehi *et al*., 2005; Walsh *et al*., 2009). We performed comparative metagenomic analysis to characterize the genetic context of known sulfur oxidation genes. To confirm the putative origin of a novel DsrAB phylotype from sedimentary RCB, we applied geneFISH (Moraru *et al*., 2010). We moreover investigated the environmental diversity of sulfur-oxidizing RCB by comparative analysis of 16S rRNA genes and SoxB. Using FISH we estimated the numerical relevance of sedimentary populations and quantified the actual relative and total abundaces of RCB over the upper 10 cm of intertidal sediments.

Results

Metagenomic analysis of uncultured sulfur-oxidizing prokaryotes in tidal sediment

We screened a large insert fosmid library (24,000 clones) that was generated from anoxic sediment layers of Janssand site (Mussmann *et al*. 2005) for genome fragments encoding DsrAB or SoxB. We identified 11 fosmid clones with sizes ranging from 30 to 43 kb (Table 1, SI Table 1). Eight fosmids carried the DsrAB gene and two fosmids carried the SoxB gene. Phylogenetic reconstruction of the deduced DsrAB and SoxB amino acid sequences affiliated them exclusively with *Alpha*- or *Gammaproteobacteria* (SI Table 2). One fosmid (WS101A12) carried both diagnostic marker genes (Fig. 1). Most conspicuously it contained an unknown clustered set of sulfur oxidation genes (Fig. 1) and obviously derived from marine RCB. Phylogenetic analysis assigned its DsrAB and SoxB sequences to *Alphaproteobacteria* (Fig. 2, Fig. 3). The DsrAB sequence was most closely related to uncultured SOP detected in sulfidic waters off the coast of Namibia (84% amino acid sequence identity, Fig. 2). The closest cultured relative was *Rhodomicrobium vannielli* (78%), a photoautotrophic, purple non-sulfur bacterium that oxidizes sulfide anaerobically to tetrathionate (Brune, 1989). The SoxB sequence of fosmid WS101A12 affiliated with the marine RCB (Fig. 3. SI Fig.1). It displayed highest sequence identity to the SoxB sequence of *Sagittula stellata* (86%). Analysis of DNA intrinsic nucleotide signatures using TaxSOM (http://soma.arb-silva.de/ Weber *et al*., submitted) provided additional evidence for a *Roseobacter* clade related affiliation of the WS101A12 genome fragment and supported *Oceanicola batensis* HTCC2597 as closest known relative.

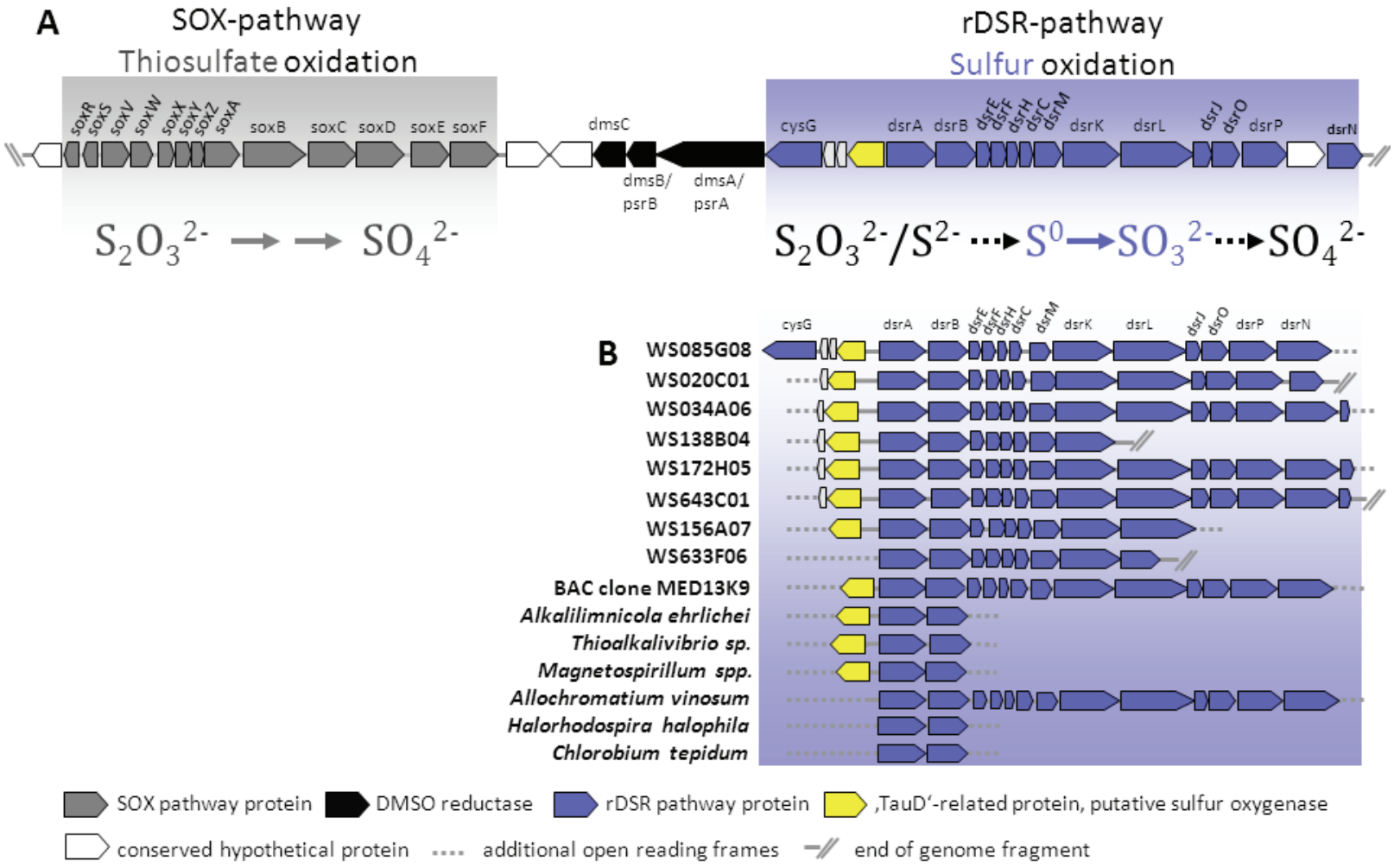

Figure 1 *Dsr* operons of Wadden Sea sediment fosmids

A. Gene arrangement on fosmid WS101A12

B. Gene neighbourhood representation of the genes *dsrAB* and the novel gene *dsrU* encoded on Wadden Sea sediment fosmids and in genomes of representative SOP. Identical colours indicate homologous genes in the corresponding genome fragments and genomes. For the fosmids and the uncultured BAC clone MED13K9 and *A. vinosum* the organization of the whole *dsr* operon is shown.

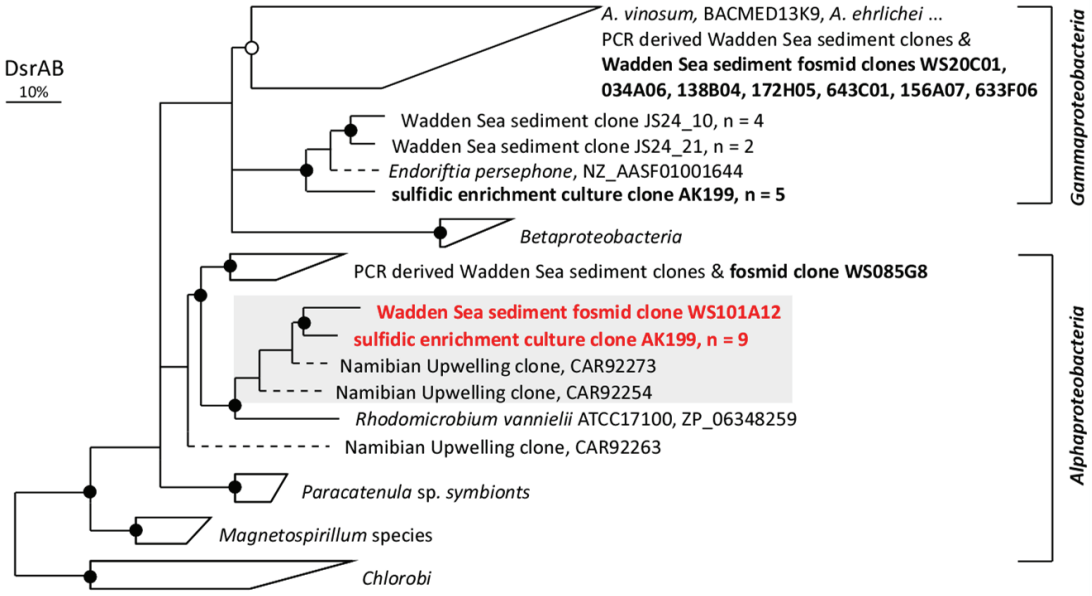

Figure 2 Maximum-likelihood tree (RAxML) of DsrAB.
Illustrated is the phylogenetic affiliation of DsrAB encoded on the Wadden Sea sediment fosmids and DsrAB retrieved from the sulfidic enrichment culture AK199 (highlighted in bold). Grey shading indicates sequences mutually originating from RCB. Bootstrapping was performed including all public available full length sequences. Circles indicate lineages with > 70% (●) and > 50% (○) RAxML bootstrap support. Multifurcations were introduced were branches could not be unambiguously resolved. The bar indicates 10% sequence divergence.

Genomic content of fosmid WS101A12

The 35 kb insert of fosmid WS101A12 contained 37 open reading frames (ORFS, Fig. 1, Table 1). It harbored the nearly entire gene set of the SOX multienzyme pathway *soxTRSVWXYZABCDEF* (ORF 1-14) including the sulfur dehydrogenase encoding genes *soxCD* (ORF 11, 12). The respective ORFs exclusively displayed *sox* genes of marine RCB as closest homologs. In addition, clone WS101A12 encoded a nearly complete *dsr* operon comprising the genes *dsrABEFHCMKLJOPN* (ORF 24-35, ORF37) and *cysG* (ORF 36). Thereof 8 ORFs (24-26, 29, 32-34, 36) displayed significant sequence similarity to homologs in *Alphaproteobacteria*. Between the *dsr* and *sox* operons ORF 17-19 encoded the three subunits of a putative dimethylsulfoxide (DMSO) reductase (*dmsABC)*. The deduced proteins displayed highest sequence similarity (77%, 79% and 62%, respectively) to DmsABC of DMSO-reducing, marine RCB.

In total, 18 of the 37 predicted ORFs possessed representative homologs in genome sequences of known RCB. We identified a novel gene (ORF 23) in the *dsr* locus upstream of *dsrAB*. Pfam analysis revealed a weak match of ORF 23 to a taurine catabolism dioxygenase ('TauD', PF02668, E-value 3.5e-6). Consistently it displayed amino acid sequence motifs typical for dioxygenases (SI Fig. 2). In *E. coli* TauD (EC 1.14.11.17) catalyzes the oxygenolytic release of sulfite during assimilation of taurine. Comparative genome analysis of the *dsr* loci revealed that homologs of ORF 23 are located upstream of *dsrAB* in nearly all recovered sediment fosmid clones (SI Table 1) and exclusively in aerobic, chemotrophic SOP (Fig. 1). In contrast, homologs are not associated with the *dsr* locus in the genomes of the facultative anaerobic, nitrate-reducing endosymbionts *Ruthia magnifica* and *Vesicomyosocius oktuanii* and phototrophic *Allochromatium vinosum* and *Halorhodospira halophila*. In genomes of the strictly anaerobic, phototrophic *Chlorobi* homologs are entirely absent.

Table 1 Overview of proteins predicted on Wadden Sea sediment fosmid WS101A12

ORF[a]	Size[b]	Predicted protein[c]	Closest homolog[d]	Identity[e]	Coverage[6]
1	268	**SoxT** YeeE/YedE family protein	*Roseovarius sp*. HTCC2601, 2E-46	53	83
2	119	**SoxR** repressor protein, transcriptional regulator	*Rhodobacterales* bacterium HTCC 2150, 2E-42	76	92
3	126	**SoxS** thioredoxin	*Rhodobacterales* bacterium HTCC2150, 1E-34	64	86
4	245	**SoxV** cytochrom c type membrane protein	*Roseobacter* sp., 2E-110	84	100
5	193	**SoxW** thioredoxin	*Sagittula stellata*, 5E-60	66	89
6	144	**SoxX** monoheme, SoxXA subunit	*Roseobacter denitrificans*, 2E-42	65	98
7	138	**SoxY** sulfur binding protein, SoxYZ subunit	*Rhodobacterales* bacterium HTCC 2654, 1E-48	77	99
8	108	**SoxZ** sulfur binding protein, SoxYZ subunit	*Roseovarius sp*. HTCC2601, 6E-49	84	99
9	286	**SoxA** diheme cytochrom c, SoxXA subunit	*Roseovarius* sp. 271, 8E-111	74	88
10	565	**SoxB** sulfate thiohydrolase/thiol esterase	*Roseovarius sp*. HTCC2601, 0.0	87	97
11	422	**SoxC** molybdoprotein, sulfur dehydrogenase subunit	*Roseobacter* sp. AzwK-3b, 0.0	86	100
12	412	**SoxD** diheme cytochrom c, sulfur dehydrogenase subunit	*Roseovarius sp*. HTCC2601, 2E-123	58	100
13	331	**SoxE**	*Roseovarius sp*. TM1035, 5E-81	69	63
14	423	**SoxF** flavocytochrom c	*Roseobacter* sp. AzwK-3b, 1E-167	75	100
15	364	YeeE/YedE family protein DUF395	*Hyphomicrobium denitrificans*, 5E-110	55	98
16	369	MRP protein (ATP/GTP-binding protein)-like protein	*Labrenzia alexandrii*, 3E-109	57	97
17	290	**DmsC**, DMSO reductase anchor subunit (Pfam8.3E-21)	*Rhodobacterales* bacterium HTCC 2150, 5E-68	62	100
18	245	**DmsB**, DMSO reductase iron-sulfur subunit	*Silicibacter pomeroyi*, 9E-120	79	100
19	946	**DmsA**, DMSO reductase chain A	*Roseobacter* sp. AzwK-3b, 0.0	77	97
20	488	**CysG** Siroheme synthase	*Rhodobacterales* bacterium HTCC 2654, 2E-144	60	94
21	72	Conserved hypothetical protein	*Rhodomicrobium vannielii*, 2E-6	50	86
22	63	Conserved hypothetical protein	*Rhodomicrobium vannielii*, 5E-13	62	92
23	312	**Taurine dioxygenase related protein** (Pfam 3.5E-6)*	*Thioalkalivibrio* sp. HL-EbGR7, 3E-69	48	92
24	435	**DsrA** dissimilatory sulfite reductase apha subunit	*Rhodomicrobium vannielii*, 0.0	75	96
25	360	**DsrB** dissimilatory sulfite reductase beta subunit	*Rhodomicrobium vannielii*, 5E-179	80	98
26	130	**DsrE** sulfurtransferase	*Rhodomicrobium vannielii*, 2E-51	73	100
27	135	**DsrF** intracellular sulfur oxidatin protein	uncultured SUP05 cluster bacterium, 3E-52	71	97
28	102	**DsrH**	*Alkalilimnicola ehrlichei*, 5E-19	49	96
29	110	**DsrC**	*Magnetospirillum gryphiswaldense*, 8E-45	74	100
30	248	**DsrM** nitrate reductase gamma subunit like protein	*Thiobacillus denitrificans*, 6E-83	66	97
31	510	**DsrK** iron-sulfur oxidoreductase	*Allochromatium vinosum*, 0.0	72	97
32	649	**DsrL** putative glutamate synthase small subunit protein	*Rhodomicrobium vannielii*, 0.0	66	98
33	157	**DsrJ** cytochrome	*Rhodomicrobium vannielii*, 1E-23	60	92
34	252	**DsrO** iron-sulfur protein	*Rhodomicrobium vannielii*, 2E-85	63	96
35	400	**DsrP** polysulfide reductase NrfD (Pfam 4.6E-58)	*Thioalkalivibrio* sp. HL-EbGR7, 1E-117	63	88
36	348	protein with similarity to glycosyl transferase	*Rhodobacterales* bacterium HTCC 2654, 5E-125	66	93
37	310	**DsrN** cobyrinic acid a,c-diamide synthase	*Sideroxydans lithotrophicus*, 6E-94	55	98

a. Open reading frames for which the closest homolog affiliates with the marine *Roseobacter* clade are shaded
b. Size of encoded protein in amino acids
c. Proteins involved in sulfur metabolism are highlighted in bold
d. Identity of amino acids to closest homolog in %
e. Coverage of ORF and the closest homolog from Blastp in %
* Novel, putative "sulfur oxygenase" encoding gene in rDSR pathway

16S rRNA and SoxB diversity of sedimentary RCB

We performed comparative phylogenetic analysis of the 16S rRNA and *soxB* gene diversity to elucidate the community composition of RCB in the sediment of Janssand site. As 16S rRNA gene sequences of *Alphaproteobacteria* were highly underrepresented in clone libraries established from bulk DNA (Lenk *et al*., 2011) we combined CARD-FISH with flow cytometry and sorted probe ROS537 hybridized cells. The clone library generated from sorted cells was significantly enriched in sequences affiliating with RCB (12 out of 30 clones). We recovered 12 full-length RC-associated sequences which grouped into 8 OTUs (based on 97% sequence identity, SI Fig. 3). These were highly diverse (77-99% overall sequence identity) and affiliated with sequences of cultured and uncultured RCB. One OTU (Wadden sea sediment clone 4432, SI Fig 3) grouped with the 16S rRNA gene sequence of *Tateyamaria pelophila* (97%), a facultative anaerobe previously isolated from the study site, which oxidizes sulfite and thiosulfate (Sass *et al*., 2009).

To better resolve the diversity of sulfur-oxidizing RCB a SoxB gene clone library was established from bulk sedimentary DNA that was previously screened for *dsrAB*-possessing SOP (Lenk *et al*., 2011). In total, 67 *soxB* sequences were recovered, which grouped into 35 OTUs (Fig. 3). The majority of sequences (40 sequences, 16 OTUs) affiliated with *Alphaproteobacteria*. In total 27 sequences (10 OTUs) formed a coherent cluster with SoxB of cultured RCB. Similar to 16S rRNA gene phylogeny, the RCB-associated SoxB OTUs were highly diverse (78%-96% amino acid sequence identity) and affiliated only distantly with sequences of known mostly planktonic representatives such as *Oceanibulbus indolifex* (OTU WS043, 86%) or the thiosulfate oxidizing *Silicibacter pomeroyi* (OTU WS037, 82%) and sulfite oxidizing *Sulfitobacter* sp. (OTU WS0470, 87%). Additional alphaproteobacterial sequences grouped with the purple non-sulfur bacterium *Rhodovulum sulfidophilum* (82%), *Labrenzia aggregata* (79%) or *Hoeflea phototrophica* (82%). Consistent with earlier findings many sequences (27 sequences, 19 OTUs) affiliated with sulfur-oxidizing *Gammaproteobacteria* (Lenk *et al*., 2011).

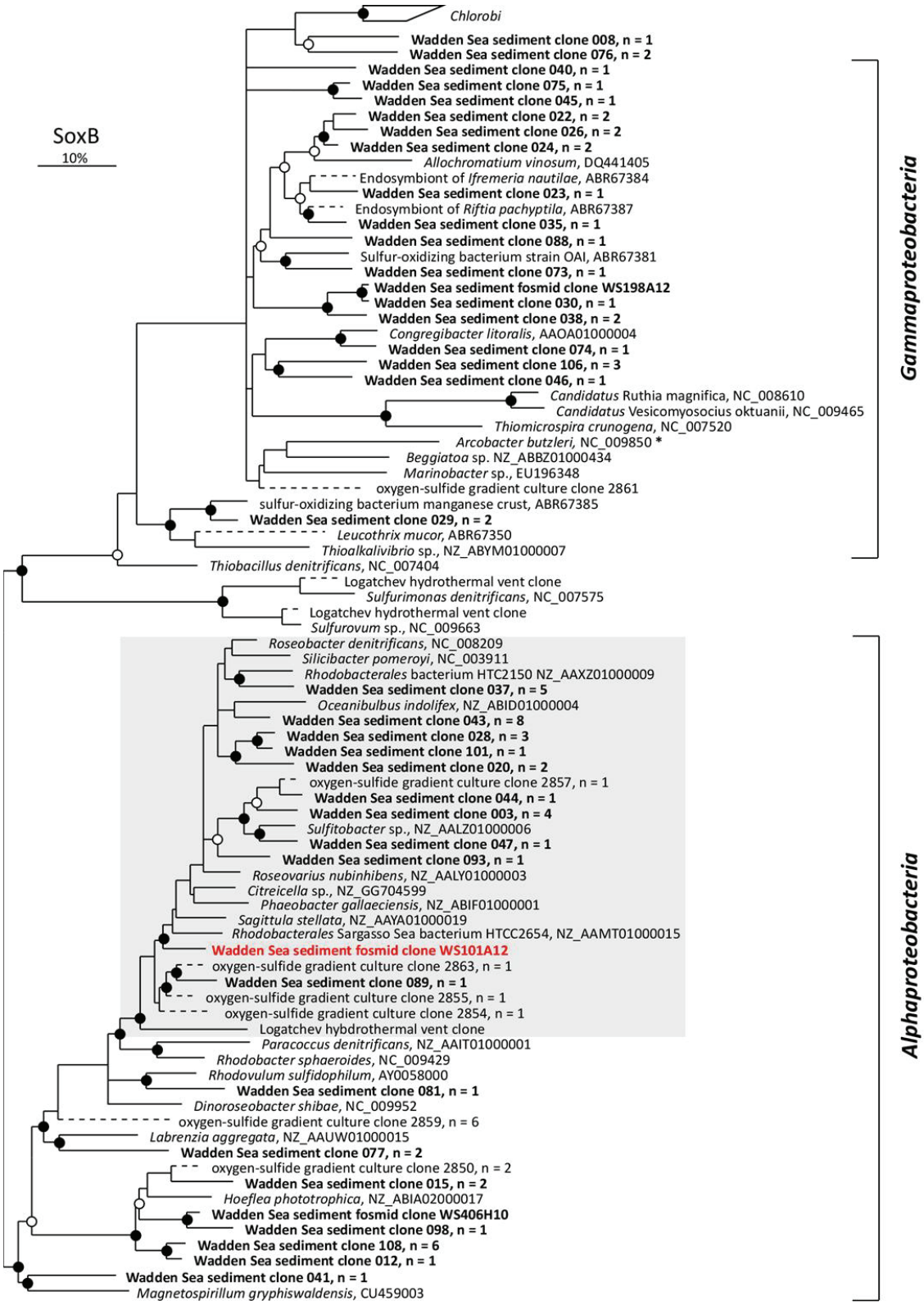
SoxB
10%
Chlorobi
Wadden Sea sediment clone 008, n = 1
Wadden Sea sediment clone 076, n = 2
Wadden Sea sediment clone 040, n = 1
Wadden Sea sediment clone 075, n = 1
Wadden Sea sediment clone 045, n = 1
Wadden Sea sediment clone 022, n = 2
Wadden Sea sediment clone 026, n = 2
Wadden Sea sediment clone 024, n = 2
Allochromatium vinosum, DQ441405
Endosymbiont of Ifremeria nautilae, ABR67384
Wadden Sea sediment clone 023, n = 1
Endosymbiont of Riftia pachyptila, ABR67387
Wadden Sea sediment clone 035, n = 1
Wadden Sea sediment clone 088, n = 1
Sulfur-oxidizing bacterium strain OAI, ABR67381
Wadden Sea sediment clone 073, n = 1
Wadden Sea sediment fosmid clone WS198A12
Wadden Sea sediment clone 030, n = 1
Wadden Sea sediment clone 038, n = 2
Congregibacter litoralis, AAOA01000004
Wadden Sea sediment clone 074, n = 1
Wadden Sea sediment clone 106, n = 3
Wadden Sea sediment clone 046, n = 1
Candidatus Ruthia magnifica, NC_008610
Candidatus Vesicomyosocius oktuanii, NC_009465
Thiomicrospira crunogena, NC_007520
Arcobacter butzleri, NC_009850 *
Beggiatoa sp. NZ_ABBZ01000434
Marinobacter sp., EU196348
oxygen-sulfide gradient culture clone 2861
sulfur-oxidizing bacterium manganese crust, ABR67385
Wadden Sea sediment clone 029, n = 2
Leucothrix mucor, ABR67350
Thioalkalivibrio sp., NZ_ABYM01000007
Thiobacillus denitrificans, NC_007404
Logatchev hydrothermal vent clone
Sulfurimonas denitrificans, NC_007575
Logatchev hydrothermal vent clone
Sulfurovum sp., NC_009663
Roseobacter denitrificans, NC_008209
Silicibacter pomeroyi, NC_003911
Rhodobacterales bacterium HTC2150 NZ_AAXZ01000009
Wadden Sea sediment clone 037, n = 5
Oceanibulbus indolifex, NZ_ABID01000004
Wadden Sea sediment clone 043, n = 8
Wadden Sea sediment clone 028, n = 3
Wadden Sea sediment clone 101, n = 1
Wadden Sea sediment clone 020, n = 2
oxygen-sulfide gradient culture clone 2857, n = 1
Wadden Sea sediment clone 044, n = 1
Wadden Sea sediment clone 003, n = 4
Sulfitobacter sp., NZ_AALZ01000006
Wadden Sea sediment clone 047, n = 1
Wadden Sea sediment clone 093, n = 1
Roseovarius nubinhibens, NZ_AALY01000003
Citreicella sp., NZ_GG704599
Phaeobacter gallaeciensis, NZ_ABIF01000001
Sagittula stellata, NZ_AAYA01000019
Rhodobacterales Sargasso Sea bacterium HTCC2654, NZ_AAMT01000015
Wadden Sea sediment fosmid clone WS101A12
oxygen-sulfide gradient culture clone 2863, n = 1
Wadden Sea sediment clone 089, n = 1
oxygen-sulfide gradient culture clone 2855, n = 1
oxygen-sulfide gradient culture clone 2854, n = 1
Logatchev hybdrothermal vent clone
Paracoccus denitrificans, NZ_AAIT01000001
Rhodobacter sphaeroides, NC_009429
Rhodovulum sulfidophilum, AY0058000
Wadden Sea sediment clone 081, n = 1
Dinoroseobacter shibae, NC_009952
oxygen-sulfide gradient culture clone 2859, n = 6
Labrenzia aggregata, NZ_AAUW01000015
Wadden Sea sediment clone 077, n = 2
oxygen-sulfide gradient culture clone 2850, n = 2
Wadden Sea sediment clone 015, n = 2
Hoeflea phototrophica, NZ_ABIA02000017
Wadden Sea sediment fosmid clone WS406H10
Wadden Sea sediment clone 098, n = 1
Wadden Sea sediment clone 108, n = 6
Wadden Sea sediment clone 012, n = 1
Wadden Sea sediment clone 041, n = 1
Magnetospirillum gryphiswaldensis, CU459003
Gammaproteobacteria
Alphaproteobacteria

Figure 3 Maximum-likelihood tree of SoxB.
A consensus tree based on Maximum-likelihood (RAxML) and PHYML was generated. Illustrated is the phylogenetic affiliation of SoxB encoded on fosmid clones WS101A12, 406H10 and 198A12 and those retrieved by PCR from Janssand intertidal sediments. Single OTUs are represented by selected clones ('Wadden Sea sediment clone'), 'n' indicates the number of sequences per OTU. SoxB that were previously obtained from an oxygen-sulfide gradient culture (Lenk *et al*., 2011) are additionally shown. Grey shading indicates OTUs that group stable with cultured RCB (SI Fig. 2). Bootstrapping was performed including all public available full-length sequences. Sequences shorter than 315 amino acids (indicated by a dashed line) were added to the tree without allowing changes in the overall tree topology. Circles indicate lineages with > 70% (●) and > 50% (○) RAxML bootstrap support. Multifurcations are shown where branches could not be unambiguously resolved. The bar indicates 10% sequence divergence.

Abundance of marine RCB in tidal sediment

We quantified RCB in the top ten cm of Janssand sediment using CARD-FISH (Fig. 4). Relative abundances reached up to 4.0 ± 0.4% of all cells in April 2005, 9.6 ± 0.5% of all cells in August 2007, and 2.6 ± 0.7% of all cells in May 2009 (corresponding to 1.6×10^8 cells ml^{-1}, 2.5×10^8 cells ml^{-1} and 4.0×10^7 cells ml^{-1}, respectively). In deeper layers RCB accounted for 3.1% and 0.8% of all cells. Dual hybridization with probe ALF968 (*Alphaproteobacteria*) revealed that RCB constituted the majority of the sedimentary *Alphaproteobacteria* (on average 60% in April 2005, Fig. 4, SI Fig. 4). Detection rates for *Alphaproteobacteria* were consistent with data previously reported by Ishii and colleagues (2004), who found maximum relative abundances of up to 6% (3.2×10^8 cells ml^{-1}) in September 2002.

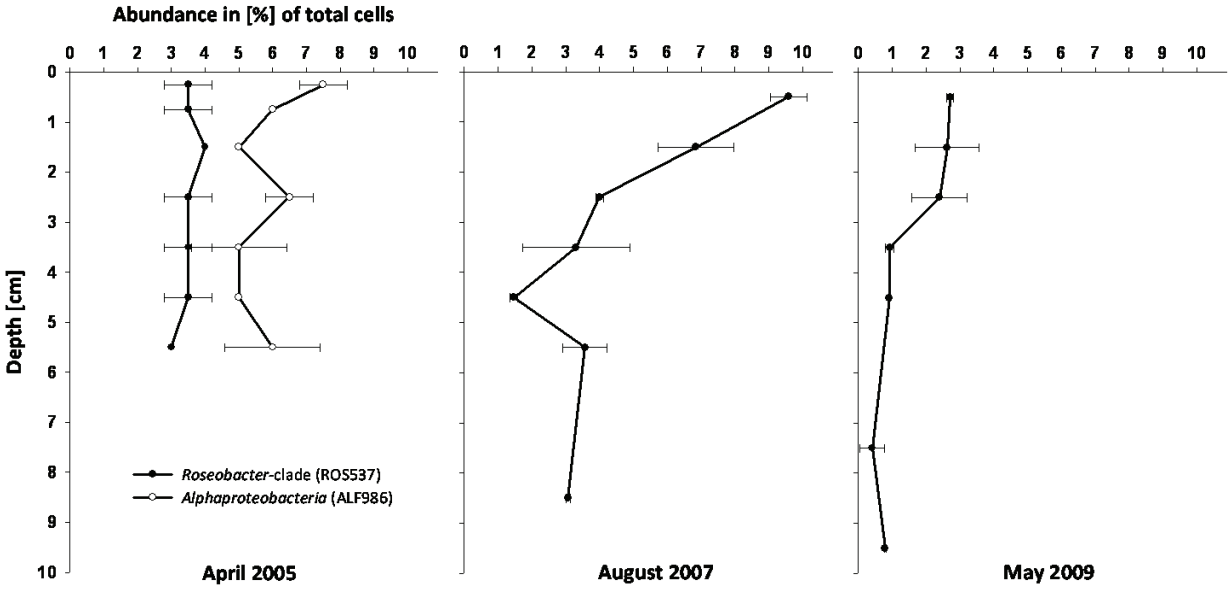

Figure 4 Relative abundances of marine RCB along vertical sediment profiles in April 2005, August 2007 and May 2009.
CARD-FISH was performed using probe ROS537. In addition, the relative abundances of total *Alphaproteobacteria* were assessed for the April 2005 sample using probe ALF968.

Enrichment and molecular characterization of dsr-possessing RCB

To test the hypothesis, whether the presence of the rDSR locus in the RCB-affiliated fosmid WS101A12 was based on horizontal gene transfer or whether it truly belonged to RCB, we screened for 16S rRNA of RCB and alphaproteobacterial rDSR in an anaerobic, sulfidic enrichment from muddy, anoxic sediments at site Koenigshafen (island of Sylt). FISH revealed that RCB accounted for 8% of all cells in the highly reduced surface sediment of 0–1 cm depth at the site. Accordingly, inoculation of anoxic sediment into reduced mineral medium enabled enrichment of sedimentary RCB. After repeated transfers, FISH

analysis showed that RCB dominated the enrichment and accounted for approximately two third of all cells, whereas *Gamma-* and *Epsilonproteobacteria* were less abundant (SI Fig. 5). From this enrichment, the 16S rRNA genes and *dsrAB* were amplified, cloned and sequenced.

Sequencing of 55 16S rRNA gene clones revealed three different operational taxonomic units (OTUs, based on >98% sequence identity, Table 2). Consistent with our FISH based findings, those affiliated with *Alpha-*, *Gamma-* or *Epsilonproteobacteria*. The alphaproteobacterial phylotype grouped in the *Roseobacter* clade (Table 2). It affiliated most closely with the benzoate degrading, nitrate reducing strain TH1 (98% sequence identity), and with *Donghicola eburneus*, an acetate utilizing aerobe (96%). The 16S rRNA gene sequence of *Rhodomicrobium vannielii* displayed only 86% sequence identity. DGGE analysis with RCB specific primers supported the presence of only one RCB-associated phylotype (SI Fig. 6) most closely related to strain TH1 and identical to the clone sequences (Table 2). Supply of different sulfur and carbon sources confirmed utilization of benzoate by the enriched RCB as revealed by FISH. However, pronounced growth occurred under aerobic conditions with the organosulfur compounds taurine and dimethylsulfoxide (SI Fig. 7).

Analysis of 32 *dsrAB* clones uncovered two different phylotypes (based on 90% nucleic acid sequence identity, Fig. 2) that were only distantly related to each other (72%). One phylotype was most closely related to the DsrAB encoded on the RCB-affiliated fosmid clone WS101A12 (93% amino acid sequence identity). The second phylotype affiliated with gammaproteobacterial DsrAB sequences previously retrieved from Janssand sediment (85%). *Endoriftia persephone* represented the closest known sulfur oxidizer (83%).

Table 2 Taxonomic affiliation of 16S rRNA gene sequences from the sulfidic enrichment culture

OTU	Phylum/Division	Closest described relative[a]	Identity[b]
AK199_DGGE band	*Alphaproteobacteria*	Benzoate degrading strain TH1, AJ133762	99
AK199_clone 2030	*Alphaproteobacteria*	Benzoate degrading strain TH1, AJ133762	99
AK199_clone 2300	*Gammaproteobacteria*	*Marinobacter* guineae, AM503093	97
AK199_clone 2040	*Epsilonproteobacteria*	*Sulfurimonas autotrophica*, AB088431	96

a. Validly described species/strain as determined by Maximum Likelihood phylogenetic reconstruction (RAxML)
b. 16S rRNA gene sequence identity in %

In situ localization of dsrA in RCB (geneFISH)

We applied gene-targeted fluorescence *in situ* hybridization (geneFISH) to directly link RCB and the *dsrA* gene at a single cell level. The protocol provides a novel *in situ* technique for linking gene presence and cell identity in uncultured microorganisms (Moraru *et al*., 2010). A polynucleotide probe that targeted the *dsrA* phylotype of fosmid WS101A12 and the alphaproteobacterial enrichment derived phylotype (SI Table 3) was hybridized to the sulfidic enrichment culture. GeneFISH of *dsrA* clearly localized the gene in cells that were simultaneously hybridized to the 16S rRNA targeting probe ROS537 specific for RCB (Fig. 5A). Cells not hybridized to probe ROS537 yielded no *dsrA* gene signals. Hybridization with the control probe NonPolyPr350 (negative control, non-target probe) proved the absence of unspecific binding as it did not result in any visible signal (Fig. 5B).

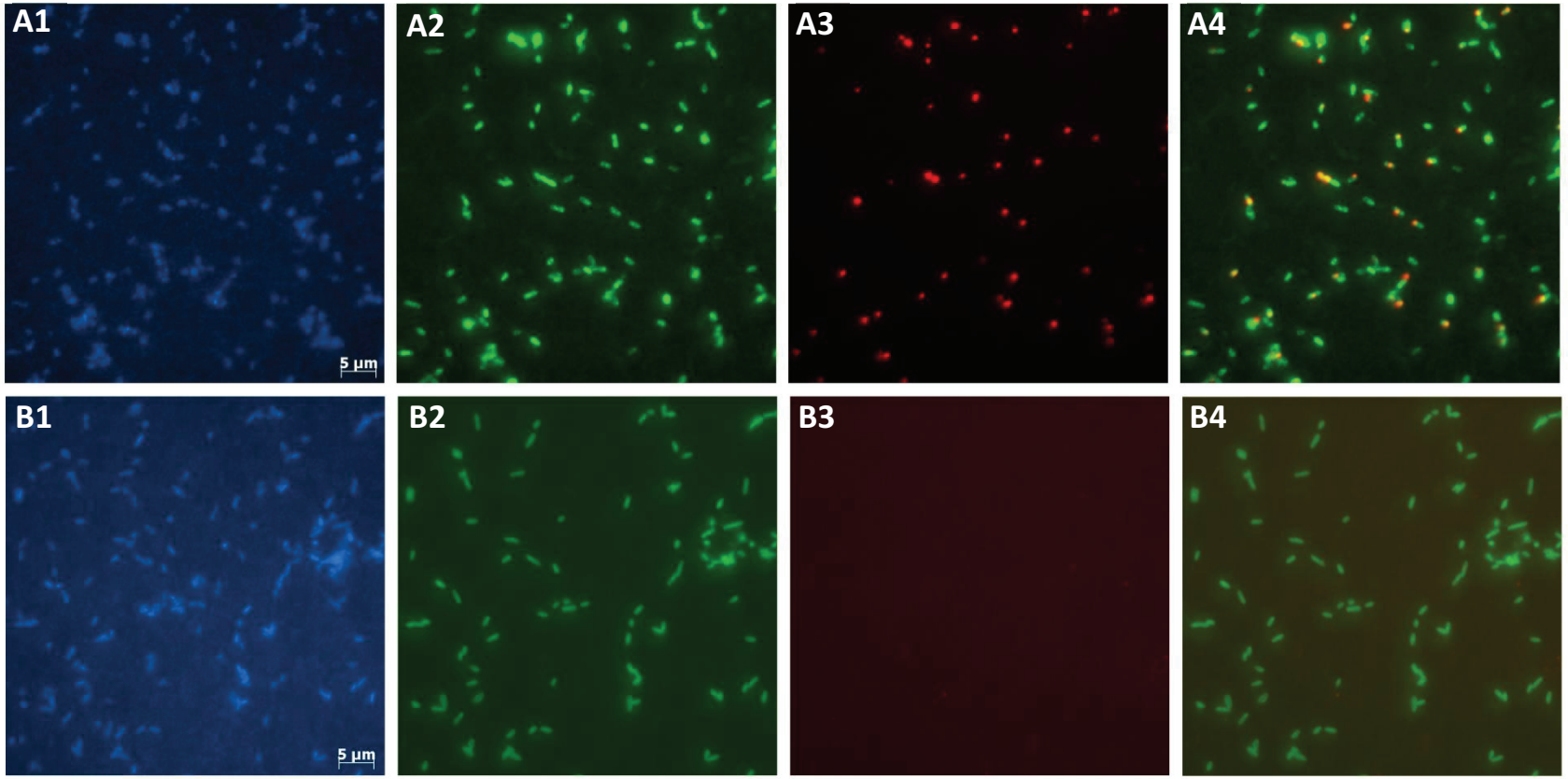

Figure 5 Micrographs of marine RCB possessing *dsrA*.
Images illustrate a dual colour hybridization of the 16S rRNA of RCB (green) and the *dsrA* targeting polynucleotide probe stained (red). The overlay image (**A4**) demonstrates that the signal for *dsrA* (red, **A3** and **A4**) is localized in the fluorescent RCB (green, **A2**, **A4**). *Gammaproteobacteria* are stained with DAPI and are not fluorescent (**A1**). In addition, no fluorescent signals were obtained from hybridization of the NonPoly350 probe (B3) to the enrichment culture which indicates the absence of non-specific binding (**B1**-**B4**)
A1. and **B1.** DNA staining (DAPI) of total cells
A2. Fluorescent signals of 16S rRNA specific probe hybridized to *Roseobacer*-clade bacteria
A3. Fluorescent signals of *dsrA*-targeted probe
A4. Overlay images of 16S rRNA-FISH and *dsrA*-FISH
B2. Fluorescent signals of 16S rRNA specific probe hybridized to *Gammaproteobacteria*
B3. Fluorescent signals of negativecontrol probe NonPoly350
B4. Overlay images of 16S rRNA-FISH and NonPoly350 probe-FISH

Discussion

It is widely known that marine RCB generally account for substantial proportions of coastal bacterioplankton communities. Here, we provide evidence that RCB also reach high abundances in coastal sediments. Using FISH we demonstrated that RCB thrive in numbers between 10^6 to 10^8 cells ml^{-1} throughout the upper 10 centimeters of the sediment and thus reach 10-1000 fold higher abundances than pelagic RCB in bacterioplankton communities. Their detection in permanently anoxic layers suggests the prevalence of facultative anaerobic members. This is consistent with the finding, that *T. pelophila,* a previously isolated relative from the study site, exhibits a respective lifestyle (Sass *et al*., 2009). Similarly, Alonso and Pernthaler (2005) demonstrated aerobic and anaerobic assimilation of glucose among pelagic RCB in the North Sea. Interestingly, the abundances of the sedimentary populations exceed reported maxima of the North Sea pelagial by factor 1000 (e.g. 2.5×10^8 cells ml $sediment^{-1}$ vs. 2.8×10^5 and 3.9×10^5 cells ml^{-1} (Eilers *et al*., 2001; Pernthaler *et al*., 2002). Our results complement earlier findings of Gonzalez and Moran (1999) who revealed that 3-11% of the total community 16S rRNA gene pool in coastal surface sediments were derived from RCB. The high cell numbers throughout the different seasons support that RCB constitute a substantial and stable part of the sediment microbial community. Likewise, results from 16S rRNA gene and SoxB analysis further support that prevailing populations comprise highly diveres sediment autochthonous RCB which are only distantly related to known representatives of the clade.

rDSR pathway in Roseobacter clade bacteria

So far, only genes of the SOX multienzyme pathway were found among marine RCB (Meyer *et al*., 2007; Moran *et al*., 2007). However, our metagenomic approach revealed a *dsr* operon in an uncultured RCB. Using cultivation and single geneFISH we could link the 16S rRNA and the DsrA gene by simultaneously visualizing both genes via geneFISH in a RCB from a sulfidic enrichment culture. Consistent with the methodological hybridization efficiency 40% of all RCB displayed a *dsrA* signal (Moraru *et al*. 2010). Congruent phylogenies of DsrAB and 16S rRNA and DGGE analysis supported the presence of only one *dsrAB*-possessing RCB in the enrichment culture that at the 16S rRNA level is closely related to the anaerobic, nitrate reducing strain TH1 from the Black Sea (Zengler, 1999). Interestingly, DsrAB sequences of the sedimentary RCB phylotypes were most closely related to several unknown DsrAB sequences from anoxic, sulfidic waters of the Namibian coast (Lavik *et al*., 2009). This is consistent with a high abundance of RCB in these waters that were otherwise dominated by autotrophic sulfur-oxidizing *Gamma*- and *Epsilonproteobacteria* (Stuehrmann, 2009). It is tempting to speculate whether respective DsrAB sequences belong to pelagic RCB that mediate sulfide detoxification via the rDSR pathway in oceanic oxygen minimum zones.

Co-localization of dsr and the sox operon including soxCD

The co-occurrence of entire *dsr* and *sox* operons and their tight genomic clustering is novel for SOP. This gene arrangement is similar to metabolic islands described for sulfate reducing organisms (Mussmann et al. 2005) and might display an ancient gene set among sulfur oxidizers due to the unique co-occurrence of *soxCD* and *dsr* genes. So far, the rDSR pathway and SoxCD have been thought to be mutually exclusive as *dsr* genes have only been detected in SOP that possess a 'truncated' Sox multi enzyme system (Meyer *et al*., 2007; Frigaard and Dahl, 2009; Loy *et al*., 2009). SoxCD has a sulfur dehydrogenase activity and

allows the oxidation of thiosulfate to sulfate without accumulation of elemental sulfur (Appia-Ayme *et al*., 2001; Friedrich *et al*., 2003). In s*oxCD*-deficient organisms, the rDSR pathway is thought to replace the sulfur dehydrogenase activity of SoxCD (Friedrich *et al*., 2005). As a common feature, these organisms transiently form elemental sulfur from the oxidation of sulfide or thiosulfate. Accordingly, in *Allochromatium vinosum* thiosulfate is oxidized via an incomplete Sox system (Hensen *et al*., 2006; Grimm *et al*., 2008), while sulfide is suggested to be oxidized via sulfide-quinone reductase (Reinartz *et al*., 1998, Frigaard and Dahl, 2009). The accumulating zero-valent sulfur species that form intracellularly are then further oxidized to sulfite by the rDSR complex (Pott and Dahl, 1998; Dahl *et al*., 2005). In contrast, the presence of entire *sox* and dsr operons in fosmid WS101A12 provides flexibility unique among SOP and would allow oxidizing thiosulfate either directly to sulfate or via elemental sulfur/polysulfide that can be stored. However, a direct oxidation via the complete Sox enzyme system is energetically favorable as it releases 8 electrons per mole of thiosulfate. In contrast, only two electrons are yielded upon intermediary sulfur globule formation when SoxCD is lacking (Friedrich *et al*., 2000). Thiosulfate could be utilized from external sources as it has been shown to accumulate in sediment of Janssand site (Kamyshny *et al*., 2010). Likewise, it might arise intracellularly from the reaction of intermediary formed sulfite and sulfur species (Rohwerder and Sand, 2003).

Given their putative functions the encoded rDSR enzymes could fulfill a crucial role in the oxidation of external supplied sulfur/polysulfide (Franz *et al*., 2007) as high concentrations of zero-valent sulfur species accumulate in sulfidic pore waters of the study site (Jansen *et al*., 2009; Kamyshny *et al*., 2010). Alternatively, tidal induced oxygen limitation (Jansen *et al*., 2009; Kamyshny *et al*., 2010) might restrict the organisms' capacity for sulfide oxidation and lead to the transient accumulation of sulfur as observed in *Thiobacillus thioparus* (Van den Ende and Van Gemerden, 1993) which are then further metabolized by rDSR.

Generally, co-regulation, spatial co-localization of the gene products and coordinated horizontal gene transfer of metabolic islands are believed to promote the operon structure of genes working in the same biological pathways (Osbourn and Field, 2009). Similarly, the genomic proximity of the *sox* and *dsr* operons detected on WS101A12 might reflect the functional relatedness of encoded pathways and promotes their putative interaction in the exchange of intermediates (Yin *et al*., 2010).

We can only speculate whether the RCB detected in the sulfidic enrichment also possess the complete *sox* operon. Further isolation efforts should facilitate the detection of SoxCD genes in this organism.

DMSO reduction

DmsABC genes and the use of DMSO as electron acceptor for growth on organic compounds have been reported for several RCB (Swingley *et al*., 2007; Wagner-Doebler *et al*., 2009; Gonzalez *et al*., 2003; Sass *et al*., 2009). The clustering of *sox, dsr* and *dms* genes in fosmid WS101A12 could mirror the adaptation for growth with different sulfur compounds and reflect close interactions of the deduced proteins (Osbourn and Field, 2009). Currently, we can only speculate whether DMSO reduction is functionally coupled to the oxidation of sulfur as demonstrated for *Rhodovulum* and *Chlorobium* species (Vogt *et al*., 1997). Results from the sulfidic enrichment culture provided evidence for the respiratory reduction of DMSO to DMS by the *dsr*-possessing RCB (SI Fig. 7). In this respect, it is noteworthy to mention that

enzymes of inorganic sulfur oxidation pathways are hypothesized to contribute to the oxidation of organosulfur compounds (Kelly, 1999; Schaefer *et al*., 2010). In particular, *dsr*-possessing organisms were previously proposed to contribute to DMS/DMSP breakdown in open ocean waters (Sabehi *et al*., 2005). However, enzymes, which oxidize DMS and derived inorganic sulfur compounds in cultured *dsr*-possessing *Thiobacilli* and *Thiocapsa* are not yet characterized (Schaefer *et al*., 2010). Our findings further support that the cell internal cycling of organic and inorganic sulfur compounds is tightly coupled.

A novel gene in the rDSR pathway

We identified an ORF that encoded typical dioxygenase motifs upstream of *dsrAB* in the RCB-related fosmid WS101A12 and nearly all other Wadden Sea sediment fosmids. As comparative genomic analyses confirmed its tight coupling to the *dsr* operon in aerobic chemotrophic SOP, a catalytic activity in the rDSR pathway is likely. Therefore, we propose the designation "*dsrU*" for this ORF. Similar to the function of TauD in *E*. *coli* it might be involved in the release of sulfite from an organic donor (Eichhorn *et al*., 1997) and thus represents a candidate enzyme for the interaction with DsrAB in the release of sulfite from the rDSR complex (Dahl *et al*., 2005). Alternatively, the derived protein might catalyze the oxidation of elemental sulfur with molecular oxygen similar to a glutathione-dependent sulfur dioxygenase (EC1.13.11.18) in *Acidithiobacillus* spp. (Rohwerder and Sand, 2003). As homologs occur in all genomes of chemotrophic, aerobic rDSR encoding SOP, we hypothesize that the derived enzyme could explain a key mechanism of sulfur oxidation in these widely distributed organisms. However, genetic studies are desirable to address this question. As the recovered Wadden Sea sediment fosmids are representative for diverse *dsrAB*-possessing *Alpha*- and *Gammaproteobacteria,* inhabiting Janssand site (Lenk *et al*., 2011), the encoded dioxygenases provides additional evidence for a contribution of respective populations to sedimentary sulfur oxidation.

Conclusion

Our study provides first evidence for a previously underestimated role of RCB in marine sediments. Overall, the acquisition of *dsr* genes suggests that RCB employ more diverse pathways for sulfur oxidation than previously known for members of the clade and for SOP in general. In the tidal sediments, different RCB populations might engage in sedimentary sulfur oxidation via the Sox and rDsr pathway. While we previously found that most *dsr*-possessing members of *Gammaproteobacteria* couple sulfur oxidation to autotrophic carbon fixation, the sedimentary RCB likely constitute lithoheterotrophic SOP. This is consistent with an earlier publication, where we did not detect any CO_2 incorporation of RCB (Lenk *et al*., 2011) and is in line with the general absence of autotrophic CO_2-fixing pathways in RCB (Tang, *et al*., 2009; www.roseobase.org). Instead, prevailing RCB likely grow on organic compounds and exploit reduced inorganic sulfur compounds as energy source. This would provide a competitive advantage in the highly sulfidic, organic matter rich surface sediments where numerous bacteria compete for carbon sources (Sorokin *et al*., 2003; Moran *et al*., 2004). We suggest that the gene set of genome fragment WS101A12 represents a special adaptation to the environmental conditions encountered in the intertidal sand flat. Here, the two sulfur oxidation pathways would facilitate the organism to utilize a variety of sulfur compounds as electron donors upon their availability in the sediment. Facing fluctuating oxygen concentration, the encoded DMSO reductase would provide the organism with the flexibility to use DMSO as alternative electron acceptor.

Our comparative genomic approach expands the range of proteins involved in rDSR-mediated sulfur oxidation by a putative dioxygenase that could explain a key step in aerobic chemotrophic sulfur oxidation. Future studies should elucidate, which sulfur compounds are utilized by *dsr*-possessing RCB and how they are channeled into the rDSR pathway. Similar to previously identified groups of sulfur-oxidizing *Gammaproteobacteria* (Lenk *et al*., 2011), the *sox*- and *dsr*-possessing RCB detected in this study contribute to sedimentary sulfide detoxification. Accordingly, future studies might infer the distribution and role of RCB in pelagic oxygen minimum zones.

Experimental Procedures

Sampling

Sediment samples originated from two intertidal sites located in the East and North Frisian German Wadden Sea. Sediment cores from the Janssand intertidal sand flat (53°44'07" N, 007°41'57" E), located in the backbarrier tidal area of the island Spiekeroog, were taken during repeated sampling campaigns in April 2005, August 2007 and May and June 2009. Sediment from a muddy intertidal site of Koenigshafen at the island of Sylt (55.02544° N, 8.4317° E) was sampled in October 2008. For FISH intact sediment cores were sliced, fixed and further processed immediately in the field or upon arrival in the lab within few hours as described previously (Ishii *et al*., 2004). Similarly, intact cores were sampled for DNA extraction and selected sediment horizons were frozen at – 20°C until further use.

Fosmid library screening

A fosmid library that had previously been established from Janssand sediment of 5–12 cm depth (Mussmann *et al*., 2005) was screened for the presence of *dsrAB* and *soxB* using primer pairs DSR1F/DSR4R (Loy *et al*., 2009), rDSR240F/rDSR808R (Lenk *et al*., 2011) and soxB432F/1446B (Petri *et al*., 2001) according to PCR conditions previously published. Clones possessing a respective gene were chosen for full-length sequencing of the insert (~40 kb). Shotgun sequencing and assembly were conducted at the MPI for Molecular Genetics, Berlin. The fosmid insert sequences were determined by a shotgun approach. Short insert shotgun libraries were generated with 1.5 and 2.5 kb inserts (Rabus *et al*., 2002). End-sequencing was performed on recombinant plasmids using BigDye 3.1 chemistry and 3730XL capillary sequencers (ABI, Darmstadt, Germany) up to a 10-fold sequencing coverage at least. Reads were assembled using PhredPhrap (http://www.phrap.org) and imported into Consed (Gordon *et al*., 1998) edited and verified. Finishing experiments were performed by primer-walking on fosmid and bridging shotgun clones to improve sequence quality and gap closure. ORF prediction was performed with METAGENE (Noguchi *et al*., 2006). Metagenomic analysis of predicted protein coding sequences was performed with jCOAST (Richter *et al*., 2008) using the software MicHanThi (Quast, 2006) for automatic annotation followed by manual refinement of annotated ORFS.

Enrichment of sedimentary RCB

RCB were enriched from sediment sampled in October 2008 at a muddy intertidal site of the island Sylt (site Koenigshafen). Initially, 2.5 ml of sediment from the anoxic layer of 3–4 cm depth were inoculated into 50 ml sulfate free, sodium bicarbonate buffered, artificial seawater medium (Widdel and Bak, 1992) containing 1 mM sulfide, 5 mM acetate and 5 mM nitrate. The redox indicator resazurin was added to the medium and the headspace contained a N_2/CO_2 (80/20 v/v) atmosphere. Gas production indicated

the presence of nitrate reducers after one month. Subsequently 1 ml of the enrichment was transferred to 9 ml of fresh medium followed by a MPN series of 15 tubes. The highest positive dilution in which growth was observed was subjected to a novel MPN series for 2 more times. Growth in the last MPN was observed in the 10^{-6} dilution. After three months and the third passage of repeated MPN series, FISH revealed high proportions of cells targeted by probe ROS537 in the highest positive dilution (10^{-6}). Subsamples were taken for construction of *dsrAB* and 16S rRNA gene clone libraries. Fresh inocula were transferred monthly into 50 ml serum bottles to maintain the enrichment culture. The growth on benzoate and sulfur compounds was assessed after the second monthly transfer. Respectively, 200 µl of the culture were incubated anaerobically upon inoculation into 20 ml hungate tubes containing 10 ml of nitrate (5 mM) amended ASW medium supplemented with benzoate (2 mM), thiosulfate (5 mM), acetate (5 mM) or biogenic elemental sulfur, respectively and a headspace of N_2/CO_2 (80/20 v/v). The biogenic sulfur was purified from cultures of *Chlorobaculum parvum* and kindly provided by Clelia Dona. Coloration of the redox indicator resazurin indicated substrate utilization in the anaerobic enrichments. Oxic amendments with DMSO (5 mM) and taurine (5 mM) were incubated in nitrate-free ASW by replacement of 10% of the headspace with filter-sterilized air. FISH was performed to assess the total number of RCB after three weeks of incubation.

DGGE analysis, amplification of 16S rRNA and dsrAB genes from the enrichment culture

DGGE analysis was performed with primers GC-ROSEO536Rf and GRb735r specific for RCB according to the protocol of Rink *et al*. (2007). For construction of 16S rRNA clone libraries aliquots of fresh cell suspension were subjected to repeated freeze and thaw cycles. Subsequently, 1 µl served as template for PCR. Thermal cycling for the 16S rRNA gene was performed using universal bacterial primers GM3F/ GM4R (Muyzer *et al*., 1995). Thermocycling included 4 min 95°C, followed by 20 cycles of 30 sec at 95°C, annealing for 30 sec at 44°C and elongation for 2 min at 72°C. A final elongation for 10 min at 72°C was included. For amplification of the *dsrAB* gene thermocycling was conducted as described previously for primers rDSR240F/403R and rDSR240F/808R (Lenk *et al*, 2011). All PCR products were cloned with the TOPO TA Cloning Kit (pCR4-TOPO, Invitrogen, Karlsruhe, Germany) and sequenced with the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturers' instructions. Partial and full length sequences of selected clones were generated using vector primers M13F/M13R.

CARD-FISH on sediment samples and enrichment cultures

Sediment cores sampled in April 2005, August 2007 and May 2009 (‘Janssand’) and October 2008 (‘Koenigshafen’) were sliced, fixed and processed as described previously (Lenk *et al*., 2011). The *in situ* abundance of RCB was assessed by CARD-FISH using probe ROS537 (Eilers *et al*., 2001) following the protocol of Ishii *et al*. (2004). Probe match analysis against the SILVA SSU Ref database release 102 revealed 92% coverage for sequences of the marine *Roseobacter*-clade. Only 3% of the sequences targeted by ROS537 represented non-target organisms outside the *RC* affiliating with *Alpha-*, *Gamma-* and *Deltaproteobacteria*, respectively. Within the intertidal sediment of Koenigshafen RCB abundances were assessed in the anoxic horizons of 1–2 cm and 7–8 cm depth. Dual colour hybridizations with probe ROS537 and ALF968 was performed according to Pernthaler and Amann (2004). For quantification of RCB in the enrichment culture, 1 ml of medium was fixed with formaldehyde (final concentration of 3.7%) for 1 h at room temperature. Cells were harvested by centrifugation in a table top centrifuged for 20 min at

14.000 rpm, washed with sterile phosphate buffer saline (PBS) and resuspended in 1 ml of PBS:Ethanol. Subsamples were transferred to glass slides. Probes ROS537, EPS549 (Lin *et al*., 2006), GAM42a (Manz *et al*., 1992), ALF968 (Glöckner *et al*., 1999) and NON-EUB (Wallner *et al*., 1993) were chosen for analysis (see also www.microbial-ecology.net/probebase). FISH was performed with Cy3 labelled probes as described earlier (Llobet-Brossa *et al*., 1998) using a standard hybridization time of 3 h and formamide concentrations according to literature.

Flow cytometry based sorting of sedimentary RCB and amplification of 16S rRNA gene

Surface sediment (0-3cm) of Janssand site was sampled in June 2009 and preserved for FISH. A subsample of 2 × 0.5 ml was sonicated (Lenk *et al*., 2011) and subjected to density gradient centrifugation according to Fazi and colleagues (2005). The purified cell fraction was hybridized with ROS537 and sorted using a MoFlow flow cytometer (Cytomation Inc., Fort Collins, Colo.) using the protocol of Sekar and colleagues (2004). Hybridized (ROS537-positive) cells were selected based on green fluorescent signals and side angle light scatter (SSC) by plotting SSC versus green fluorescence in a bivariant dot plot diagram. In total 133.000 target cells were sorted. To inspect the purity of the sorted cell fraction a subsample was counterstained with DAPI (1µg ml^{-1}). Microscopic analysis confirmed a green fluorescent signal for 97% of all cells. Amplification of the 16S rRNA gene was performed by filter-PCR (Sekar *et al*., 2004). Cloning and sequencing of the amplification product was performed as described previously (Lenk *et al*., 2011).

Amplification of soxB genes from the sediment

The clone library of the *soxB* gene was established using DNA of previously sampled surface sediment (0–3 cm) from Janssand site (Lenk *et al*., 2011). Primers and PCR conditions for amplification of an approximately 1000 bp fragment were chosen according to Petri and colleagues (2001) using a number of 30 PCR cycles. Generally, PCR reactions contained 50 pmol of each primer, 6.25 nmol of each dNTP, 1 × Master Taq Buffer and 1 U of Taq DNA Polymerase (Eppendorf, Hamburg, Germany) and were adjusted to a total volume of 25 µl with sterile PCR water. Cloning and sequencing was performed as described previously (Lenk *et al*., 2011).

Phylogenetic analysis

The 16S rRNA sequences were analyzed with the ARB software (Ludwig *et al*., 2004) using the SILVA 16S rRNA SSU Reference database release 102 (Pruesse *et al*., 2007). Selected clones were chosen for almost full length sequencing (>1400 bp). Sequences were analyzed for chimera formation using Bellerophon software (Huber *et al*., 2004). Final tree reconstruction was based on 500 nearly full lengths sequences. The phylogenetic tree was constructed using the maximum likelihood method RAxML (Stamatakis *et al*., 2008) employing a position variability filter for *Bacteria* (Pruesse *et al*., 2007). Operational taxonomic units (OTUs) were grouped based on 97% sequence identity using the ARB similarity matrix. Fosmid and PCR derived sequences of *dsrAB* and *soxB* were imported into ARB databases containing a respective nucleotide/amino acid alignment. The *dsrAB* sequences were manually aligned using a previously established *dsrAB*/DsrAB reference database (Lenk *et al*., 2011). Deduced amino acid sequences of environmental SoxB of this study and public available sequences were aligned using CLUSTALX prior to import of the alignment into a *soxB*/SoxB database ARB. Reconstruction of phylogenetic trees was based

on DsrAB and SoxB amino acid sequences and conducted with ARB PhyML and RAxML (Stamatakis *et al*., 2008) using an insertion/deletion filter and the JTT amino acid substitution matrix. OTUs were grouped according to branching patterns based on 90% sequence identity.

dsrA–targeted in situ hybridization (geneFISH) coupled to 16S rRNA CARD-FISH

A *dsrA*-targeting polynucleotide probe (359 bp, GC content 69%) was designed based on the *dsrAB* sequence of Wadden Sea fosmid WS101A12 using the software PolyPro (http://www.mpibremen.de/en/method-development/PolyPro). The target region of the *dsrA* probe exhibited 92.28% sequences identity to the enrichment derived alphaproteobacterial *dsrA* phylotype. In contrast, it displayed 33.2% to 39% mismatches to the *Gammaproteobacteria*-related phylotype detected in the enrichment culture. Fosmids WS101A12 were extracted from *E.coli* cells using the Spin Miniprep Kit Qiagen) to serve as template for the synthesis of the double stranded DNA polynucleotide (dsDNA probe). A Dig-dUTP labeled polynucleotide probe was synthesized using the PCR Dig Probe Synthesis Kit (Roche). Primers *dsr*1-F GAAGTATCCCGAGTCGAAGG and *dsr*1-R GCGCCGGGCGGTGCATCTC were used for amplification at an annealing temperature of 56°C. The dsDNA probes were purified with the Gene Clean Turbo kit (Q-Biogene). Formaldehyde fixed cells of the enrichment culture were filtered on palladium/gold coated polycarbonate filters (25 mm diameter). Filters were incubated in 0.01 M HCL for 10 min at room temperature to inactivate endogenous peroxidases, followed by incubation in 10 mg/ml lysozyme for 1 h at 37°C to permeabilize cell walls. CARD-FISH of 16S rRNA with probe ROS537 and GAM42a was performed as described above following the protocol Ishii and colleagues (2004) using Alexa488-labeled tyramide for signal amplification. After hybridization of the 16S rRNA gene, horseradish peroxidase enzymes (HRP) introduced with the 16S rRNA targeting probe were inactivated. Therefore, filters were incubated in 3% H_2O_2 in PBS for 30 min followed by incubation in 0.1 M HCL for 10 min. Inactivation of the HRP was monitored on control filters subjected to an additional tyramide signal amplification step using Alexa594 dye. No deposition of the dye occurred and visible signals were absent during microscopic observation. For digestion of RNA filters were incubated in RNase solution (0.5 U μl^{-1} RNase I, Ambion), 30 µg ml-1 RNase A (Sigma), 0.1 M Tris-HCl pH 8 for 4-5 h at 37°C. Filter sections, probe *dsrA* and NonPoly350 were incubated in hybridization buffer containing 45% formamide. After initial denaturation at 75°C for 25 min hybridization lasted for 18–22 h at 50°C followed by binding of the anti-Dig HRP-conjugated antibody (Fab fragments) and signal amplification with a Alexa594-labeled tyramide. Filter sections were embedded in SlowFadeGold antifade reagent (Invitrogen), containing 1 µg/ml 4´, 6-diamidino-2-phenylindole (DAPI). Microscopy was performed on an epifluorescence microscope (Axioplan, Carl Zeiss), equipped with the following fluorescence filters: DAPI (365/10 nm excitation, 420 LP emissions, FT 395 Beam Splitter), Alexa488 (472/30 excitation, 520/35 emission, 495 Beam Splitter) and Alexa594 (562/40 excitation, 624/40 emission, 593 Beam Splitter).

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Supporting Information

Figure S1 Alignment of taurine dioxygenase (TauD)-related amino acid sequences encoded on fosmid clones and in genomes of known SOP.
Illustrated is the iron-binding motif His-X-Asp-X53-His of the taurine dioxygenase of *E. coli*. Conserved His and Asp residues are marked by asterisks (*). The grey shading indicates 90% sequence identity.

Figure S2 Alignment of alphaproteobacterial SoxB amino acid sequences.
Roseobacter-clade bacteria (grey) possess a characteristic insertion at amino acid 215 (corresponding to *Chlorobium tepidum* based alignment position 283) which is highlighted in grey. As an exception *Dinoroseobacter shibae* (*) does not possess this insertion. The order of sequences in the alignment is given according to position of sequences in the phylogenetic tree (Fig. 3).

Figure S3 Maximum-likelihood phylogeny (RAxML) of alphaproteobacterial 16S rRNA gene sequences from intertidal sediments of the Janssand.
Single OTUs (shown in bold) are represented by selected clones ('Wadden Sea sediment clone'), 'n' equals numbers of sequences per OTU. Shaded in grey are sequences that affiliate with the marine *Roseobacter*-clade. Sulfur-oxidizing bacteria and/or bacteria known to possess the SoxB gene are indicated by filled (★) and open (☆) asterisks, respectively. In addition, RCB previously isolated from coastal sediment are marked (■). RAxML bootstrap values are indicated for lineages with >70% (filled circles) or >50% (open circles) support. The bar indicates 10% sequence divergence.

Figure S4 Epifluorescence microscopy images of marine RCB identified in the intertidal sediments of Janssand.
For all images, green (Alexa 488): cells that were identified by RCB-specific probe ROS537; red (Alexa 594): *Alphaproteobacteria* that hybridized with the general probe ALF968; blue: DAPI-stained DNA. RCB are simultaneously stained in green and red. *Alphaproteobacteria* not affiliating with the *Roseobacter clade* are exclusively stained in red. The scale bar corresponds to 5 µm.

Figure S5 Epifluorescence microscopy images of RCB (ROS), *Gamma*- (GAM) and *Epsilonproteobacteria* (EPS) identified in the 10^{-6} dilution of the acetate and nitrate amended sulfidic enrichment culture.
The scale bar corresponds to 5µm.

Figure S6 DGGE analysis of the enrichment culture with RCB specific primers .

Figure S7 Utilization of benzoate and sulfur compounds by RCB of the sulfidic enrichment culture.
The number of cells hybridized to probe ROS537 was assessed after three weeks of incubation. Control, only inoculum

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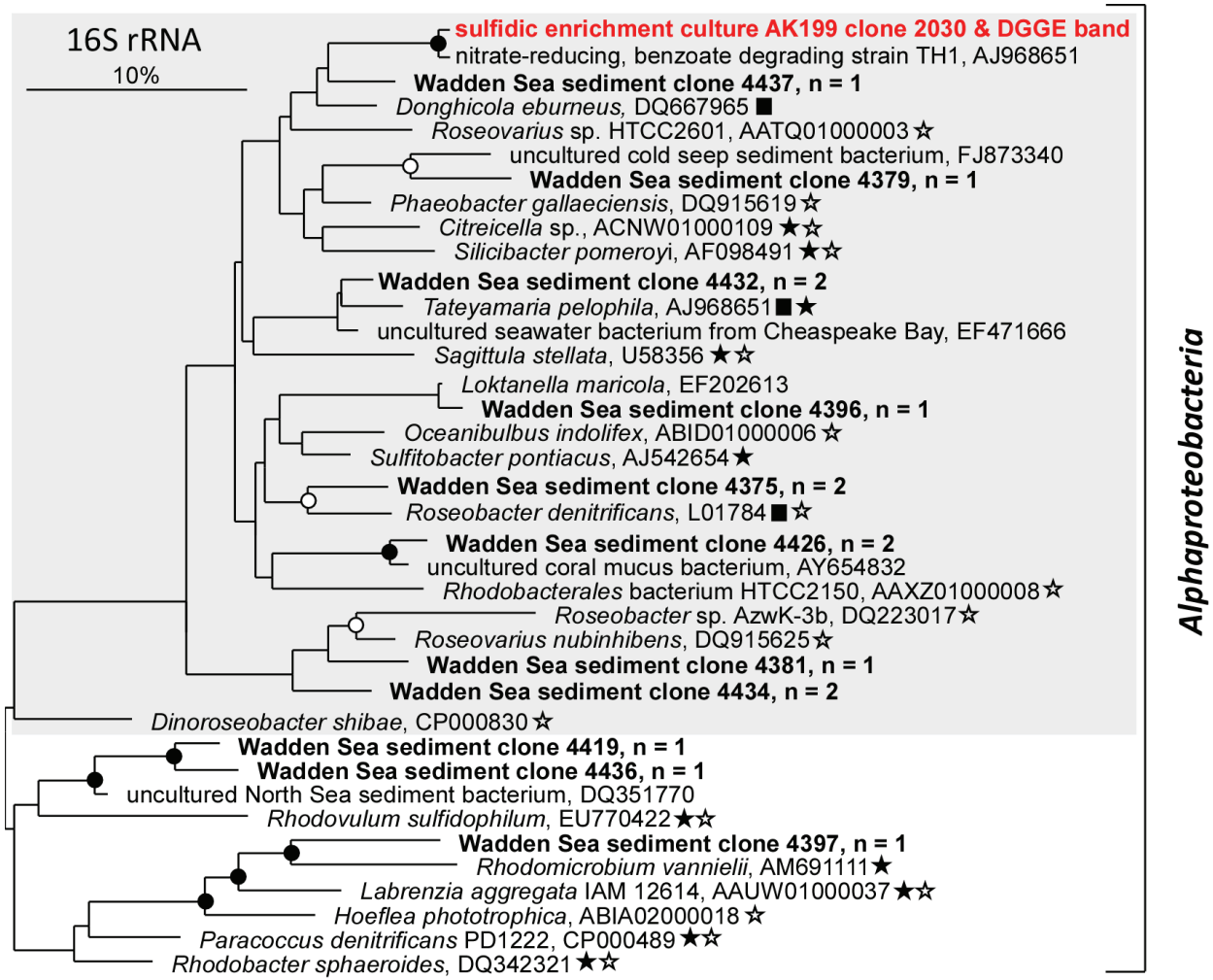
                                  180        190        200        210        220        230
                                  ....|....| ....|....  |....|.... |....|.... |....|... .|....|... .|...
Roseobacter denitrificans         MTFHWEFTLG SDRVNEIVE- GLPFAALGQN IFDAEWDEPA -ELFPPFQFF ERGGVKIAVI GQAFP
Silicibacter pomeroyi             MTFHWEFTLG TDRVTELVE- SLPFASLGQN IFDAEWDEPA -ELFKPYKFF ERGGVKVAVI GQAFP
Rhodobacterales bacterium HTCC 2150 MTFHWEFTLG SDRVRELIE- GLPFAALGQN IFDAEWDEPA -ESFESYKMM EAGGVKIGVI GQAFP
Wadden Sea sediment clone 037     MTFHWEFTLG SSRVNEIVE- DLPFAALGQN IFDAEWDEPS -ELFPPYKLF VRGGVKIAVI GQAFP
Oceanibulbus indolifex            MTFHWEFTLG SERVQEIVQ- GLPFAALGQN IFDAEWDEPA -ELFKPYEFF ERGGVKIAVI GQAFP
Wadden Sea sediment clone 043     MTFHWEFTLG SGRVNELVE- GLPFAALGQN IFDAEWDEPA -ELFKPYKFF ERGGVNIAVI GQAFP
Wadden Sea sediment clone 028     MTFHWEFTLG SDRVNELVE- GLPFAALGQN IFDAEWDEPA -ELFPPYKFF ERGGAKIAVI GQAFP
Wadden Sea sediment clone 101     MTFHWEFTLG SKRVNELVE- GLPFAALGQN IFDAEWDEPA -ELFPPYKFF ERGGAKIAVI GQAFP
Wadden Sea sediment clone 020     MTFHWEFTLG SERVNELVE- GLPFAALGQN IFDAEWDEPA -ELFPPYKFF ETGGVKVAVI GQAFP
sulfide-oxygen gradient culture clone 2857 MTFHWEFTLG SDRVNELVE- GLPFAALGQN IFDTEWDEPT -ELFPPYKLF ERGGTKIAVI GQAFP
Wadden Sea sediment clone 044     MTFHWEFTLG SERVAELVE- SLPFAALGQN IFDAEWDEPT -DLFPPYKMF ERGGTKVAVI GQAFP
Wadden Sea sediment clone 003     MTFHWEFTLG SDRVTELVE- SLPFAALGQN IFDAEWDEPT -DLFPPYKMF ERGGTKIAVI GQAFP
Sulfitobacter sp.                 MTFHWEFTLG SERVNEIVQ- GLPFAALGQN IFASEWDEPA -ELFKPYKMF ERGGTKIAVI GQAFP
Wadden Sea sediment clone 047     MTFHWEFTLG SERVQELVE- QLPFAALGQN IFDAEWDEPA -ELFPPYKMF ERGGTKIAVI GQAFP
Wadden Sea sediment clone 093     MTFHWEFTLG SDRVHELVE- NLPFAALGQN IFDAEWDEPA -ERFPPYTFF ERGGVKIAVI GQAFP
Roseovarius nubinhibens           MTFHWEFTLG SDRVTELVE- GLPYAALGQN IFDAEWDEPA -ELFKPYEFF ERGGVKIAVI GQAFP
Citreicella sp.                   MTFHWEFTLG SDRVNEIVE- GLPFAALGQN IFDAEWDEPA -ELFKPYEFF ERGGVKVAVI GQAFP
Phaeobacter gallaeciensis         MTFHWEFTLG SDRVNEIVE- GLPFAALGQN IFDAEWDEPA -ELFKPYKFF DRGGAKVAVI GQAFP
Sagittula stellata                MTFHWEFTLG SGRVQELVE- SLPFAALGQN IFDAEWDEPL PDLFPPYKFF ERGGVKIAVI GQAFP
Rhodobacterales bacterium HTCC 2654 MTFHWEFTLG SDRVNEIVE- NLPFAALGQN IFDAEWDEPT -DLFPPYKFF ESGGVKVAVI GQAFP
Wadden Sea fosmid clone WS101A12  MTFHWEFTLG SDRVHELVE- QMEFAALGQN IFDAEWDEPA -EIFKPYQMF ERGGTKIAVI GQAFP
sulfide-oxygen gradient culture clone 2863 MTFHWEFTLG SDRVRDIVN- DLPFAALGQN IFDAEWDEPA -EDFAPYQFF ERGGVKIAVI GQAFP
Wadden Sea sediment clone 089     MTFHWEFTLG SDRVRDIVS- DLPFAALGQN IFDAEWDEPA -EDFAPYKFY ESGGVKVAVI GQAFP
sulfide-oxygen gradient culture clone 2855 MTFHWEFTLG SGRVRDIVS- DLPFAALGQN IFDAEWDEPS -EDFQPYKFF ERGGVKIAVI GQAFP
sulfide-oxygen gradient culture clone 2854 MTFHWEFTLG SDRVRNIIQ- DLPFASLGQN IFDAEWEQSH -EDFKPYEFF ERGGVKVAVI GQAFP
Logatchev hydrothermal vent clone MTFHWEFTLG SERVREILA- DVSYDGLGQN IYDKEWEEPS -EDFKPYEFY ESGGIKIAVI GQAFP
Paracoccus denitrificans          MTSHWEWTYG TERVKEIVET QLKFPFLGAN IFDAEWDEPA ---FEPYKVF ERGGRRIGVI GQAFP
Rhodobacter sphaeroides           MTSHWEWTLG TERVKEIVD- ALPFPFLGAN IFDKEWDEPA ---FPPYEIF ERGGLRIAVI GQAFP
Rhodovulum sulfidophilum          MTSHWEFTLG LDRVNEIVE- GLDFAFLGAN IFDAEWDEPA ---YEPYRIF ERGGAKIAVI GQAFP
Wadden Sea sediment clone 081     MTSHWEFILG IDRVTEIVD- SLPFPFLGAN IFDNEWDEPA ---YEPYEMF ERGGAKVAVI GQAFP
Dinoroseobacter shibae            MTSHWEFTFG IDRVNDIVEN HLNFPFLGAN IFDAEWDEPA ---YEPYQMF ERGGAKIAVI GQAFP
Labrenzia aggregata               MTGHWEFTYG TDRVQEVID- SLPFAFLGSN IYDNEWDEPA ---FESWKMF ERGGSKVAVI GQAFP
Hoeflea phototrophica             MTGHWEFTLG EDRFLELVE- AMGYPFLASN IFDAEWDEPA ---FEHTAFF ERGGVNVAVI GQAMP
```

Figure S1

	pos	* *				*
Wadden Sea fosmid clone 101A12, orf23	13	**NWHTDGYYNG PGARVNAFVL HCVRPAAAGG ENCIIDPEIA YLRLRQENPE FIRALM--HPE---A MTIPEN**
Wadden Sea fosmid clone 85G8, orf06	92	SWHTDGYYNE KNRQINAVVL HCVEAASRGG ENALLDPEMV YIKLRDEDPR FIAAFE--HPE---C MTIPAN
Wadden Sea fosmid clone 20C1, orf14	86	GWHTDGYYNA GTLQVRAWLL FCAQPAAEGG ANELFDHEVA YIRLRDENPG WIRALM--ARN---A FTIPSN
Wadden Sea fosmid clone 34A6, orf18	12	NWHTDGYYNR LDEQVCGIVM HCVSEPATGG DNLILDPEIA YILMRDENPG YIAALM--QAD---A MTIPPN
Wadden Sea fosmid clone 138B4, orf10	12	NWHTDGYYNR LDQQVRGIVM HCVSEPASGG DNLFLDPEIA YILLRDENPD YIAALM--QPD---A MTIPPN
Wadden Sea fosmid clone 172H5, orf20	13	SWHTDGYYNS VEQRIRGMLL HCVSDAAQGG DNLLLDHEIC YIHLRDTNPD YIHALM--QPR---A MTIPPN
Wadden Sea fosmid clone 643C1, orf19	11	NWHTDGYYNR PQEQVRGIIM HCVSTSASGG DNLFLDPEIA YLLLRDENPD YIRAFM--QPD---A MTIPAN
Wadden Sea fosmid clone 156A7, orf14	79	NWHTDGYYNA IAAPVRAFIL HCRRPAEQGG ENSLLDPEYI YIRLRDLSVD LIAALM--EEH---A LTIPEN
Beggiatoa sp. PS (ZP 01998516)	12	HWHTDGYYNA LDKQIYGMIL HCVSPAQTGG ENALLDHEIA YIKLRDENPD YIHTLM--QPD---V MCIPAN
Thioalkalivibrio sp. (YP 002514251)	14	SWHCDGYYNP PERRIRAMLL HCVTDAAEGG ENALLDHELL YIRLRDENPG WIEALM--HPQ---A MTIPEN
Allochromatium vinosum (YP 003444269)	12	SWHTDGYYNS PEHQIHGLLL HCVHPAEEGG ANDLLDHEIA YILLRDLSPE FIRALM--HPE---C MTIPAN
Magnetospirillum magneticum AMB-1 (YP 422729)	12	TWHTDGYYNP PERIVRALLL HCAGKAAEGG ANRLMDHEML YILLRDQDPE LIRVLM--QPD---A MTIPGN
Magnetospirillum magnetotacticum MS-1 (ZP 00053118)	12	TWHTDGYYNP PERTVRALLL HCAGRAAEGG ANRLMDHEML YIQLRDESPD MIRALM--EPD---A MTIPGN
Magnetococcus sp. MC-1 (YP 864577)	12	QWHTDGYYNE PERTIRGMAL HCARQAEQGG ENDLLDHEIM YIRLRDQNPE HIRALM--AED---V LTIPAR
Alkalilimnicola ehrlichei MLHE-1 (YP 742488)	12	NWHTDGYYNP PERRVRGLIL HCVRPAREGG VNRLLDHRLV WQALAVSRPD ALKALQ--HPR---A MTIPPD
uncultured SUP05 cluster (EEZ80625)	13	HWHTDGYYND PDKQIHALNL HVVQKAEKGG ENQLMDHEIA YILLREKNPD FVRALM--QNN---V MMIPAG
Candidatus Ruthia magnifica (YP 904216)	11	GWHTDGYYNT IEQCIRAFSL FCITPANCGG ENQWIDQQMV YLQLRESNPD VTKALT--HTQ---A MSIPEH
Endoriftia persephone (ZP 02537955)	44	AWHTDGYYNQ SEEQIHGLLL HCVEPAAKGG ENALLDHEIV YLQIRDYQPA YIQALM--HPQ---A MTIPAN
Candidatus Vesicomyosocius okutanii (YP 001219765)	16	GWHTDGYYNK TDQRIRAFSL FCVTPASYGG ENKWINQQIV YLQLRESNPD VAMALT--HAQ---A MSIPEH
Sideroxydans litotrophicus (YP 003524312)	11	KWHTDGYYNP PERTIRGMIL HCVRNASSGG ENQLMDHELA YLLLRDEDPQ HIQALM--QPD---A MTIPER
Thiobacillus denitrificans (YP 314816)	12	NWHTDATYYG SNHTIQALFL LCKRPALEGG SNKVLDHEVL YIHLRDKAPE ALDVLM--NRD---C FNYRNP
Thiobacillus denitrificans (YP 316249)	12	NWHTDGYYNA LDRRILGMTL HCAQDAEAGG ENALLDHEIA YIQLRDTDPD YVAALM--QPD---A MTIPAR
Ralstonia eutropha, a-ketoglutarate dioxygenase (YP 025400)	11	LWHSDSSFQQ PAA--RYSML SAVVVPPSGG DTEFCDMRAA YDALPRDLQS ELEGLRAEHYAL--N SRFLLG
Escherichia coli, taurine dioxygenase (NP 414902)	97	NWHTDVTFIE TPP--AGAIL AAKELPSTGG DTLWTSGIAA YEALSVPFRQ LLSGLRAEH-DF--R KSFPEY

Figure S2


16S rRNA
10%
sulfidic enrichment culture AK199 clone 2030 & DGGE band
nitrate-reducing, benzoate degrading strain TH1, AJ968651
Wadden Sea sediment clone 4437, n = 1
Donghicola eburneus, DQ667965 ■
Roseovarius sp. HTCC2601, AATQ01000003 ☆
uncultured cold seep sediment bacterium, FJ873340
Wadden Sea sediment clone 4379, n = 1
Phaeobacter gallaeciensis, DQ915619 ☆
Citreicella sp., ACNW01000109 ★☆
Silicibacter pomeroyi, AF098491 ★☆
Wadden Sea sediment clone 4432, n = 2
Tateyamaria pelophila, AJ968651 ■★
uncultured seawater bacterium from Cheaspeake Bay, EF471666
Sagittula stellata, U58356 ★☆
Loktanella maricola, EF202613
Wadden Sea sediment clone 4396, n = 1
Oceanibulbus indolifex, ABID01000006 ☆
Sulfitobacter pontiacus, AJ542654 ★
Wadden Sea sediment clone 4375, n = 2
Roseobacter denitrificans, L01784 ■☆
Wadden Sea sediment clone 4426, n = 2
uncultured coral mucus bacterium, AY654832
Rhodobacterales bacterium HTCC2150, AAXZ01000008 ☆
Roseobacter sp. AzwK-3b, DQ223017 ☆
Roseovarius nubinhibens, DQ915625 ☆
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Wadden Sea sediment clone 4434, n = 2
Dinoroseobacter shibae, CP000830 ☆
Wadden Sea sediment clone 4419, n = 1
Wadden Sea sediment clone 4436, n = 1
uncultured North Sea sediment bacterium, DQ351770
Rhodovulum sulfidophilum, EU770422 ★☆
Wadden Sea sediment clone 4397, n = 1
Rhodomicrobium vannielii, AM691111 ★
Labrenzia aggregata IAM 12614, AAUW01000037 ★☆
Hoeflea phototrophica, ABIA02000018 ☆
Paracoccus denitrificans PD1222, CP000489 ★☆
Rhodobacter sphaeroides, DQ342321 ★☆
Alphaproteobacteria

Figure S3

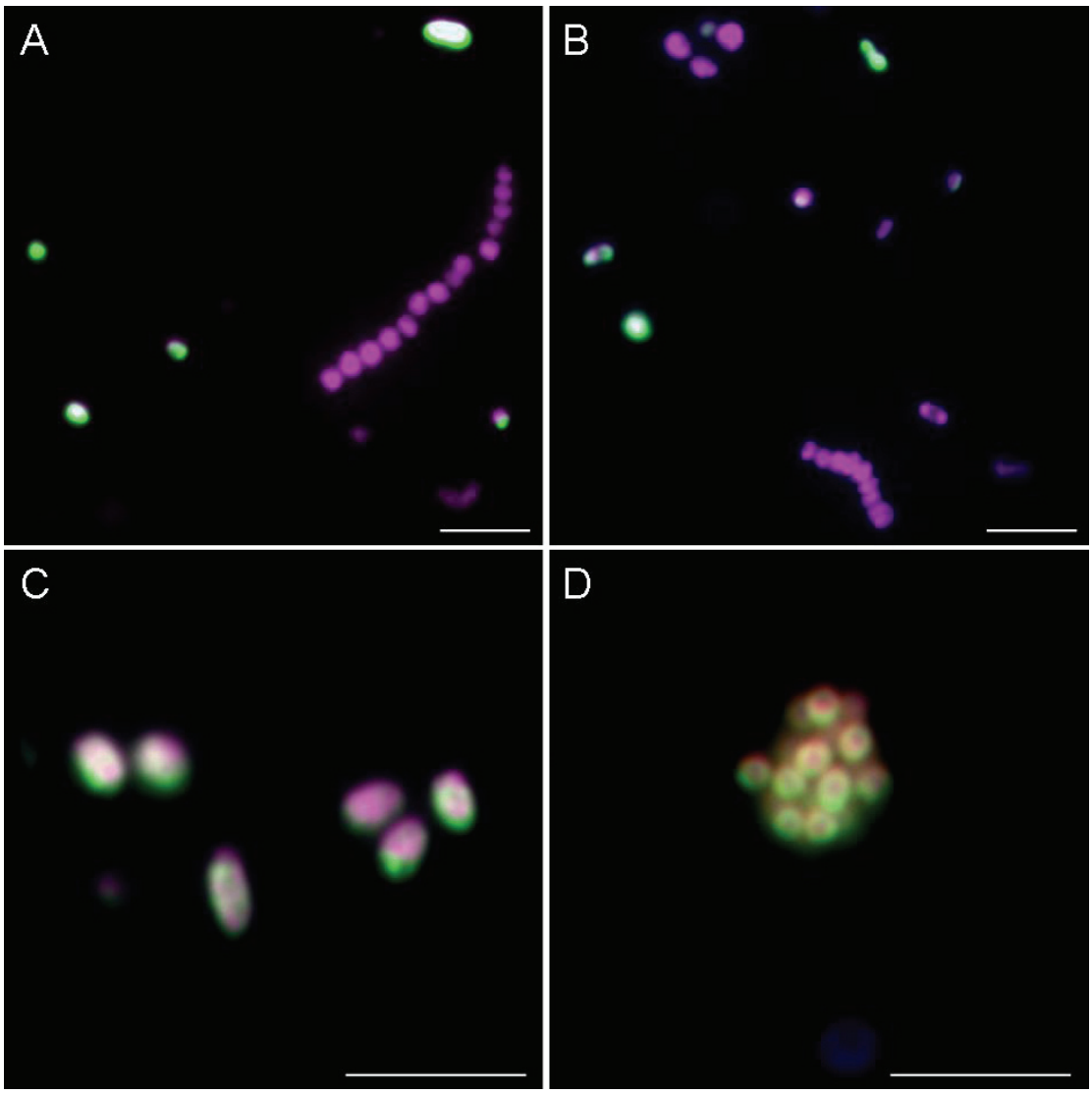

Figure S4

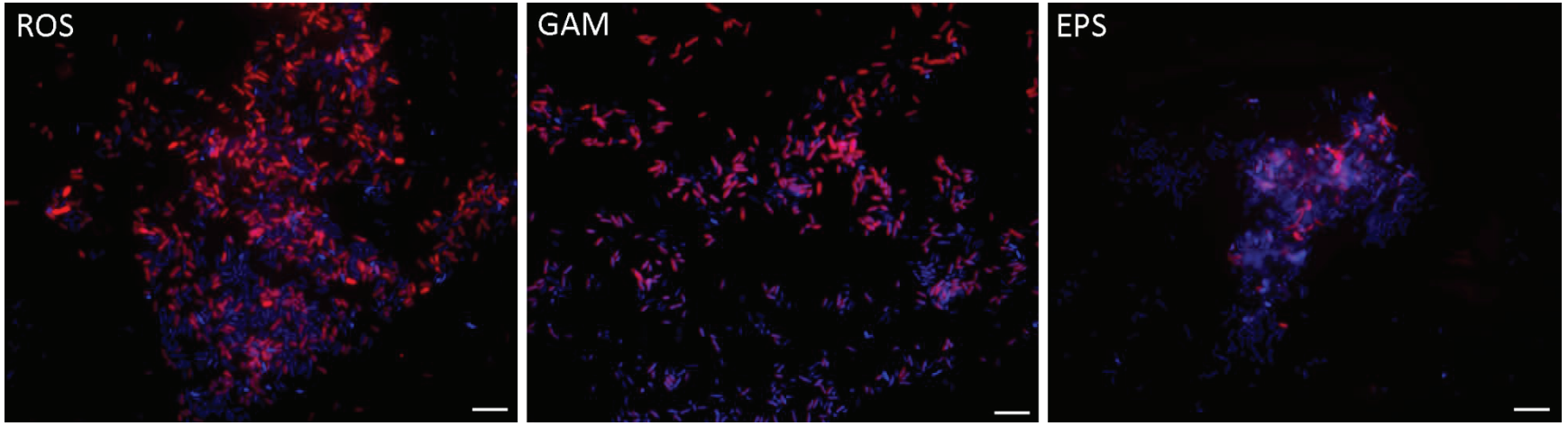

Figure S5

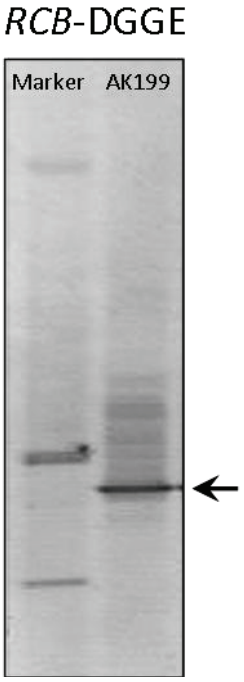

Figure S6

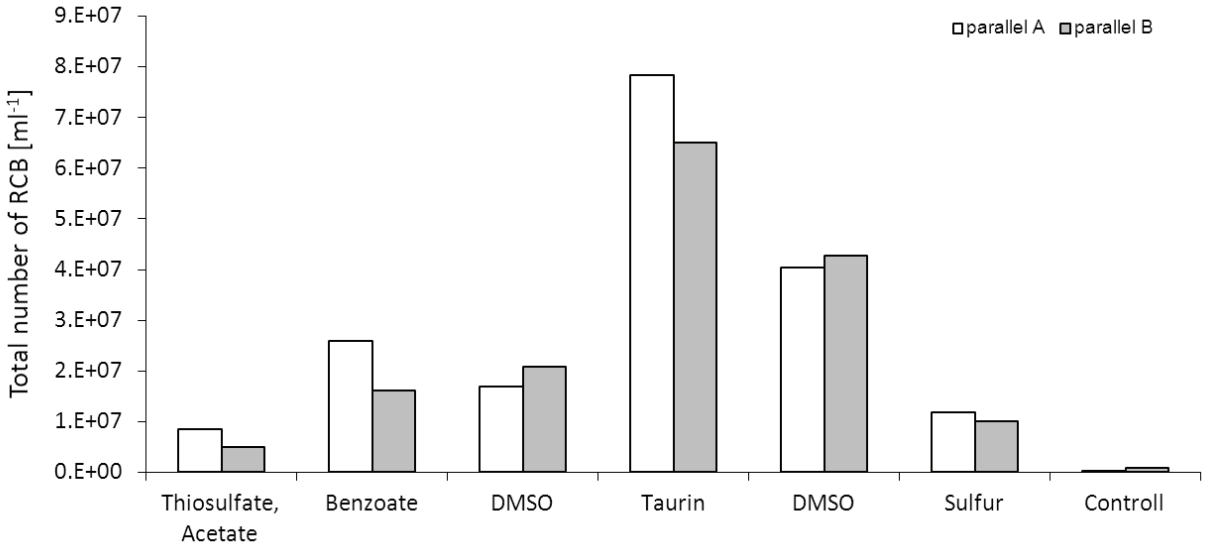

Figure S7

Table S1 Proteins predicted on additional DsrAB and SoxB encoding Wadden Sea fosmids

WS085G08, Alphaproteobacteria

ORF	AA	Product	Gene	EC
1	389	RND family efflux transporter MFP subunit		
2	1041	cation/multidrug efflux pump		
3	209	carboxymuconolactone decarboxylase		
4	468	Siroheme synthase, CysG	*cysG*	
5	67	conserved hypothetical protein		
6	67	conserved hypothetical protein		
7	262	**taurine catabolism dioxygenase (TauD)-related protein**		1.-.-.-
8	418	**reverse-type dissimilatory sulfite reductase (rDSR), alpha subunit (DsrA)**	*dsrA*	1.8.99.3
9	338	**reverse-type dissimilatory sulfite reductase (rDSR), beta subunit (DsrB)**	*dsrB*	
10	119	**DsrE sulfur transferase protein**	*dsrE*	2.8.1.-
11	130	**DsrF intracellular sulfur oxidation protein**	*dsrF*	
12	103	**DsrH**	*dsrH*	
13	110	**DsrC**	*dsrC*	2.8.1.-
14	196	**DsrM nitrate reductase gamma subunit like protein**		1.7.99.4
15	511	**DsrK iron-sulfur oxidoreductase**	*dsrK/hmeD*	
16	642	**DsrL putative glutamate synthase small subunit protein**	*dsrL*	
17	130	**DsrJ cytochrome**	*dsrJ*	
18	252	**DsrO iron-sulfur protein**	*dsrO/hmeA*	
19	412	**DsrP polysulfide reductase NrfD**	*dsrP/hmeB*	
20	474	**DsrN cobyrinic acid a,c-diamide synthase**	*dsrN*	
21	348	quinolinate synthetase complex, alpha subunit		
22	273	nicotinate-nucleotide diphosphorylase (carboxylating)		
23	518	L-aspartate oxidase		
24	451	aldehyde dehydrogenase family protein		
25	362	stage II sporulation E family protein		
26	123	protein containing sulfate transporter/antisigma-factor		
27	150	anti-sigma regulatory factor, serine/threonine protein kinase		
28	62	hypothetical protein		
29	226	two component transcriptional regulator, winged helix family		
30	487	signal transduction histidine-protein kinase BaeS		
31	146	secreted protein		
32	134	death-on-curing family protein		
33	402	hypothetical protein		
34	327	biotin/acetyl-CoA-carboxylase ligase		
35	209	acyl-phosphate glycerol-3-phosphate acyltransferase		
36	600	DegV family protein		
37	111	protein containing PspC domain		
38	100	weak similarity to glutaredoxin 2		
39	459	L-serine dehydratase 1		
40	300	peptidase M23B		
41	532	peptide chain release factor 3 (RF-3)		
42	411	Na^+ driven multidrug efflux pump		

WS020C01, Gammaproteobacteria

ORF	AA	Product	Gene	EC
1	325	**DsrN cobyrinic acid a,c-diamide synthase**	*dsrN*	
2	406	**DsrP polysulfide reductase NrfD**	*dsrP/hmeB*	
3	269	**DsrO iron-sulfur protein**	*dsrO/hmeA*	
4	126	**DsrJ cytochrome**	*dsrJ/hmeE*	
5	653	**DsrL putative glutamate synthase small subunit protein**	*dsrL*	
6	511	**DsrK iron-sulfur oxidoreductase**	*dsrK/hmeD*	
7	247	**DsrM nitrate reductase gamma subunit like protein**	*dsrM/hmeC*	1.7.99.4
8	110	**DsrC**	*dsrC*	2.8.1.-
9	102	**DsrH**	*dsrH*	
10	134	**DsrF Intracellular sulfur oxidation protein**	*dsrF*	
11	130	**DsrE sulfur transferase protein**	*dsrE*	2.8.1.-
12	357	**reverse-type dissimilatory sulfite reductase (rDSR), beta subunit (DsrB)**	*dsrB*	
13	407	**reverse-type dissimilatory sulfite reductase (rDSR), alpha subunit (DsrA)**	*dsrA*	1.8.99.3
14	260	**taurine catabolism dioxygenase (TauD)-related protein**		1.-.-.-
15	62	conserved hypothetical protein		
16	238	conserved hypothetical protein		
17	233	CRISPR-associated protein, Cas6-related		
18	114	sulfur relay protein, TusE/DsrC/DsvC family		
19	430	cyclopropane-fatty-acyl-phospholipid synthase		2.1.1.-
20	332	geranylgeranyl reductase		
21	231	membrane protein		
22	58	hypothetical protein		
23	486	transposase IS4 family protein		
24	85	hypothetical protein		
25	99	putative transposase		
26	88	hypothetical protein		
27	139	hypothetical protein		
28	112	hypothetical protein		
29	753	serine/threonine protein kinase		
30	53	hypothetical protein		
31	271	transposase		
32	51	hypothetical protein		
33	300	conserved hypothetical protein		
34	492	transposase IS66		
35	331	phage integrase family protein		
36	253	integrase/recombinase		

WS034A06, Gammaproteobacteria

ORF	AA	Product	Gene	EC
1	249	capsular polysaccharide biosynthesis protein I		
2	103	weak similarity to prophage repressor		
3	155	Rhodanese domain protein		
4	221	two component transcriptional regulator, LuxR family		
5	523	GAF sensor signal transduction histidine kinase		
6	103	**DsrR**	*dsrR*	
7	455	**Intracellular sulfur oxidation protein DsrN**	*dsrN*	
8	402	**DsrP polysulfide reductase NrfD**	*dsrP/hmeB*	
9	248	**DsrO iron-sulfur protein**	*dsrO/hmeA*	
10	159	**DsrJ cytochrome**	*dsrJ/hmeE*	
11	648	**DsrL putative glutamate synthase small subunit protein**	*dsrL*	
12	496	**DsrK iron-sulfur oxidoreductase**	*dsrK/hmeD*	
13	242	**DsrM nitrate reductase gamma subunit like protein**	*dsrM/hmeC*	1.7.99.4
14	111	**DsrC**	*dsrC*	2.8.1.-
15	98	**DsrH**	*dsrH*	
16	136	**DsrF Intracellular sulfur oxidation protein**	*dsrF*	
17	130	**DsrE sulfur transferase protein**	*dsrE*	2.8.1.-
18	357	**reverse-type dissimilatory sulfite reductase (rDSR), beta subunit (DsrB)**	*dsrB*	
19	421	**reverse-type dissimilatory sulfite reductase (rDSR), alpha subunit (DsrA)**	*dsrA*	1.8.99.3
20	313	**taurine catabolism dioxygenase (TauD)-related protein**		1.-.-.-
21	67	conserved hypothetical protein		
22	228	CRISPR-associated protein, Cas6-related protein		
23	67	conserved hypothetical protein		
24	112	sulfur relay protein, TusE/DsrC/DsvC family		2.8.1.-
25	168	hypothetical protein		
26	223	oxidoreductase, 2OG-Fe(II) oxygenase family		
27	130	conserved hypothetical protein		
28	116	hypothetical protein		
29	143	thioesterase/acetyltransferase domain protein		
30	197	putative dioxygenase (BACMed13K19 3E-61)		
31	361	conserved hypothetical protein		
32	183	conserved hypothetical protein		
33	64	conserved hypothetical protein		
34	144	Sel1 domain protein repeat-containing protein		
35	377	DsrS	*dsrS*	
36	87	Glutaredoxin, GrxC		
37	463	Siroheme synthase	*cysG*	
38	215	two component LuxR family transcriptional regulator		
39	75	similarity to redoxin domain protein		

WS138B4, Gammaproteobacteria

ORF	AA	Product	Gene	EC
1	35	hypothetical protein		
2	496	**DsrK iron-sulfur oxidoreductase**	*dsrK/hmeD*	
3	243	**DsrM nitrate reductse gamma subunit like protein**	*dsrM/hmeC*	1.7.99.4
4	111	**DsrC**	*dsrC*	2.8.1.-
5	98	**DsrH**	*dsrH*	
6	136	**DsrF Intracellular sulfur oxidation protein**	*dsrF*	
7	130	**DsrE sulfur transferase protein**	*dsrE*	2.8.1.-
8	357	**reverse-type dissimilatory sulfite reductase (rDSR), beta subunit (DsrB)**	*dsrB*	
9	421	**reverse-type dissimilatory sulfite reductase (rDSR), alpha subunit (DsrA)**	*dsrA*	1.8.99.3
10	309	**taurine catabolism dioxygenase (TauD)-related protein**		1.-.-.-
11	67	conserved hypothetical protein		
12	229	CRISPR-associated protein, Cas6-related		
13	69	conserved hypothetical protein		
14	113	sulfur relay protein, TusE/DsrC/DsvC family		
15	169	conserved hypothetical protein		
16	223	oxidoreductase, 2OG-Fe(II) oxygenase family		
17	111	conserved hypothetical protein		
18	126	hypothetical protein		
19	143	acetyltransferase		
20	197	protein containing Prolyl 4-hydroxylase, alpha subunit domain		1.14.-.-
21	361	conserved hypothetical protein		
22	188	conserved hypothetical protein		
23	64	conserved hypothetical protein		
24	165	Sel1 domain protein repeat-containing protein		
25	378	DsrS	*dsrS*	
26	463	Siroheme synthase	*cysG*	
27	224	two-component transcriptional regulator, LuxR family		
28	174	uracil-DNA glycosylase superfamily protein		
29	608	excinuclease ABC subunit C		
30	194	CDP-diacylglycerol-glycerol-3-phosphate 3-phosphatidyltransferase		2.7.8.5
31	136	conserved hypothetical protein		
32	417	DNA-directed DNA polymerase		
33	508	two-component sensor		
34	469	glutamyl-tRNA synthetase		6.1.1.17
35	563	glutaminyl-tRNA synthetase		6.1.1.18
36	147	Streptomyces cyclase/dehydrase		
37	363	uptake hydrogenase small subunit		
38	595	hydrogenase 2 large subunit		

WS172H5, Gammaproteobacteria

ORF	AA	Product	Gene	EC
1	534	acriflavin resistance protein		
2	349	Secretion protein HlyD		
3	607	ABC transporter, ATP-binding/permease protein		
4	127	weak similarity to prophage repressor		
5	155	Rhodanese domain protein		
6	222	two component transcriptional regulator, LuxR family		
7	514	GAF sensor signal transduction histidine kinase		
8	113	**DsrR**	*dsrR*	
9	459	**DsrN cobyrinic acid a,c-diamide synthase**	*dsrN*	
10	403	**DsrP polysulfide reductase NrfD**	*dsrP/hmeB*	
11	252	**DsrO iron-sulfur protein**	*dsrO/hmeA*	
12	169	**DsrJ cytochrome**	*dsrJ/hmeE*	
13	647	**DsrL putative glutamate synthase small subunit protein**	*dsrL*	
14	490	**DsrK iron-sulfur oxidoreductase**	*dsrK/hmeD*	
15	246	**DsrM nitrate reductse gamma subunit like protein**	*dsrM/hmeC*	1.7.99.4
16	110	**DsrC**	*dsrC*	2.8.1.-
17	102	**DsrH**	*dsrH*	
18	135	**DsrF Intracellular sulfur oxidation protein**	*dsrF*	
19	130	**DsrE sulfur transferase protein**	*dsrE*	2.8.1.-
20	358	**reverse-type dissimilatory sulfite reductase (rDSR), beta subunit (DsrB)**	*dsrB*	
21	418	**reverse-type dissimilatory sulfite reductase (rDSR), alpha subunit (DsrA)**	*dsrA*	1.8.99.3
22	309	**taurine catabolism dioxygenase (TauD)-related protein**		1.-.-.-
23	63	conserved hypothetical protein		
24	224	CRISPR-associated protein, Cas6-related		
25	112	DsrC-like protein		2.8.1.-
26	69	conserved hypothetical protein		
27	224	oxidoreductase, 2OG-Fe(II) oxygenase family		
28	111	conserved hypothetical protein		
29	146	galactoside O-acetyltransferase		
30	120	protein containing Forkhead-associated domains		
31	52	hypothetical protein		
32	202	similarity to 2OG-Fe(II) oxygenase		
33	352	conserved hypothetical protein		
34	145	conserved hypothetical protein		
35	70	conserved hypothetical protein		
36	149	Sel1 domain protein, repeat-containing protein		
37	358	ATPase associated with various cellular activities AAA_3		
38	446	conserved hypothetical protein		
39	374	DsrS	*dsrS*	
40	183	hypothetical protein		
41	84	Glutaredoxin, GrxC		
42	97	protein containing DUF427		
43	430	conserved hypothetical protein		
44	322	methyltransferase		

45	127	conserved hypothetical protein		
46	121	transmembrane protein		
47	569	peptidase M61 domain-containing protein		
48	276	type II and III secretion system protein		
49	170	conserved hypothetical protein		
50	141	GCN5-related N-acetyltransferase		
51	386	cupin superfamily protein		
52	193	Adenylosuccinate lyase		

WS643C01, Gammaproteobacteria

ORF	AA	Product	Gene	EC
1	28	hypothetical protein		
2	154	Rhodanese domain protein		
3	225	two component transcriptional regulator, LuxR family		
4	399	GAF sensor signal transduction histidine kinase		
5	103	**DsrR**	***dsrR***	
6	455	**DsrN cobyrinic acid a,c-diamide synthase**	***dsrN***	
7	408	**DsrP polysulfide reductase NrfD**	***dsrP/hmeB***	
8	248	**DsrO iron-sulfur protein**	***dsrO/hmeA***	
9	171	**DsrJ cytochrome**	***dsrJ/hmeE***	
10	654	**DsrL putative glutamate synthase small subunit protein**	***dsrL***	
11	496	**DsrK iron-sulfur oxidoreductase**	***dsrK/hmeD***	
12	245	**DsrM nitrate reductse gamma subunit like protein**	***dsrM/hmeC***	1.7.99.4
13	111	**DsrC**	***dsrC***	2.8.1.-
14	96	**DsrH**	***dsrH***	
15	135	**DsrF Intracellular sulfur oxidation protein**	***dsrF***	
16	130	**DsrE sulfur transferase protein**	***dsrE***	2.8.1.-
17	338	**reverse-type dissimilatory sulfite reductase (rDSR), beta subunit (DsrB)**	***dsrB***	
18	421	**reverse-type dissimilatory sulfite reductase (rDSR), alpha subunit (DsrA)**	***dsrA***	1.8.99.3
19	290	**taurine catabolism dioxygenase (TauD)-related protein**		1.-.-.-
20	67	conserved hypothetical protein		
21	237	CRISPR-associated protein, Cas6-related protein		
22	67	conserved hypothetical protein		
23	111	sulfur relay protein, TusE/DsrC/DsvC family		
24	169	conserved hypothetical protein		
25	224	oxidoreductase, 2OG-Fe(II) oxygenase family		
26	107	conserved hypothetical protein		
27	116	hypothetical protein		
28	148	galactoside O-acetyltransferase		
29	786	weak similarity to ATPase,		
30	203	putative dioxygenase (BACMed13K19 1E-62)		
31	361	conserved hypothetical protein		
32	185	conserved hypothetical protein		
33	720	two-component hybrid sensor and regulator		
34	157	protein containing NUDIX hydrolase, core domain		
35	551	phosphate uptake regulator, PhoU		
36	353	tISRso5 ISRSO5-transposase protein		
37	298	RimK-like ATP-grasp domain protein		
38	356	HPr kinase		
39	318	phosphohistidine phosphatase, SixA		
40	228	Phosphoglycerate/bisphosphoglycerate mutase		
41	147	metallophosphoesterase-like protein		

WS156CA7, Gammaproteobacteria

ORF	AA	Product	Gene	EC
1	154	weak similarity to peptidoglycan-binding protein		
2	80	weak similarity to deoxyribodipyrimidine photolyase-related protein		
3	484	weak similarity to repeat containing protein		
4	266	protein containing DUF169		
5	138	protein containing DUF35		
6	420	acetyl-CoA acetyltransferase		
7	281	hypothetical protein		
8	71	hypothetical protein		
9	194	conserved hypothetical protein		
10	62	hypothetical protein		
11	67	conserved hypothetical protein		
12	227	CRISPR-associated protein, Cas6-related		
13	239	conserved hypothetical protein		
14	263	**taurine catabolism dioxygenase (TauD)-related protein**		1.-.-.-
15	434	**reverse-type dissimilatory sulfite reductase (rDSR), alpha subunit (DsrA)**	*dsrA*	1.8.99.3
16	358	**reverse-type dissimilatory sulfite reductase (rDSR), beta subunit (DsrB)**	*dsrB*	
17	130	**DsrE sulfur transferase protein**	*dsrE*	2.8.1.-
18	137	**DsrF Intracellular sulfur oxidation protein**	*dsrF*	
19	100	**DsrH**	*dsrH*	
20	110	**DsrC**	*dsrC*	2.8.1.-
21	248	**DsrM nitrate reductse gamma subunit like protein**	*dsrM/hmeC*	1.7.99.4
22	498	**DsrK iron-sulfur oxidoreductase**	*dsrK/hmeD*	
23	650	**DsrL putative glutamate synthase small subunit protein**	*dsrL*	
24	503	similarity to repeat-containing protein		
25	151	transposase protein (IS200-family)		
26	3352	protein containing peptidase S8 and S53, Na-Ca exchanger/integrin-beta4		
27	241	weak similarity to Fibronectin type III domain protein		

WS633F06, Gammaproteobacteria

ORF	AA	Product	Gene	EC
1	583	ssDNA exonuclease RecJ		
2	359	conserved hypothetical protein, secreted		
3	108	small multidrug resistance protein		
4	341	signal peptide protein		
5	374	signal peptide peptidase SppA, 36K type		
6	113	secreted protein		
7	221	transcriptional repressor, LexA family		
8	415	DNA-directed DNA polymerase		
9	135	conserved hypothetical protein		
10	75	hypothetical protein		
11	305	putative TPR repeat-containing protein		
12	225	Crp/Fnr family transcriptional regulator		
13	191	lipoprotein		
14	186	hypothetical protein		
15	255	aspartyl/asparaginyl beta-hydroxylase		1.14.11.-
16	484	conserved hypothetical protein		
17	510	secreted protein		
18	72	hypothetical protein		
19	74	hypothetical protein		
20	112	secreted protein		
21	78	hypothetical protein		
22	348	alpha/beta hydrolase fold protein		
23	251	conserved hypothetical protein		
24	419	**reverse-type dissimilatory sulfite reductase (rDSR), alpha subunit (DsrA)**	*dsrA*	1.8.99.3
25	357	**reverse-type dissimilatory sulfite reductase (rDSR), beta subunit (DsrB)**	*dsrB*	
26	130	**DsrE sulfur transferase protein**	*dsrE*	2.8.1.-
27	133	**DsrF Intracellular sulfur oxidation protein**	*dsrF*	
28	103	**DsrH**	*dsrH*	
29	110	**DsrC**	*dsrC*	2.8.1.-
30	246	**DsrM nitrate reductse gamma subunit like protein**	*dsrM/hmeC*	1.7.99.4
31	511	**DsrK iron-sulfur oxidoreductase**	*dsrK/hmeD*	
32	340	**DsrL**	*dsrL*	

WS406H10, Alphaproteobacteria

ORF	AA	Product	Gene	EC
1	32	hypothetical protein		
2	143	**SoxS thioredoxin**	*soxS*	
3	213	**SoxV cytochrom c type membrane protein**	*soxV*	
4	194	**SoxW thioredoxin**	*soxW*	
5	170	**SoxX monoheme, SoxXA subunit**	*soxX*	
6	158	**SoxY sulfur binding protein, SoxYZ subunit**	*soxY*	
7	107	**SoxZ sulfur binding protein, SoxYZ subunit**	*soxZ*	
8	298	**SoxA diheme cytochrom c, SoxXA subunit**	*soxA*	
9	567	**SoxB sulfate thiohydrolase/thiol esterase**	*soxB*	
10	399	**SoxC molybdoprotein, sulfur dehydrogenase subunit**	*soxC*	1.8.3.-
11	366	**SoxD diheme cytochrom c, sulfur dehydrogenase subunit**	*soxD*	
12	254	**SoxE**	*soxE*	
13	429	**SoxF flavocytochrom c**	*soxF*	1.8.2.-
14	147	secreted protein containing DUF1791		
15	366	YeeE/YedE family protein		
16	357	vitamin-B12 independent methionine synthase family protein		
17	107	hypothetical protein		
18	212	similarity to transcriptional regulator, TetR family		
19	438	TRAP dicarboxylate transporter, DctM subunit		
20	186	TRAP dicarboxylate transporter, DctQ subunit,		
21	343	TRAP dicarboxylate transporter- DctP subunit		
22	936	xanthine dehydrogenase family protein, molybdopterin-binding subunit		
23	620	pyruvate synthase		
24	335	2-oxoglutarate ferredoxin oxidoreductase subunit beta		
25	110	hypothetical protein		
26	530	conserved hypothetical protein		
27	199	conserved hypothetical protein		
28	789	mechanosensitive ion channel family protein		
29	271	conserved hypothetical protein		
30	142	conserved hypothetical protein		
31	59	hypothetical protein		
32	266	conserved hypothetical protein CHP00245		
33	201	ABC transporter, ATP-binding protein,		
34	173	glutathione-dependent formaldehyde-activating, GFA		
35	608	phosphogluconate dehydratase		4.2.1.12
36	238	6-phosphogluconolactonase		3.1.1.31
37	490	glucose-6-phosphate 1-dehydrogenase		
38	302	permease		
39	152	Sel1 domain-containing protein repeat-containing protein		
40	46	hypothetical protein		

WS198A12, Gammaproteobacteria

ORF	AA	Product	Gene	EC
1	308	weak similarity to glycosyl transferase, group 2 family		
2	416	glycosyl transferase group 1		2.4.-.-
3	266	similarity to methionyl-tRNA formyltransferase		
4	263	N-acetylglucosaminyl phosphatidylinositol deacetylase		3.5.1.89
5	492	polysaccharide biosynthesis protein		
6	244	Methyltransferase type 11		2.1.1.-
7	325	polysaccharide deacetylase		3.-.-.-
8	371	asparagine synthase (glutamine-hydrolyzing)		
9	121	hypothetical protein		
10	328	NAD-dependent epimerase/dehydratase		
11	270	weak similarity to Methyltransferase type 11		2.1.1.-
12	158	putative membrane protein of eicosanoid and glutathione metabolism		
13	607	asparagine synthase (glutamine-hydrolyzing)		
14	396	protein containing Glycosyl transferase, group 1 domain		2.4.-.-
15	100	conserved hypothetical protein		
16	277	similarity to HPr kinase		
17	216	similarity to fibronectin type III domain protein		
18	175	bacterioferritin		
19	69	protein containing BFD-like [2Fe-2S]-binding region domain		
20	259	rhamnolipids biosynthesis 3-oxoacyl-(acyl-carrier-protein) reductase		
21	470	secreted protein		
22	462	methylamine utilization protein MauG,		
23	178	similarity to TonB-dependent receptor protein		
24	97	Fructose-1,6-bisphosphate aldolase		
25	474	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	*cbbL*	
26	118	Ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit	*cbbS*	
27	277	CbbQ/NirQ/NorQ domain protein		
28	791	Rubisco activation protein CbbO	*cbbO*	
29	232	Phosphoglycolate phosphatase (PGPase)		3.1.3.18
30	587	**SoxB sulfate thiohydrolase/thiol esterase**	*soxB*	
31	127	conserved hypothetical protein		

Table S2 Taxonomic assignment of Wadden Sea sediment fosmids according to DsrAB phylogeny and SoxB phylogeny

Fosmid	Affiliation	Closest related sequences[a]	[%][b]	Closest known relative	[%][b]
DsrAB					
WS101A12	*Alphaproteobacteria*	Enrichment culture clone AK199	93	*Rhodomicrobium vannielii* (ZP06348259)	78
WS085G08	*Alphaproteobacteria*	Wadden Sea sediment clone d1_B6	99	*Rhodomicrobium vannielii* (ZP06348259)	77
WS643C01	*Gammaproteobacteria*	Wadden Sea sediment clone JS37_8	99	*Thiothrix nivea* (EU155048)	85
WS138B04	*Gammaproteobacteria*	Wadden Sea sediment clone JS24_31	93	*Thiothrix nivea* (EU155048)	83
WS034A06	*Gammaproteobacteria*	Wadden Sea sediment clone JS24_31	95	*Thiothrix nivea* (EU155048)	83
WS156A07	*Gammaproteobacteria*	Wadden Sea sediment clone JS37_13	97	*Olavius algarvensis* Gamma 1 symbiont (AASZ01004984)	83
WS020C01	*Gammaproteobacteria*	Wadden Sea sediment clone JS24_43	99	*Thiobios zoothamnicolii* (ACI42952)	88
WS633F06	*Gammaproteobacteria*	Wadden Sea sediment clone d1_A2	99	*Olavius algarvensis* Gamma 1 symbiont (AASZ01004984)	86
WS172H05	*Gammaproteobacteria*	Wadden Sea sediment clone d1_A2	84	*Olavius algarvensis* Gamma 1 symbiont (AASZ01004984)	87
SoxB					
WS101A12	*Alphaproteobacteria*	*Rhodobacterales* bacterium HTCC 2654 NZ_AAMT01000015	84	*Sagittula stellata* (NZ_AAYA01000019)	86
WS406H10	*Alphaproteobacteria*	Wadden Sea sediment clone 098	92	*Hoeflea phototrophica* (NZABIA02000017)	75
WS198A12	*Gammaproteobacteria*	Wadden Sea sediment clone 030	100	*Thiorhodococcus minor* (EF618606)	72

a. Closest related sequence inferred from Maximum Likelihood phylogenetic reconstruction (RAxML)
b. Amino acid sequence identity in %

Table S3 Parameter of the *dsrA*-targeted polynucleotide probe applied in geneFISH

Sequences[a]	Affiliation[b]	Tm °C at 975mM Na	Tm °C at 1718mM Na	% Identity[c]	% MM[d]	Target
WS101A12	*Alphaproteobacteria*	74.5	76.6	100	0	target
Enrichment clone AK199_3a	*Alphaproteobacteria*	66.7	68.8	92.2	7.8	target
Namibia clone	*Alphaproteobacteria*	52.8	54.9	78.2	21.8	nontarget
R. vannielii	*Alphaproteobacteria*	50.5	52.7	76.0	24.0	nontarget
Enrichment clone AK199_1	*Gammaproteobacteria*	41.6	43.8	67.1	32.9	nontarget

a. *dsrA*-sequences analyzed for probe match with *dsrA* targeted polynucleotide probe
b. Taxonomic affiliation based on reconstruction of DsrAB phylogeny as determined by Maximum Likelihood method (RAxML)
c. Number of identical nucleotides between *dsrA*-sequences and polynucleotide probe in %
d. Number of mismatches between *dsrA*-sequences and polynucleotide probe in %

4. Acetate Assimilating Microbes in Coastal Sediments

Contributions to the study:

Sabine Lenk: conducted MAR-FISH analysis of June 2009 and October 2010 samples; conducted VFA extraction, conducted fluorescence activated cell sorting and nanoSIMS analysis, performed data analysis and processing, developed the concept of the manuscript, wrote the manuscript

Katrice Zerjatke: conducted MAR-FISH analysis of October 2008 samples; assisted in MAR-FISH analysis of June 2009 and October 2009 samples

Marc Mußmann: performed beta-imaging analysis

Sabine Lenk, Rudolf Amann and Marc Mussmann: designed research, discussed data, conceived the manuscript, edited the manuscript

Patterns of Acetate Incorporation in Permeable Intertidal Sediments Revealed at the Single Cell Level

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Running title: Acetate assimilating microbes in permeable coastal sediments

Key words: Acetate, FISH, permeable sediments, microautoradiography

Summary

The mineralization of deposited organic matter is central to carbon cycling in coastal sediments. During aerobic and anaerobic degradation organic acids such as acetate are prominent intermediates. While it is a widely held assumption that in marine, anoxic surface sediments acetate is mainly consumed by sulfate reducing organisms, the identity and abundance of microbes that actively participate in benthic carbon turnover is still largely unknown at the single cell level. We combined microautoradiography and fluorescence *in situ* hybridization (MAR-FISH) to study the incorporation of ^{14}C-acetate by defined clades of the microbial community of an intertidal sandflat. Up to 17% of all cells (1.9×10^8 cells ml^{-1}) incorporated ^{14}C-acetate under oxic conditions. In contrast, only 6–10% of all cells incorporated acetate under anoxic conditions. Beta-imaging of the upper 10 mm of intact sediment cores revealed highest incorporation in the top 4 mm of the sediment and support a mainly oxygen-dependent assimilation. Consistently, the proportion of substrate incorporating cells rapidly decreased with depth. Members of the *Gammaproteobacteria* were the most abundant acetate consumers in the oxygenated surface layer accounting for 67% of all acetate-incorporating cells. In addition, we observed acetate incorporation by *Roseobacter* clade bacteria (RCB). No significant incorporation by sulfate reducing members of the *Deltaproteobacteria* could be detected. To confirm our results we developed a protocol to perform nano-scale secondary ion mass spectrometry (nanoSIMS) on acetate-assimilating microbes. We combined density gradient centrifugation, CARD-FISH and flow cytometry. Preliminary nanoSIMS measurements of cells from ^{13}C-acetate incubated sediment supported our MAR-FISH results. Distinct ^{12}C/^{13}C carbon isotope ratios indicated that individual gammaproteobacterial cells assimilated more ^{13}C-acetate than individual RCB. Our study provides a first high-resolution analysis of single cells that contribute to acetate turnover in marine sediments

Introduction

Permeable sandy sediments cover vast areas of the continental shelf. Particularly in tidal zones they act as giant natural filters that mineralize infiltrated organic matter efficiently (de Beer *et al*., 2005; Anschutz *et al*., 2009). In contrast to muddy sediments, they are characterized by increased oxygen penetration depths and enhanced benthic primary production of microphytobenthos communities (Billerbeck *et al*., 2007; Evrard *et al*., 2010). Advective processes transport suspended particles into the surface layers and provide organic carbon and nutrients to complex sediment microbial communities (Ishii *et al*., 2004; Hunter *et al*., 2006; Musat *et al*., 2006). While the flow of organic carbon through the benthic food web has previously been investigated (Cook *et al*., 2007; Chipman *et al*., 2010; Evrard *et al*., 2010), studies that identify microorganisms actively involved in crucial mineralization processes are still scarce. A key intermediate in the degradation of organic matter is acetate (Christensen and Blackburn, 1982; Wellsbury and Parkes, 1995). It forms anaerobically as major product from fermentation of hydrolyzed macromolecular material and constitutes a primary substrate for terminal oxidation processes, particularly sulfate reduction (Sørensen *et al*., 1981; Christensen, 1984; Parkes *et al*., 1989; Finke *et al*., 2007; Valdemarsen *et al*. 2010). Accordingly, it has previously been shown to stimulate rates of oxygen consumption and sulfate reduction in permeable sediments (Werner *et al*., 2006). Sulfate reducing prokaryotes (SRP) use acetate as energy and/or carbon source during chemoorganoheterotrophic or chemolithoheterotrophic growth (Rabus *et al*., 2006). Similarly methanogens are at the end of the anaerobic food chain and use acetate as energy source. Due to rapid turnover organic fermentation products do not accumulate keeping concentration in the natural habitat low. In coastal sediments acetate pore water concentrations of 5 – 100 µM have been reported (Finke *et al*., 2007; Sawicka *et al*., 2009). Previous studies on coastal sites found enhanced microbial incorporation of acetate particularly after deposition of phytoplankton blooms (Meyer-Reil, 1987; Wu *et al*., 1997).

So far, stable isotope probing (SIP) has provided novel insights into the identity of bacteria that degrade simple carbon compounds such as glucose and fatty acids (Boschker *et al*., 2001; MacGregor *et al*., 2006; Webster *et al*., 2006; Miyatake *et al*., 2009; Webster *et al*., 2010). Although the technique proved a powerful tool in obtaining the rRNA gene sequences of metabolically active (nucleic acid synthesizing) organisms, it has several limitations. The general PCR bias, for example, can lead to under- or overrepresentation of phylogenetic groups. Similarly, contamination of the heavy nucleic acid fraction with nucleic acids from the light fraction can cause false-positive results. Usually, long incubation times and high substrate concentrations are required which can lead to selective enrichment of certain populations and cross-feeding of organisms that are not primary substrate consumers (Dumont, 2005; Wagner *et al*., 2006). While experiments might be optimized so that the incubation conditions resemble those *in situ* more closely (Friedrich, 2006), SIP does generally not allow for an enumeration of substrate assimilating organisms. Thus, even though a number of studies already traced the fate of acetate in marine sediments, nothing is known about the abundance of acetate assimilating microbes. In addition, most studies focused on muddy sites and sulfate reducing conditions (Boschker *et al*., 2001; MacGregor *et al*., 2006; Webster *et al*., 2006; Miyatake *et al*., 2009; Webster *et al*., 2010).

Here, we investigated the incorporation of acetate in sandy sediments of a coastal intertidal flat situated in the German Wadden Sea (site Janssand). The site is characterized by high nutrient concentrations,

high aerobic respiration rates and intense sulfate reduction (Billerbeck *et al*., 2006; Al-Raei *et al*., 2009). As tidal cycles lead to periodic exposure and inundation of the flat, the surface layers of the sediment face fluctuating oxygen concentrations. Using microautoradiography (MAR), beta-imaging, and nano-scale secondary ion mass spectrometry (nanoSIMS) we studied the assimilation of acetate in oxic and anoxic slurries and intact cores. The assimilation of isotopically labeled acetate was analyzed at the single cell-level by combining whole-cell fluorescence *in situ* hybridization coupled catalyzed reporter deposition (CARD-FISH) with MAR and by halogen *in situ* hybridization coupled secondary ion mass spectrometry (HISH-SIMS). We quantified (i) the proportion of the total microbial community that actively assimilates acetate in different sediment layers and we identified (ii) phylogentic groups and specific populations that account for the observed incorporation.

Results

We adapted the MAR-FISH protocol of Alonso and Pernthaler (2005) and applied a set of 14 different oligonucleotide probes (Table 1) to trace acetate-assimilating phylogenetic groups and populations. The incorporation of acetate by the microbial community from two different layers under oxic and anoxic conditions was quantified in October 2008 and October 2009. Slurries from oxygenated surface (0–1 cm depth) and deeper anoxic layers (10–11 cm depth) were incubated with 100 µM ^{14}C-acetate under oxic and anoxic conditions, respectively. The incorporation of substrate over time was studied in oxic slurries in June 2009. In addition, combined analysis of MAR-FISH and beta-imaging revealed a depth-dependent incorporation in intact sediment cores. Here, preliminary HISH-SIMS analysis indicated differential incorporation by distinct microbial populations.

Table 1 Oligonucleotide probes applied for detection of acetate incorporation by different phylogenetic groups

Probe name	Specificity	Sequence (5' - 3')	Target site (*E. coli*)	FA[a]	MAR[b]	Reference[c]
GAM42a[c]	*Gammaproteobacteria*	GCC TTC CCA CAT CGT TT	1027 – 1043 (23S)	35	+	Manz, *et al.*,1992
NOR5-730[c]	NOR5/OM60 clade *Gammaproteobacteria*	TCG AGC CAG GAG GCC GCC	730 – 747	50	-	Eilers *et al.*, 2001
NOR5-1238[c]	NOR5/OM60 clade *Gammaproteobacteria*	CCC TCT GTG CGT TCC ATT	1238 – 1255	50	-	Yan *et al.*, 2009
WS-Gam209[c]	some Wadden Sea *Gammaproteobacteria*	CTA CTA GTG CCA GGT CCG	209 – 227	25	-	Lenk *et al.*, 2011
WS-Gam446[c]	some Wadden Sea *Gammaproteobacteria*	ACC CGC AAC TGT TTC CTC	446 – 462	20	-	Lenk *et al.*, 2011
WS-Gam1030[c]	some Wadden Sea *Gammaproteobacteria*	CCT GTC AAC CAG TTC CCG	1030 – 1048	25	-	Lenk *et al.*, 2011
WS-Gam843	some Wadden Sea *Gammaproteobacteria*	CTG CAC CAC TGA GAC CTC	843 – 861	20	-	Lenk *et al.*, 2011
ROS537	*Roseobacter* clade bacteria	CAA CGC TAA CCC CCT CC	537 – 553	35	+	Eilers *et al.*, 2000
DELTA495a DELTA495b DELTA495c	*Deltaproteobacteria*	AGT TAG CCG GTG CTT CCT AGT TAG CCG GCG CTT CCT AAT TAG CCG GTG CTT CCT	495 – 512	35	+	Loy *et al.*, 2001
DSS658	*Desulfobacteraceae*	TCC ACT TCC CTC TCC CAT	658 – 675	35	-	Manz, *et al.*, 1998
CF319a	*Bacteroidetes*	TGG TCC GTG TCT CAG TAC	319 – 336	35	-	Manz, *et al.*,1996
PLA46	*Planctomycet*ales	GAC TTG CAT GCC TAA TCC	46 – 63	30	-	Neef, *et al.*, 1998
NON338	control probe	ACT CCT ACG GGA GGC AGC	338 – 355	10	-	Wallner, *et al.*, 1993

a. Formamide (v/v) in hybridization buffer at 46 °C hybridization temperature

b. Microautoradiography combined FISH: '+' incorporation detected; '-' no incorporation observed

c. Competitor and helper oligonucleotides were used according to reference, see also www.microbial-ecology.net/probebase for further information.

Bulk acetate incorporation in sediment cores

In June 2009, three intact sediment cores were sampled for volatile fatty measurements and assessment of bulk acetate incorporation rates. *In situ* concentrations of volatile fatty acids strongly varied throughout the upper 10 cm of the profile and between replicate cores. Concentration of acetate and lactate did not exceed 10 µM. Concentrations of formate were below 5 µM (SI Fig. 1).

Bulk incorporation rates in the ^{14}C-acetate percolated core (Fig. 1) were highest at the surface reaching 7.6 ± 0.7 µmol l^{-1} h^{-1} (0–1cm). They steeply decreased to 1.6 ± 0.2 µmol l^{-1} h^{-1} in the layer immediately below (1–2cm) and accounted for only 0.2 ± 0.01 µmol l^{-1} h^{-1} in 9–10cm depth.

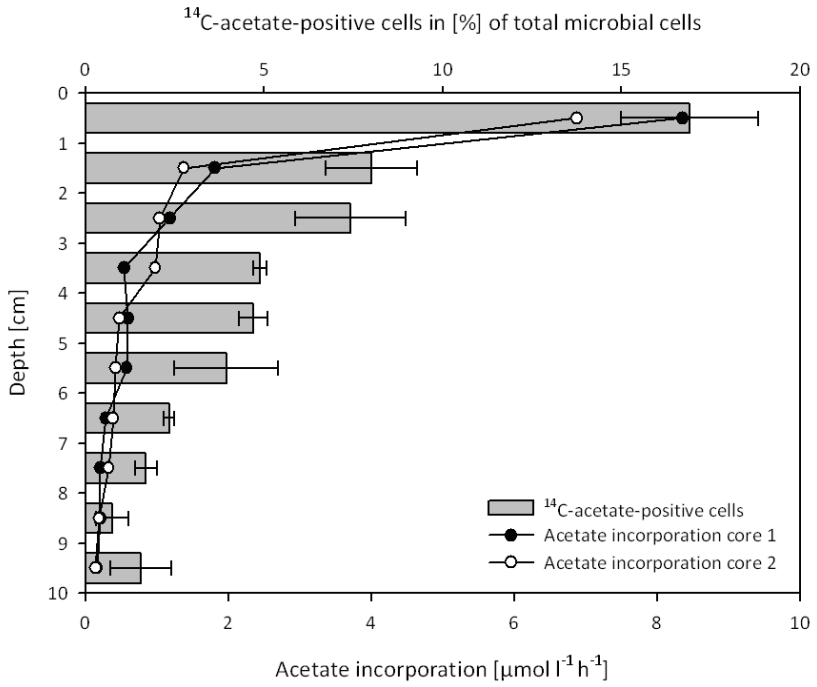

Figure 1 Total acetate incorporation and relative abundances of ^{14}C-acetate assimilating microbial cells over the vertical sediment profile in June 2009.

Total incorporation (-●-) is measured on the bottom axis and relative abundance (■ bars) on the top axis. Bars illustrate mean values of duplicate incubations. Error bars indicate data range.

To obtain a higher vertical resolution of acetate incorporation, small sediment cores were percolated with ^{14}C-acetate spiked seawater in the field. Acetate incorporation was measured by beta-imaging in two replicates of vertical slices of the upper cm (Fig. 2). Beta-imaging analysis revealed that acetate incorporation was highest in the top 4 mm of the sediment. Below 4 mm a sharp decrease to approximately 50% of the highest radiation measured was observed.

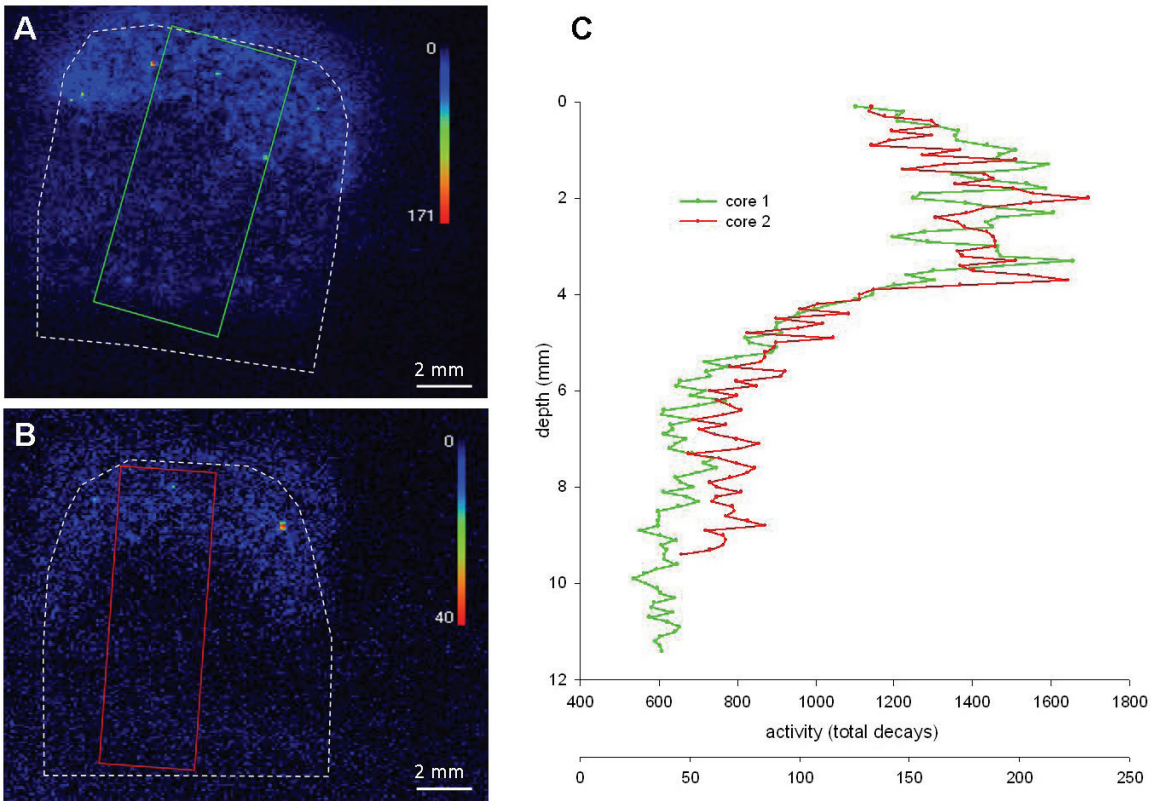

Figure 2 Acetate incorporation in the upper 10 mm of the sediment in June 2009 revealed by beta-imaging analysis. Green and red frames indicate scanned areas and refer to plots in the right panel. Measurements were not quantitative.

Acetate incorporation by the microbial community

The assimilation of acetate by the microbial community differed greatly between oxic and anoxic conditions and depth layers. Consistent with bulk incorporation the relative abundance of acetate assimilating cells strongly decreased with depth (Fig. 1). At the surface 17% of all cells incorporated acetate compared to 8% in the layer below (1–2 cm) and 2% in the deepest sediment layer (9–10 cm).

Parallel to the core incubation we assessed the bulk acetate incorporation and the relative abundance of acetate-incorporating cells over time in oxic slurries (SI Fig. 2). A significant increase of acetate incorporation occurred only in the first hour of incubation. The concentration of assimilated acetate amounted to 12.9 ± 0.6 µmol l^{-1} after 0.5 h and reached 18.4 ± 0.7 µmol l^{-1} after 1 h and 17.2 ± 1.1 µmol l^{-1} after 6 h. A maximum of 11 ±0.4% and 11 ±2% of all cells were labeled after 0.5 h and 1 h, respectively, compared to 14 ± 0.1% after 6 h. The proportion of labeled cells was comparable to those detected at the surface of the core (13.6 ± 0.1% of total cells in the slurry after 6 h compared to 17 ± 2% of total cells in the core after 8 h). The calculated cell-specific assimilation rate amounted to 0.46 ± 0.1 fmol acetate $cell^{-1}$ h^{-1}.

In slurry incubations from October 2008 and 2009 a higher proportion of cells incorporate acetate under oxic conditions, regardless whether the sediment originated from 0–1 or 10–11cm depth (Fig. 3).

Respectively, 16% and 17% of all cells from 0–1 cm and 12% and 15% of cells from 10–11 cm depth were labeled. Overall, the relative number of acetate-positive cells after oxic incubations was similar in originally anoxic and oxic sediments. After incubation of sediment from the oxic surface under anoxic conditions 12% of all cells were labeled, compared to 16% of all cells when incubated oxically (October 2009, SI Fig. 3). In anoxic incubations of sediment from 10–11 cm depth only 6% and 10% of all cells incorporated the substrate (Fig. 3). After prolongation of incubation time to 24 h under anoxic conditions (October 2009) a similar proportion of acetate-positive cells was detected as when incubated 6 h (10 ± 1% of all cells labeled, SI Fig. 3).

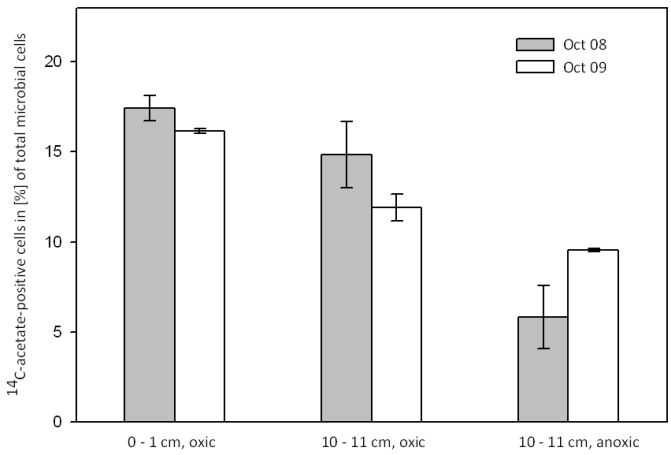

Figure 3 Relative abundances of ^{14}C-acetate incorporating microbial cells in slurries of October 2008 and October 2009.

Sediment from 0-1 cm and 10-11 cm depth was incubated oxically. Sediment from 10-11 cm depth was also incubated anoxically. Bars illustrate mean values of duplicate incubations.

Acetate assimilation by members of the Gammaproteobacteria

We assessed the proportion of acetate-assimilating phylogenetic groups in the top 2 cm of the core incubation (Fig. 1). *Gammaproteobacteria* accounted for 60% (0–1 cm) and 57% (1–2 cm) of all acetate-incorporating cells, respectively (Table 2, additional layers not assessed). Respectively, 19% (1.1×10^8 cells ml^{-1}) and 12% (5.2×10^7 cells ml^{-1}) of all *Gammaproteobacteria* were labeled. A similar proportion of substrate incorporating *Gammaproteobacteria* was detected in the oxic slurry of the time-series experiment (Table 2).

In the October slurries, *Gammaproteobacteria* accounted for the majority of acetate-assimilating cells under oxic conditions. They comprised 51% of all labeled cells in October 2008 (Fig. 4) and 67% of all labeled cells in October 2009 (Table 2). Under anoxic conditions less *Gammaproteobacteria* incorporated acetate accounting for 25-39% of all labeled cells. Between 9% and 12% of all gammaproteobacterial cells assimilated acetate under oxic conditions versus 8% under anoxic conditions.

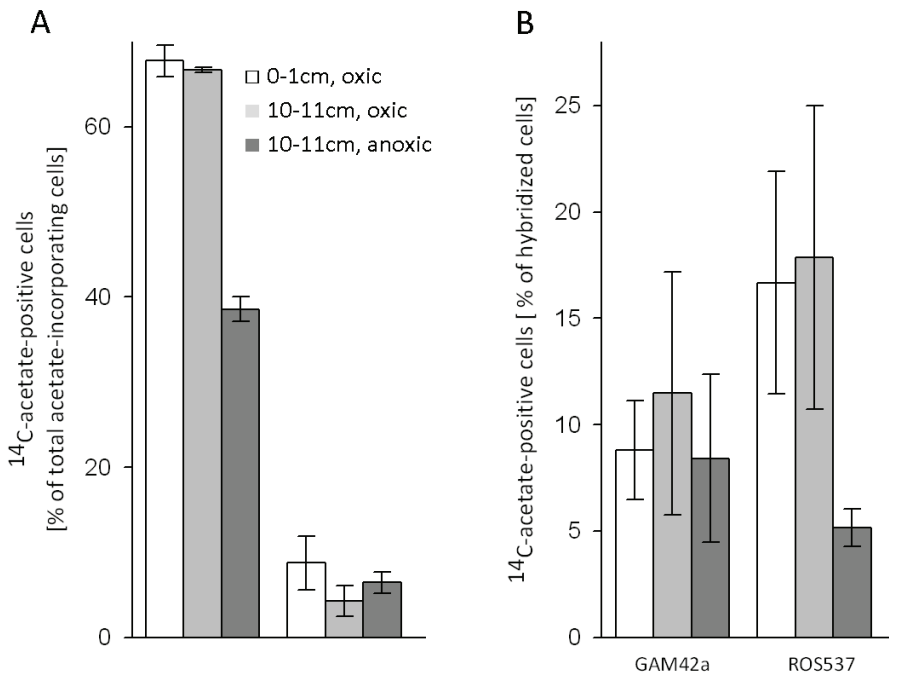

Figure 4 Relative abundances of ^{14}C-acetate incorporating members of Gammaproteobacteria and RCB in slurries of October 2008.

The proportions of ^{14}C-acetate-positive target cells (GAM42a, ROS537) are given in percent of A) total ^{14}C-acetate incorporating micro-bial cells and B) total target cells hybridized to the probe. Bars illustrate mean values of duplicate incubations. GAM42a, *Gammapro-teobacteria,* ROS537, RCB

Table 2 Relative abundances of ^{14}C-acetate incorporating phylogenetic groups in incubations of June and October 2009

Incubation	Target cells	^{14}C-acetate positive target cells as % of total ^{14}C-acetate positive cells		^{14}C-acetate positive target cells as % of hybridized target cells	
Slurry - Oct 09		0–1cm, oxic	10–11cm, anoxic	0–1cm, oxic	10–11cm, anoxic
	GAM42a	51 ± 1	25 ± 2	12 ± 1	8 ± 1
	ROS537	3 ± 0.4	*not detected*	13 ± 1	*not detected*
Core - June 09		0–1 cm	1–2cm	0–1 cm	1–2cm
	GAM42a	60 ± 1	57±2	19± 0.2	12±0.6
	ROS537	7 ± 1	4±1	40± 8	13±6
	DEL495	6 ± 2	5±1	8 ± 1	4±0.3
Slurry - June 09		0–1 cm, oxic		0–1 cm, oxic	
	GAM42a	47 ± 0.3		20 ± 4	

GAM42a - *Gammaproteobacteria*; ROS537- *Roseobacter* clade bacteria; DEL495 - *Deltaproteobacteria*; DSS658 *Desulfosarcina*-related cells

Acetate assimilation by additional phylogenetic groups

Roseobacter clade bacteria (RCB) constituted 7% of all acetate consuming cells at the top centimeter of the core (Table 2). Here, 40% (1.2×10^7 cells ml^{-1}) of all RCB were labeled. In contrast, they accounted for only 4% of all acetate consuming cells in 1–2cm depth were a smaller fraction of 13% (4.1×10^6 cells ml^{-1}) of all RCB was labeled.

In the oxic slurries RCB accounted for 3 to 9% of labeled cells (Fig. 4, Table 2). Up to 18% of all RCB were labeled under oxic but only 5% under anoxic conditions (October 2008). No labeled RCB cells were detected in the anoxic slurries of October 2009. We could not detect assimilation of acetate by *Desulfosarcina*-relatives (probe DSS658) in incubations of October 2008, October 2009 (slurries oxic, anoxic) and June 2009 (core). Similarly, the proportion of acetate incorporating *Deltaproteobacteria* was low (Table 2). Cells of other abundant groups such as *Bacteroidetes*, *Planctomycetes* and *Gammaproteobacteria* of the NOR5/OM60 clade did not incorporate acetate.

Acetate assimilation of single cells revealed by nanoSIMS

As varying size and number of silver precipitates around individual cells of *Gammaproteobacteria* and some RCB already indicated high variability in the amounts of assimilated acetate, we attempted to visualize the differences in substrate incorporation at higher resolution. To compare the amount of incorporated ^{13}C-acetate by distinct populations we performed semi-quantitative analysis using nanoSIMS (Fig. 5).

Generally, high particle background and low abundance of target cells hamper nanoSIMS of sediment microorganisms (N. Musat., pers. communication). To enable an efficient analysis, *Gammaproteobacteria* and RCB cells were detached from ^{13}C-acetate incubated sediment, separated and flow-sorted. We extracted target cell fractions of high purity by combining density gradient centrifugation with fluorescence activated cell sorting and obtained samples free of contaminating background particles (SI Fig. 4). In addition, flow cytometry allowed dense spotting of target cells on membrane filters, which enabled the analysis of several cells in a single scan. Sorted target populations were identified by a good fluorine signal in the majority of cells (Fig. 5). Occasionally cells exhibited weak fluorine signals, but the high sorting specificity (95-97%) and virtual absence of organic background particles allowed an identification of target cells based on $^{12}C^{14}N^-$ and $^{32}S^-$ (Fig. 5). Among the analyzed target populations approximately 40% of *Gammaproteobacteria* and 18% of RCB incorporated ^{13}C-acetate. Gammaproteobacterial cells were more enriched in ^{13}C (values of 0.03 – 0.09) than RCB (values of 0.013–0.022). These data are only preliminary and based on analysis of 28 cells of *Gammaproteobacteria* and 17 cells of RCB.

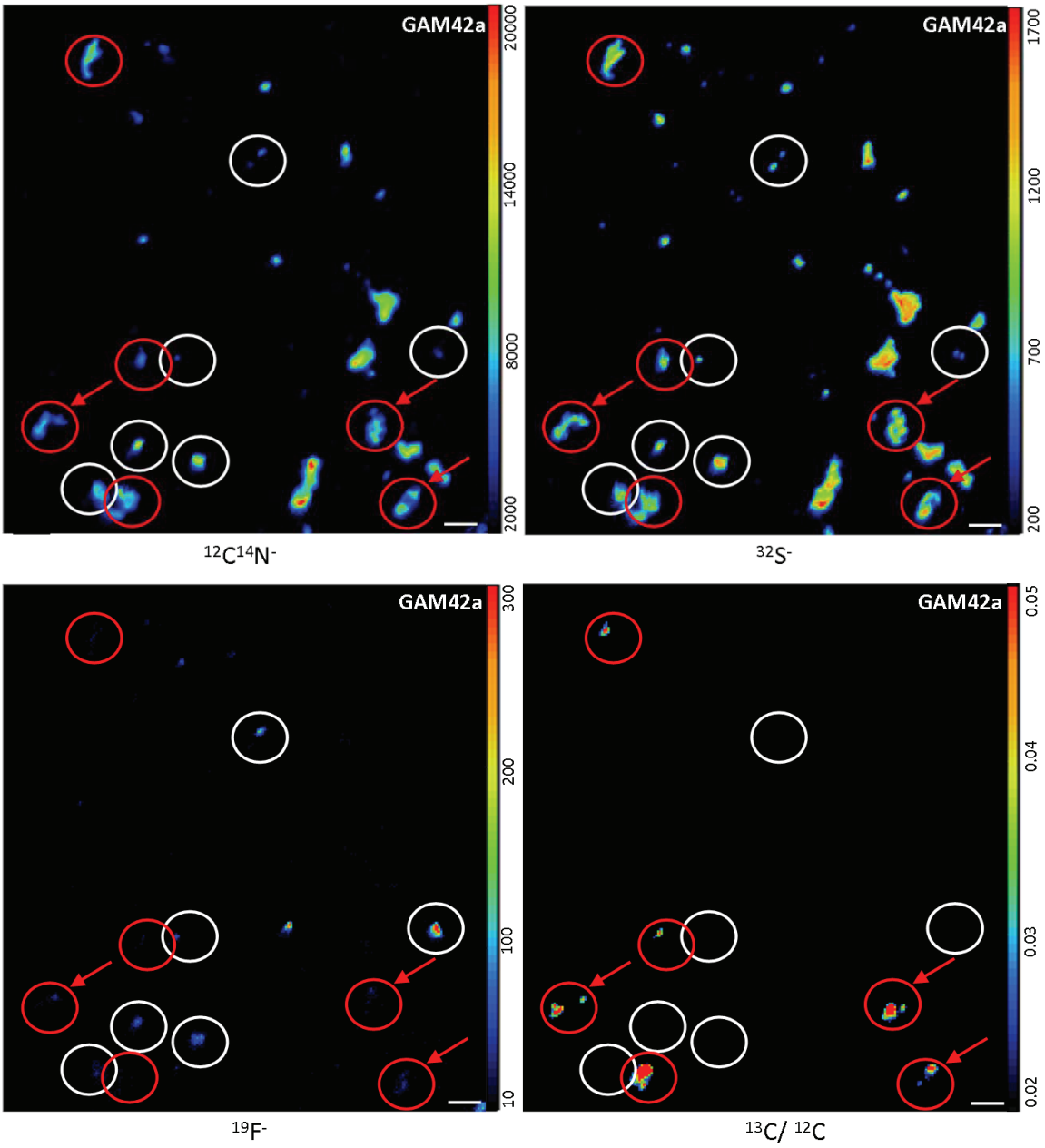

Figure 5A ^{13}C-acetate incorporation by individual cells of *Gammaproteobacteria* (GAM42a) revealed by nanoSIMS. The abundances of $^{12}C^{14}N^-$, $^{32}S^-$, $^{19}F^-$ and the $^{13}C/^{12}C$ ratios are shown. Circles indicate cells that were enriched in ^{13}C-acetate (red circles) or not (white circles). Red arrows indicate cells with visible fluorine signal.

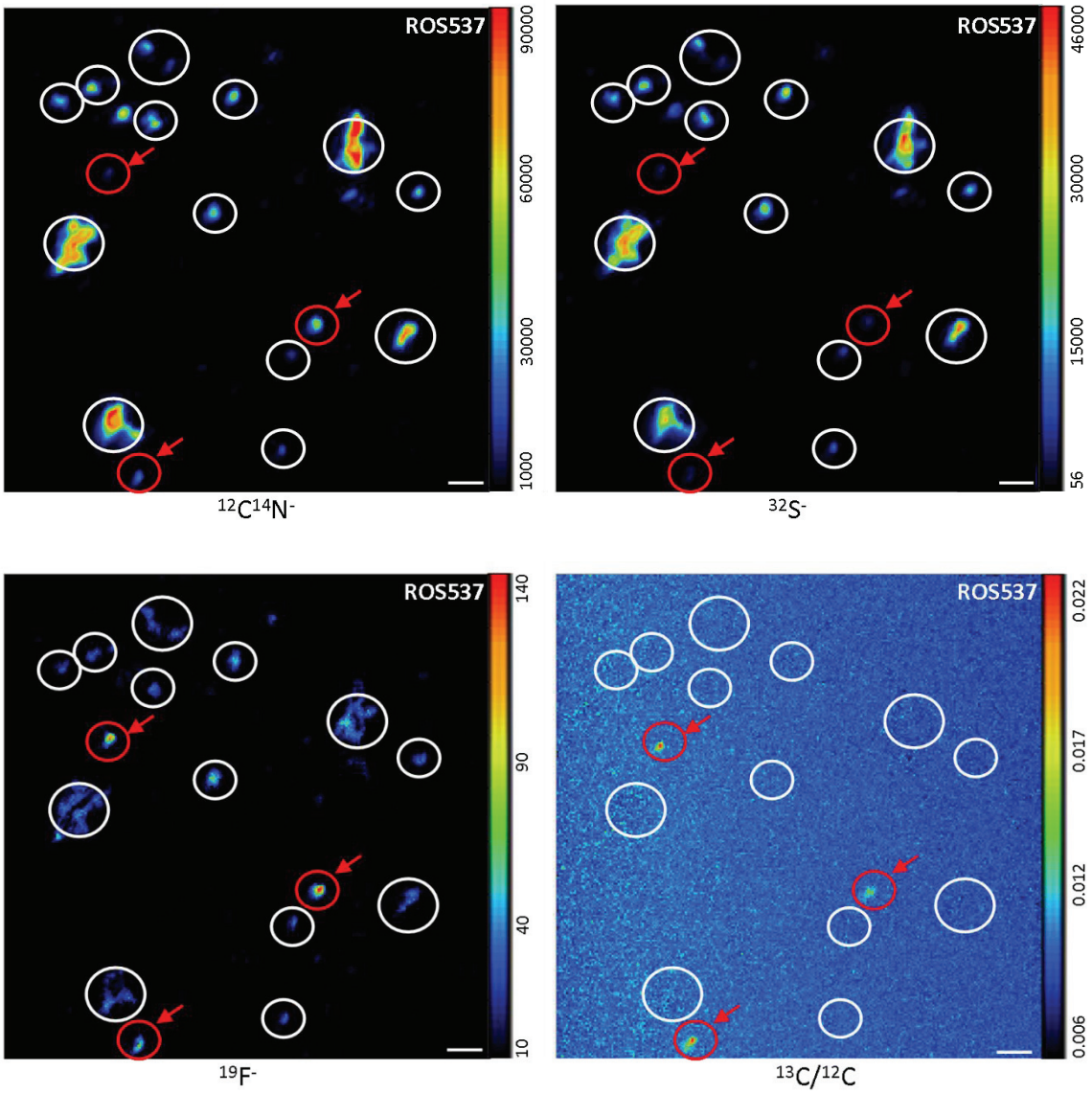

Figure 5B ^{13}C-acetate incorporation by individual RCB (ROS537) revealed by nanoSIMS.
The abundances of $^{12}C^{14}N^-$, $^{32}S^-$, $^{19}F^-$ and the $^{13}C/^{12}C$ ratios are shown. Circles indicate cells that were enriched in ^{13}C-acetate (red circles) or not (white circles). Red arrows indicate cells with visible fluorine signal.

Discussion

We combined MAR-FISH and nanoSIMS to study key transformations of coastal carbon cycling. Here, we identified and quantified microorganisms that participate in the turnover of acetate in a coastal intertidal sandflat.

Specificity of acetate incorporation in sediment

Within marine sediment acetate has been found to adsorb to organics and solid surfaces (Wellsbury and Parkes, 1995). This retractile acetate is of only limited availability to microbial metabolism but might cause false positive background radioactivity. We combined slurry incubations with core percolation experiments of ^{14}C-acetate to study the assimilation of acetate by the microbial community. We found

no evidence for complexation of ^{14}C-acetate in inactivated sediment samples. Scintillation counts in the prefixed controls accounted for <1% of those measured in active samples. In addition, no silver grain precipitates were detected in the prefixed controls after MAR-analysis. Accordingly, we can exclude the occurrence of false-positive signals due to unspecific binding of ^{14}C-labeled acetate to sediment particles.

We conducted initial MAR-FISH experiments on sediment of October 2008 four weeks after sampling. To exclude that storage had introduced significant alteration of community activity, particular with respect to sulfate reducing prokaryotes, we repeated the incubations on fresh sediment in October 2009. Acetate incorporation patterns in both slurry experiments were similar. We therefore have no evidence for artifacts that might have been introduced by the storage of the Ocotber 2008 sample.

Contribution of sediment microbial community to overall acetate assimilation

Acetate incorporation was generally highest under oxic conditions as they can be naturally found at the sediment surface (Jansen *et al*., 2009). The stimulated surface incorporation is consistent with earlier substrate incorporation measurements of marine sediments (Meyer-Reil, 1987). Here, MAR-FISH analysis revealed that the proportion of acetate-positive cells in the top centimeter of the core (comprising 1.9×10^8 cells ml^{-1}, 0–1 cm) is twice as high compared to the layer immediately below (comprising 9.2×10^7 cells ml^{-1}, 1–2 cm). Accordingly the total amount of incorporated acetate was significantly lower below 1 cm depth. Our results demonstrate that microbial activity rapidly decreased with depth and is most likely strongly influenced by the availability of oxygen. Our high resolution beta-imaging analysis even visualized that the maximum incorporation of acetate is restricted to the top 4 mm of sediment. These findings are consistent with the fact that oxygen penetrates only few millimeters into the sediment in cores incubated under stagnant laboratory conditions (Polerecky *et al*., 2005). Similarly, a strongly reduced oxygen penetration to a few millimeters depth is also encountered during exposure of the sand flat at low tide (Jansen *et al*., 2009). Interestingly, we found a similar microbial activity in sediment originating from the oxic surface and such originating from anoxic layers of several cm depths when incubated under oxic conditions. This supports the presence of a facultative anaerobic microbial community that is well adapted to fluctuating oxygen concentrations. Similarly, Alonso and Pernthaler (2005) found pelagic bacteria of aerated North Sea waters to incorporate organic substrate under anaerobic conditions. At Janssand site oxygen usually does not penetrate deeper than 3–4 cm into the sediment (Jansen *et al*., 2009). However, tide induced waves, storms and animal activity can cause a frequent reworking of the sediment which leads to exposure of otherwise anoxic sediment layers. Our findings demonstrate that the prevailing sediment bacteria can rapidly adapt to sudden oxygenation events.

Consistent with early studies that combined autoradiography with acridine orange direct counts (Novitsky, 1983; Novitsky, 1987) we detected only a small proportion of bacteria that actively participate in substrate turnover. Overall, up to 18% of the sediment microbial community assimilated acetate. Similar to Novitsky (1983) who found highest heterotrophic activity at the narrow sediment-water interface, the highest proportion of substrate assimilating bacteria at Janssand site was restricted to the uppermost millimeters of the sediment. The detection rate seems comparatively low in spite of the vast proportion of sediment bacteria which did not respond to substrate addition Generally, the discrepancy

in the measured activity of a microbial community and the total counts of microorganisms determined by fluorescent staining is explained by the fact that a large fraction of marine bacteria is dormant (alive but inactive) or dead (Zweifel and Hagström, 1995; Gasol *et al*., 1999). As we used nucleic acids staining to assess total cell counts we can not exclude that a certain proportion of detected bacteria is dead. However, FISH on Janssand surface sediment using the general probe EUB for *Bacteria* reported detection rates of >80% of DNA stained cells (Ishii *et al*., 2004) and even abundances equaling those detected by DNA staining (Arnds, 2006; Lenk, unpublished data). As ribosome-containing, FISH visualized cells are generally assumed to be active (Lew *et al*., 2010), we presume that the non-responsiveness to acetate addition in our incubations results from the presence of numerous dormant cells, cells of very low activity and substrate preferences other than acetate rather then the presence of a large proportion of dead cells.

The fraction of active cells rapidly incorporated acetate (SI Fig. 2). Substrate incorporation within the time-course experiment ceased after 1 h of incubation. As slurries were thoroughly shaken, it seems unlikely that oxygen limitation inhibited further uptake. In contrast, a rapid respiration of added acetate could have induced substrate limitation. As it was no goal to perform carbon budgeting, no $^{14}CO_2$ was trapped. Therefore we can not yet estimate the proportion of supplied acetate that is respired or remains unaffected by microbial attack.

The fast substrate incorporation indicated that acetate consumption is tightly coupled to its formation *in situ*. This is supported by *in situ* concentrations of acetate that were low compared to concentrations reported for muddy sites (Sawicka *et al*., 2009). A rapid microbial turnover likely explains these low concentrations as it generally accounts for lower organic carbon content of sandy sediments. The cell-specific acetate assimilation rate of 0.46 fmol cell^{-1} h^{-1} is close to the range of uptake rates of 1 – 13 fmol cell^{-1} h^{-1} reported for activated sludge communities (Nielsen and Nielsen, 2002; Nielsen *et al*., 2003). It is also comparable to *in situ* leucine incorporation rates of 0.3 fmol cell^{-1} h^{-1} detected for heterotrophic North Sea bacterioplankton cells during a phytoplankton bloom (Alonso and Pernthaler, 2006).

Acetate assimilating populations

Gammaproteobacteria accounted for the majority of acetate assimilating cells (47-67%) under oxic conditions, although they account for only 20–30% of the total microbial community (Lenk *et al*., 2011). Such a disproportional high contribution of the group to substrate utilization has been observed in earlier experiments that traced the incorporation of dissolved inorganic carbon (Lenk *et al*., 2011). Here, carbon fixation by *Gammaproteobacteria* was suggested to be coupled to a sulfur-oxidizing physiology. Similarly, the use of acetate as a carbon source is known for sulfur-oxidizing *Thiomargarita* (Schulz and de Beer, 2002) or *Thiothrix* (Nielsen *et al*., 2000) and heterotrophic RCB (Sorokin *et al*., 2005). Some of the acetate-incorporating cells detected in this study could thus be heterotrophic or mixotrophic populations that oxidize inorganic sulfur compounds.

Generally, we could not resolve the identity of acetate-incorporating gammaproteobacterial populations in detail. As for the oxic incubations none of the specific Wadden Sea sediment populations or the abundant NOR5/OM60 group was labeled in the anoxic slurries. This is at least partially consistent with the proposed autotrophic lifestyle of some of these bacteria (Lenk *et al*., 2011).

In addition, we detected acetate incorporation by bacteria of the marine *Roseobacter* clade. Previous FISH counts revealed substantial numbers of these bacteria in oxic and anoxic layers of Janssand sediment that can account for up to 10% of all cells (Lenk *et al*., in prep). Facultative anaerobic representatives have been isolated from the site (Sass *et al*., 2009) and detected in North Sea surface waters (Alonso and Pernthaler, 2005). Here we provide evidence that the prevailing sedimentary *Roseobacter* clade populations are active and participate in the turnover of specific sediment substrates. This is in line with a previous enrichment of sedimentary RCB on sulfide, acetate and nitrate amended mineral medium (Lenk *et al*., in prep). Our data support that their activity is mainly restricted to oxic conditions which is consistent with an aerobic lifestyle of most members of the group (Wagner-Doebler and Biebl, 2006).

Using MAR-FISH we successfully quantified the acetate incorporating populations. In addition, nanoSIMS visualized the relative amount of substrate incorporated by single cells and thus provided detailed insights into the activities of the two different phylogenetic groups. Accordingly, acetate-assimilating *Gammaproteobateria* accounted for up to 10^8 cells ml^{-1} in the surface sediment and also incorporated more substrate per cell, displaying $^{13}C/^{12}C$ ratios of 0.03–0.09 compared to acetate-incorporating RCB that accounted for maximal 10^7 cells ml^{-1} and displayed $^{13}C/^{12}C$ ratios of 0.013–0.022. The workflow used for sample preparation successfully reduced particle background and thus provides a protocol for studies of comparable complex assemblages.

We could detect no acetate incorporation by sulfate reducing *Desulfosarcina*-relatives among the *Desulfobacteraceae*. This was unexpected as acetate has been shown to stimulate sulfate reduction in Wadden Sea sediment (Werner *et al*., 2006). However, stimulation by acetate might rely on secondary effects as also prolonged incubation to 24 h did not result in an increase of the number of labeled cells. Thus, although acetate is considered as substrate for SRP, they might exhibit preferences for other organic acids such as lactate and propionate. This is supported by findings of Miyatake and colleagues (2009) who detected a low labeling level of SRP in acetate amended SIP experiments after 24 h of incubation but instead found them to be important consumers of propionate. Similarly, lactate was found to enhance bacterial sulfate reduction in sediment of Janssand site (Kamyshny *et al*., 2009). Alternatively, substrates other than VFA such as hydrogen might be utilized during autotrophic growth on CO_2 (Valdemarsen and Kristensen, 2010). As, heterotrophic SRP generally oxidize most of the consumed organic substrate for energy conservation (Rabus *et al*., 2006), prolonged incubation times might be necessary to trace the small amount of acetate assimilated into cell mass. This is in agreement with the fact, that SIP experiments which consequently hint at acetate assimilation by SRP include extended incubation times with higher substrate concentrations (Webster *et al*., 2006) or repeated substrate amendment (Webster *et al*., 2010). The steep decrease of acetate incorporation over depth in intact cores and the general lower uptake in anoxic sediment slurries additionally suggest that a large amount of substrate is fed into energy metabolism under anoxic conditions.

Sequences of additional phylogenetic lineages, including *Acidobacteria, Actinobacteria*, *Bacteroidetes*, *Firmicutes* and the JS candidate division 1 have previously been recovered from acetate amended sulfate reducing enrichment slurries (Webster *et al*., 2006). As we found no evidence for an assimilation of

acetate by *Bacteroidetes*, detection of these phyla could result from cross feeding. While additional probes were not applied in our MAR analysis, respective phylogenetic groups have been detected in 16S rRNA gene clone libraries of Janssand sediment (Lenk *et al*., 2011) and nearby sites (Webster *et al*., 2007). Their *in situ* quantification and substrate utilization remains a subject of future studies.

Conclusions

Here we provided first insights into acetate incorporation patterns of marine sediment bacteria at single cell level using MAR-FISH and nanoSIMS. Preliminary data yielded comparable results from both approaches. However, for statistical evaluation analysis of additional cells by nanoSIMS is essential.

We found the prevailing sediment bacteria to exhibit highest activities in oxygenated surface sediment where they rapidly assimilate freshly added organic carbon and are well adapted to temporally changing oxygen concentrations. These findings agree with the fact that advective flushing infiltrates organic matter and oxygen into the surface layer of tidal sands (Billerbeck *et al*., 2006) where it promotes intense aerobic respiration particular during flood tide. While exposure of the sand at low tide might temporally decrease aerobic microbial activity in the less oxygenated surface layers, populations from anoxic horizons are also capable to rapidly response to sudden oxygenation events.

We identified members of the *Gammaproteobacteria* as most abundant acetate assimilating organisms. As the group generally accounts for high proportions in coastal sediments worldwide (Ravenschlag *et al*., 2001; Hunter *et al*., 2006; Musat *et al*., 2006; Kim *et al*., 2008; Bi-Wei *et al*., 2009) its members likely exhibit a central role in sedimentary carbon turnover. Future studies might further investigate the activity of SRP and quantify the amount of respired acetate in different sediment layers. Complementary, the assimilation of additional fatty acids should be traced. Similarly, further studies might address the role of chemolithoheterotrophs. To date little is known about their abundance and contribution to sedimentary sulfur oxidation.

Experimental Procedure

Sampling

The investigated intertidal sand flat is situated in the backbarrier tidal area of the German Wadden Sea (53°43'N 07°41'E). A detailed description of the site and sediment characteristics is provided by Jansen and colleagues (2009). Microautoradiography combined CARD-FISH (MAR-FISH) was performed on sediment sampled in October 2008, June 2009 and October 2009. In addition, volatile fatty acid extraction, beta-imaging and nanoSIMS were conducted on sediment sampled in June 2009. Cores of 20 cm length (except for beta-imaging) were taken, stored at *in situ* temperature and transported to the laboratory within 24 h after sampling.

Slurry incubations October 2008 and October 2009

Sediment cores sampled in October 2008 were stored at 4°C and incubation were conducted 4 weeks after sampling. Sediment cores were adapted to room temperature 12 h before the start of incubations. Cores were sliced and 1 ml of sediment from 0–1cm and 10–11cm depth was transferred to 5 ml glass vials and mixed with 1 ml of sterile filtered oxic seawater containing 200 µM acetate. Samples were pre-incubated under mild agitation to facilitate oxygen penetration into the sediment. After 2 h [1, 2-^{14}C]

acetate (specific activity 118 mCi/mmol (Hartmann Analytic, Braunschweig) was added to a final concentration of 25 µM and incubated for additional 6 h. For the anoxic incubations sediment from 10-11 cm depth was transferred to glass vials containing 1 ml of sterile filtered seawater flushed with N_2/CO_2 (80/20 v/v). After addition of unlabeled acetate (200 µM final), vials were closed with butyl rubber stoppers. After pre-incubation, the acetate tracer was added under continuous flushing with N_2. Vials were closed with butyl rubber stoppers and incubated for additional 6 h. Sediment fixed with formaldehyde prior to incubation served as a negative control. All incubations were performed in duplicates at 20°C in the dark.

In October 2009 incubation were repeated with freshly sampled sediment cores and performed within 48 h after sampling. Experiments were conducted as described above with the following modifications. No pre-incubation was conducted. The [1, 2-^{14}C] acetate (specific activity 100 mCi/mmol (Hartmann Analytic, Braunschweig) was added to a final concentration of 100 µM. For the anoxic incubations sediment from 10–11 cm depth was transferred to glass vials containing 1 ml of sterile filtered porewater, extracted from sediment of 8–12 cm depth flushed with N_2/CO_2 (80/20 v/v). All incubations were stopped with 1.8% formaldehyde and fixed overnight at 4 °C. Sediment portions of 0.5 ml were collected, washed 3 times in sterile 1 × PBS and centrifuged according to Ishii and colleagues (2004) and stored in PBS:Ethanol (2:3) at -20°C until further processing.

Core and slurry incubations June 2009

For measurement of depth and time dependent acetate incorporation intact sediment cores were collected in June 2009 and incubations were started in the laboratory within 24 h after sampling. An intact sediment core was percolated with 50 ml of sterile filtered pore water containing 100 µM [1, 2-^{14}C] acetate (specific activity 100 mCi/mmol (Hartmann Analytic, Braunschweig) to displace natural pore water as described previously and incubated for 8 h (Lenk *et al*., 2011). Subsequently, [1, 2-^{14}C] acetate was eliminated from the cores by percolating 2 × 50 ml of sterile filtered sea water through the sediment, followed by slicing of the core into 1 cm layers. From each layer 0.5 ml were fixed for FISH as described above. An additional sediment core labeled with ^{13}C-acetate was incubated in parallel (see below). To assess time-dependent acetate incorporation in the surface sediment, respectively 0.5 ml portions of mixed sediment from 0–3 cm depth of another sediment core were transferred to 5 ml glass vials and mixed with 0.5 ml of sterile filtered oxic seawater containing 100 µM [1, 2-^{14}C] acetate (specific activity 100 mCi/mmol (Hartmann Analytic, Braunschweig). Incubations were stopped after 30 min, 1 h and 6 h, respectively, as described above.

MAR-FISH analysis

To study the substrate incorporation by single sediment bacteria we applied the MAR-FISH protocol developed by Alonso and Pernthaler (2005) with the following specifications. To detach cells from sediment particles ultrasonic treatment was performed on ice for 7 × 30 sec (amplitude 30%, pulse 20) including 6 × 30 sec break intervals using a SonoPlusHD70 ultrasonic probe (Bandelin Electronic, Berlin, Germany). For filter preparation, 10 µl of the sonicated samples were diluted in 90 µl of PBS. In total, 10–15 µl of the diluted sample were suspended in 5 ml of PBS and filtered on 0.2 µm GTTP filters applying a gentle vacuum of 200 mbar. Dilution of samples was optimized to ensure an even distribution of

maximum 20–30 cells per microscopic field on the filter and a low background of sediment particles. To determine the fraction of ^{14}C-acetate incorporating cells filters were stained with the DNA stain SYBR Green I (Molecular Probes, USA). An exposure time of four days was required to detect a maximum number of acetate-incorporating cells. In sediment that was fixed with formaldehyde prior to incubation (negative control), no 'false positive' cell-associated silver grain precipitates could be detected. To assess the proportion of ^{14}C-acetate positive cells relative to the total microbial community at least 1000 SYBR Green stained cells and the number of corresponding MAR signals associated with cells signals were counted. To identify acetate-incorporating phylogenetic groups and populations duplicate samples of the October 2008 incubations were evaluated with every FISH probe (Table 1). Approximately 300 MAR signals and the corresponding probe specific signals were counted to assess the fraction of substrate assimilating *Gammaproteobacteria*, *Roseobacter* clade bacteria, *Deltaproteobacteria* and *Desulfosarcinales*-related target cells. As an exposure time of four days resulted in a number of strong MAR-signals which obscured the corresponding fluorescent signal of the cell, two days of exposure were chosen to identify acetate-incorporating cells hybridized to a probe. All preparations were inspected manually under an Axioplan epifluorescence microscope (Zeiss, Jena, Germany) at 1000 × magnification.

Bulk measurements of acetate incorporation and cell-specific incorporation rates

The amount of incorporated [1, 2-C^{14}] acetate was assessed by liquid scintillation counting. After sonication of the FISH fixed sample 10 µl of supernatant were mixed immediately with the scintillation cocktail (Ultima *Gold* XR, Packard). The incorporated radioactivity of the individual samples was assessed using a liquid scintillation counter (Tri-Carb 2900 Packard, Perkin Elmer, USA). Counts were corrected by those of the prefixed, dead controls (negative controls). Acetate incorporation rates were calculated based on the total acetate content added to the sample, the amount of [1, 2-C^{14}] acetate added and the amount of [1, 2-C^{14}] acetate incorporated into biomass during the incubation time.

Beta-imaging of sediment slices

In June 2009 mini-cores of approximately 4 cm in length were recovered with plastic syringes (diameter ~ 1cm). Sediment was retained in the mini-cores by one layer of whatman paper and pore water was withdrawn. Unlabeled acetate (20 µM) and [1,2-C^{14}] acetate (activity of 0.1 µCi) were mixed with 8 ml filtered sea water and were percolated through the mini cores within approximately 20-30 seconds to saturate pores. Cores were then incubated in the dark under *in situ* temperature for 30 min and then washed and preserved with 1% formaldehyde dissolved in sea water. In the laboratory the cores were washed with PBS and then embedded with heated, liquid agarose (1% in sea water) to maintain structure of the sediment. After solidification of agarose the upper cm was vertically sliced in 1 mm sections and transferred to glass slides, dried at room temperature and used for 2D beta-imaging on a beta-imager (Biospace Mesures, Paris, France). Scintillation scans were performed for 52 h (core 1) and 6 h (core 2).

Volatile fatty acid analysis

Concentrations of volatile fatty acids were analyzed in two parallel sediment cores sampled in June 2009. Sediment from 0–1, 1–2, 2–3, 3–4, 4–6, 6–8 and 8–10 cm depth was sampled. High-performance liquid chromatography (HPLC), collection and analysis of porewater were performed as described by Sawicka and colleagues (2009). In brief, sediment samples from respective depths were centrifuged in Sphinex^{R}

filters at 4000 rpm at 4°C for 15 min. Collected porewater was filtered into 1-ml brown borosilicate glass vials that were pre-combusted at 480°C for 4 h to minimize possible contamination. The acids were derivatized with *p*-nitrophenyl hydrazine, separated by HPLC using a LiChrosphere 80/100 (Knauer, Berlin, Germany) column at 25°C, and the concentrations were determined from the absorption on a UV/VIS detector (Linear) at 400 nm.

Flow sorting and nanoSIMS analysis

An intact sediment core sampled in June 2009 was percolated with 50 ml of sterile filtered pore water containing 100 µM [1, 2-^{13}C] acetate (99% ^{13}C, Sigma-Aldrich), incubated for 8 h and fixed for FISH as described above. Sediment from 0-3cm depth was mixed; portions of 0.5 ml were suspended in 1 ml PBS and sonicated. Sediment-cell suspensions were diluted with 9 ml of PBS and vigorously vortexed. Portions of 1 ml were dispersed into 2 ml Eppendorf tubes. Afterwards, 1 ml of Nycodenz density gradient medium was placed underneath the slurry followed by centrifugation according to the protocol of Fazi and colleagues (2005). Purified cell-factions were collected and densely filtered on 0.2 µm GTTP membrane filters. No agarose embedding and lysozyme treatment of filters was performed. CARD-FISH with probe GAM42a and ROS537 was conducted as previously published by Ishii and colleagues (2004) including a hybridization time of 18 h followed by washing and signal amplification (CARD) for 20 min using Alexa$_{488}$-labeled tyramides. Subsequently, cells were resuspended from membrane filters according to Sekar and colleagues (2004). Therefore filter sections were placed in 2 ml Eppendorf tubes containing 1.5 ml of 150 mM NaCl and 0.05% Tween80 and horizontally incubated on a shaker at 250 rpm for 30 min at 37°C. Afterwards samples were vigorously vortexed for 15 min at 2500 rpm. The resuspended cell fraction was subjected to a MoFlo Flow Cytometer (Cytomation Inc., Fort Collins, Colo.). Target cells were identified based on green fluorescent signals and side angle light scatter (SSC) by plotting SSC versus green fluorescence in a bivariant dot plot diagram (SI Figure 3). Sorted target cells were spotted on palladium/gold coated GTTP filters (25 mm diameter). A subsample was counterstained with DAPI (1µg ml^{-1}). Microscopic inspection proved high purity of the sorted target cell fraction (SI Figure 3). For nanoSIMS, filters were incubated H_2O_2 (final conc. 3% in 1 × PBS) for 15 min at room temperature to inactivate introduced peroxidases. Halogen *in situ* hybridization (HISH) with probe EUBI-III (*Bacteria*) for sorted *Gammaproteobacteria* and probe ALF968 (*Alphaproteobacteria*) for sorted *Roseobacter* clade bacteria was performed according to Musat and colleagues (2008) using an OregonGreen-labeled, fluorine-containing tyramide for signal amplification. NanoSIMS analysis was conducted on a NanoSIMS50 instrument (Cameca). Fields of 20 µm × 20 µm were scanned for cells with substrate incorporation. Secondary ion images of $^{12}C^-$, ^{13}C-, $^{32}S^-$, $^{19}F^-$, $^{12}C^{14}N^-$ were recorded for each cell. Images and data were processed using the Cameca Win-Image processing software.

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Supporting Information

Figure S1 Profiles of *in situ* porewater concentrations of volatile fatty acids (June 2009).

Figure S2 Incorporation of ^{14}C-acetate in oxic slurries over time (June 2009).
Illustrated is the relative abundance of ^{14}C-acetate assimilating microbial cells in % of total microbial (□■, left axis) and the total amount of assimilated acetate (○●, right axis). Results from duplicate incubations are shown. Incorporation was corrected for the dead, prefixed control.

Figure S3 Relative abundances of ^{14}C-acetate incorporating microbial cells in vial incubations of October 2009.
Sediment from 0-1 cm and 10-11 cm depth was incubated oxically and anoxically for 6 h. In addition, sediment from 10-11 cm depth was incubated anoxically for 24 h. Bars illustrate mean values of duplicate incubations.

Figure S4 Sediment bacteria sorted with flow cytometry.
The dot plot diagram shows the side angle light scatter (SSC) plotted versus green fluorescence of target *Gammaproteobacteria* (GAM42a) or RCB (ROS537) after hybridization with clade-specific HRP-oligonucleotide probes using $Alexa_{488}$-labeled tyramides. Epifluorescence micrographs show individual cells of *Gammaproteobacteria* and RCB after flow cytometric sorting and a second hybridization with a *Bacteria*- or *Alphaproteobacteria*-targeting HRP-conjugated oligonucleotide probe using fluorine-containing tyramides. The DNA of all cells was stained with DAPI (blue). The purity of the sorted cell fraction is evident from the overlay of fluorescence-conferred probe and DAPI signals.

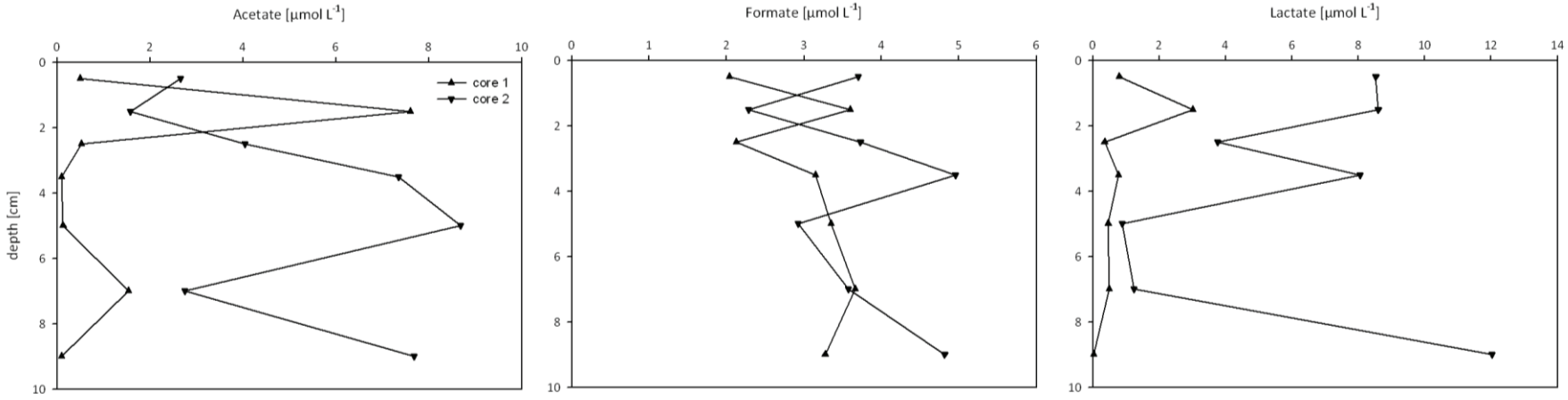

Figure S1

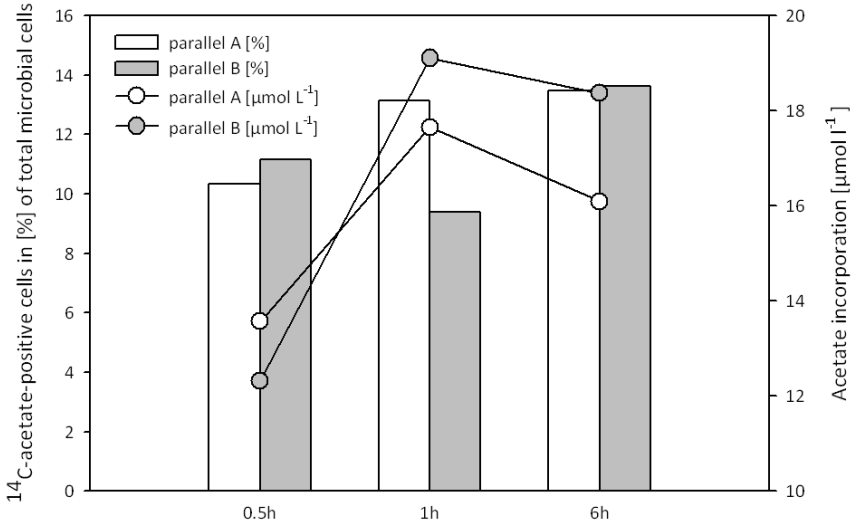

Figure S2

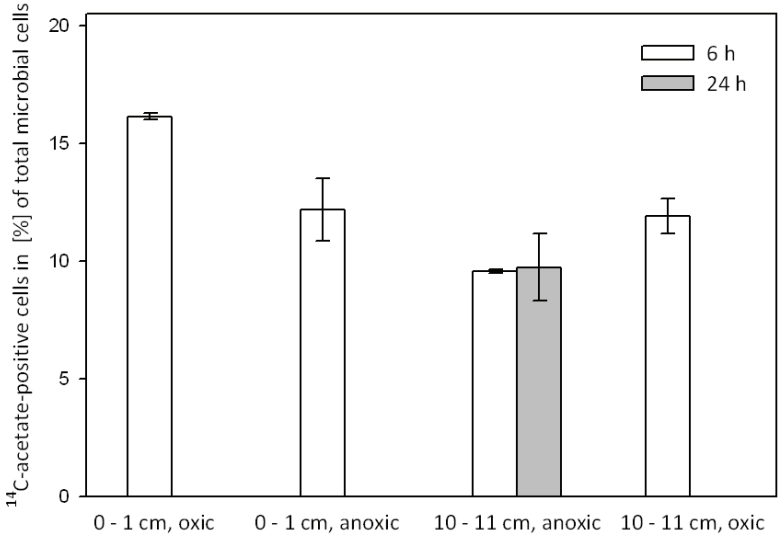

Figure S3

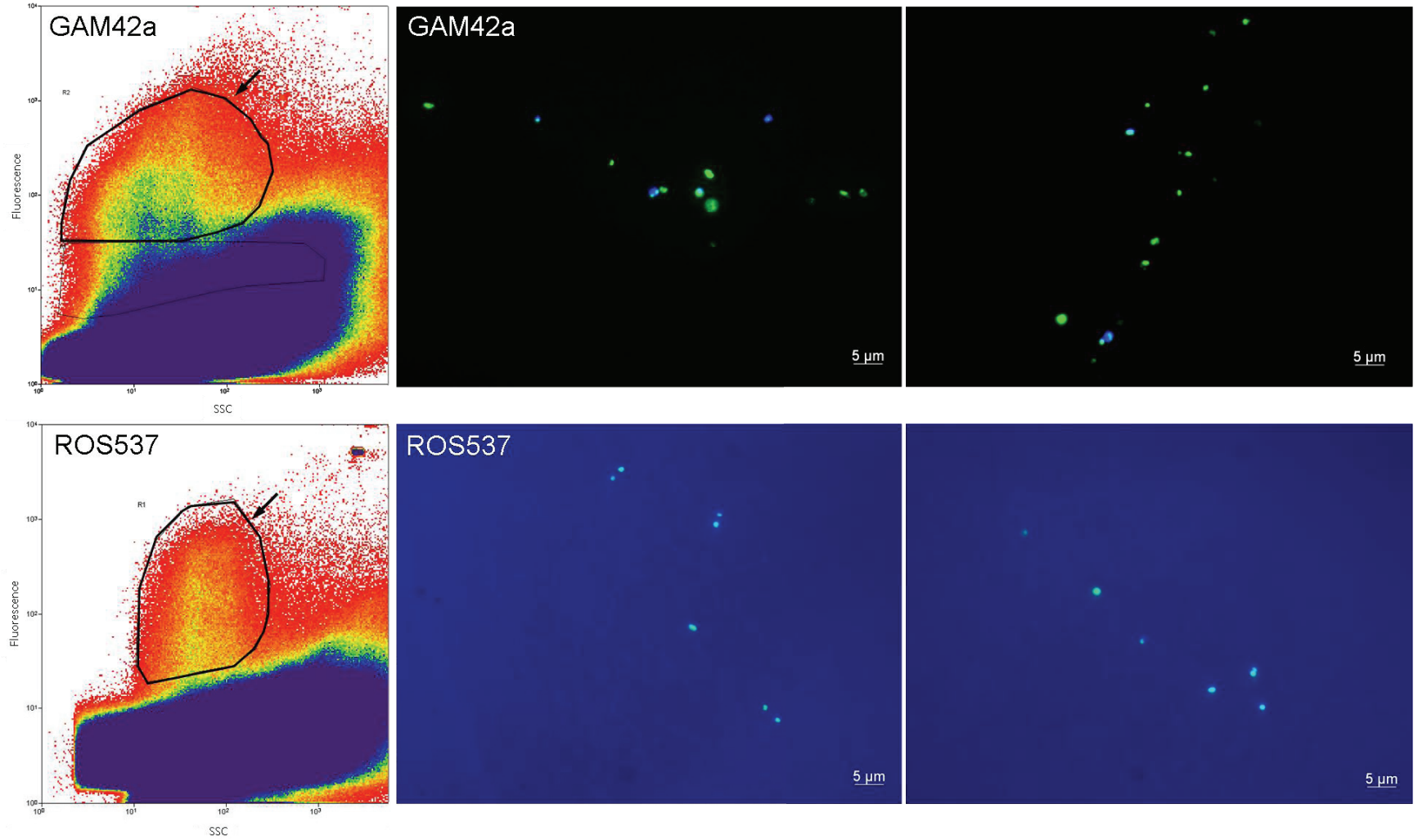
GAM42a
R2
Fluorescence
SSC
GAM42a
5 µm
5 µm
ROS537
R1
Fluorescence
SSC
ROS537
5 µm
5 µm

Figure S4

5. Sulfur-Oxidizing *Epsilonproteobacteria* in Coastal Sediments

Contributions to the study:

Sabine Lenk: established oxygen-sulfide gradient culture, performed molecular analysis of enrichment culture, intertidal sulfur pool and sediment

Heide Schulz-Vogt and Martina Meier: assisted in experimental set-up of the oxygen-sulfide gradient culture

Arcobacter spp. Dominate Oxygen-Sulfide Gradient Cultures and Sulfidic Intertidal Sulfur Pools

additional data obtained in this thesis

Running title: Sulfur-oxidizing *Epsilonproteobacteria* in coastal sediments

Key words: *Epsilonproteobacteria*, *Arcobacter*, permeable sediments, sulfur

Results and Discussion

Sulfidic water of an intertidal pool was sampled to identify and quantify SOP. To complement the culture independent investigations we enriched sedimentary SOP under laboratory conditions.

Molecular analysis of the oxygen-sulfide gradient enrichment culture

In an attempt to grow chemolithoautotrophic SOP agar tube cultures with opposing gradients of oxygen and sulfide were established and inoculated with sediment from the oxic-anoxic transition zone sampled in June 2009. These should mimic natural conditions encountered in the surface sediment. After one week microbial cells and precipitated sulfur accumulated and formed a distinct whitish layer (Fig. 1). The sulfur precipitation zones were dominated by *Arcobacter* spp. related organisms comprising 70% of DAPI stained cells (Fig. 1 and Fig. 2). Members of the marine *Roseobacter* clade constituted the second largest fraction of 12% of DAPI cells. Only 1.4% of cells were *Gammaproteobacteria*. Consistently, the derived 16S rRNA gene library was dominated by sequences related to *A. halophilus* (up to 94% sequence identity, 91% of all clones, Fig. 3) previously isolated from water of a hypersaline lagoon (Donachie et al., 2005).

A DsrAB gene library from this zone was established to identify *dsr*-possessing SOP among the *Alpha*- and *Gammaproteobacteria*. In total, 32 *dsrA* sequences were recovered (see Manuscript 1, Fig. 2) of which 31 sequences formed a single OTU most closely related to *dsrAB* of the *O. haakonmosbiensis* endosymbiont (93% amino acid sequence identity) and Wadden Sea clone d1H5 (96% sequence identity). A SoxB gene library from the sulfur precipitation zones yielded few *soxB* sequences that affiliated with *Alphaproteobacteria*, in particular with members of the marine *Roseobacter* clade (see Manuscript 2, Fig. 3).

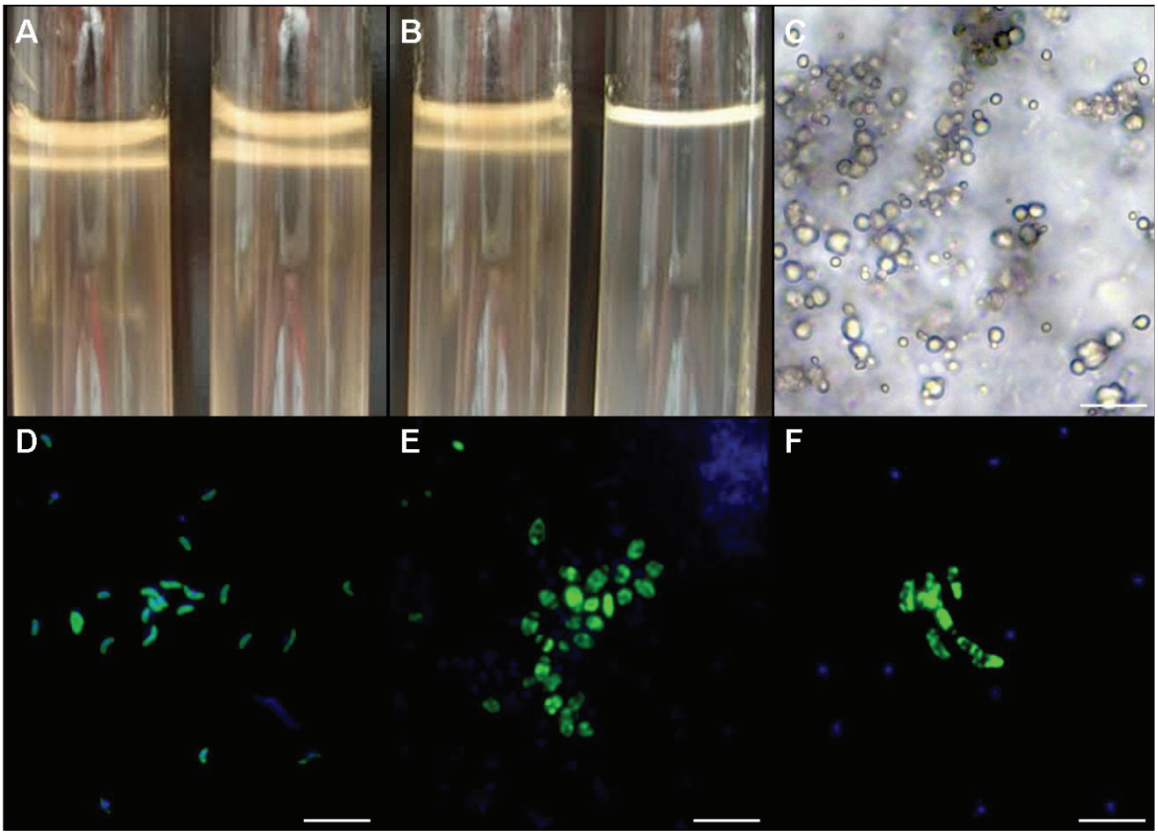

Figure 1 Bright field and epifluorescence microscope images of the oxygen-sulfide gradient culture visualizing the sulfur precipitations (A, B, C).
In tubes inoculated with Janssand sediment a thick layer of sulfur precipitations had formed after 2 weeks of incubation in the dark in contrast to the not inoculated control tube (**B**, right). FISH revealed the presence of organisms related to *Arcobacter* spp. (**D**), *Roseobacter* spp. (**E**) and *Gammaproteobacteria* (**F**). The scalebar corresponds to 5 µm.

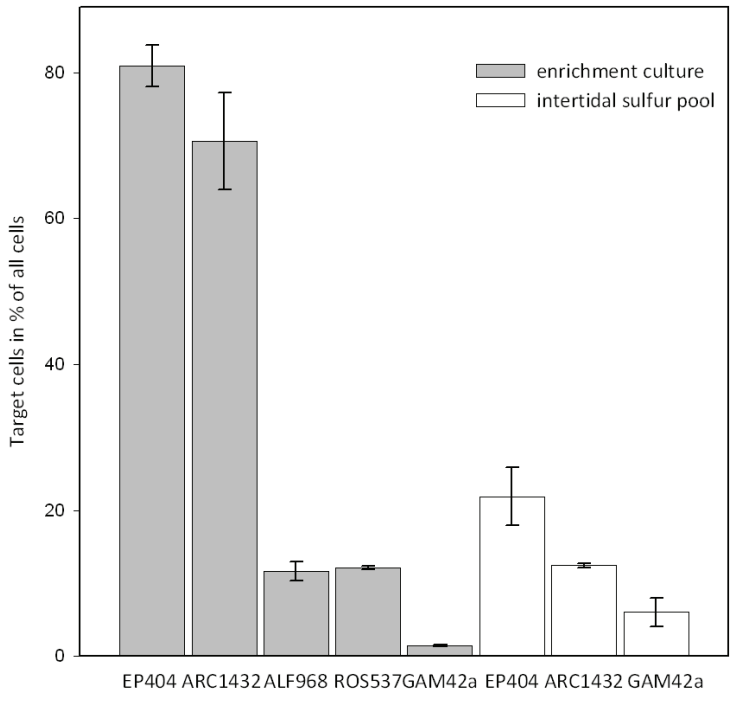

Figure 2 Community compositions in the oxygen-sulfide gradient culture and the sulfidic intertidal sulfur pool assessed by CARD-FISH.

Probes for *Epsilonproteobacteria* (EP404) in particular *Arcobacter* spp. (ARC1432), *Alphaproteobacteria* (ALF968) including marine *Roseobacter* clade bacteria (ROS537) and *Gammaproteobacteria* (GAM42a) were applied. In addition, the abundances of putative sulfur-oxidizing *Epsilonproteobacteria/Arcobacter* spp. and *Gammaproteobacteria* in the sulfidic pool are shown. Bars indicate mean values of duplicate incubations.

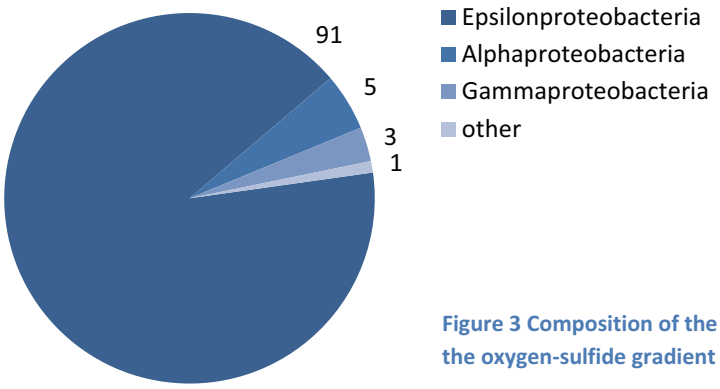

Figure 3 Composition of the 16S rRNA gene clone library from the sulfur layer in the oxygen-sulfide gradient enrichment culture.

Molecular analysis of the sulfidic intertidal sulfur pool

The community composition of a sulfidic intertidal pool was analyzed by FISH. The sulfur containing water harbored 6 ± 2% (3.0×10^5 cells ml^{-}) *Gammaproteobacteria* (Fig. 2). However, no cells were detected using the novel gammaproteobacterial population-specific probes. *Epsilonproteobacteria* (probe EP404), in particular *Arcobacter* spp. (probe ARC1430) related organisms constituted 22 ± 4% (1.2×10^6 cells ml^{-1}) and 12% ± 0.4% of DAPI cells (6.0×10^5 cells ml^{-1}) in the pool water (Fig. 2 and 3) whereas they were close to detection limit in the sediment (Fig. 3).

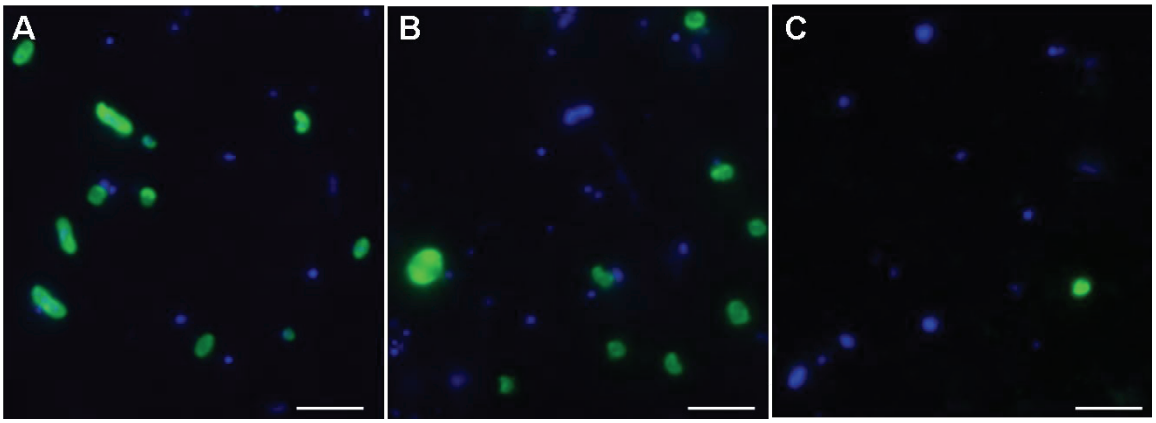

Figure 4 Epifluorescence microscope images of *Epsilonproteobacteria*.
(A) *Epsilonproteobacteria* and (B) *Arcobacter* spp. were detected in the sulfidic intertidal pool. (C) In the sediment, *Arcobacter* spp. accounted for less than 0.5% of all cells. The scalebar corresponds to 5 µm.

Visible sulfur precipitates in intertidal pools are apparent phenomena of intense sulfur cycling at the investigated sand flat (Fig. 5). As these pools represent sites with high sulfide outflow (Kamyshny *et al.*, 2010) Jansen and colleagues (2009) previously suggested the presence of motile SOP.

We identified only *Epsilonproteobacteria* such as *Arcobacter* spp. as possible SOP in the sulfidic pool. Cells of the genera *Arcobacter/Sulfurimonas* spp. are usually highly mobile and flourish in habitats such as oxygen minimum zones (Lavik *et al.*, 2009; Grote *et al.*, 2007), hydrothermal vents (Campbell *et al.*, 2006) and cold seeps (Omoregie *et al.*, 2008), where high amounts of sulfide reach the water column. Similarly, we observed *Arcobacter* spp. in the sulfide-oxygen transition zone of our gradient cultures. The

organisms are well known to enrich in liquid media with opposing gradients of oxygen and sulfide, where they form elemental sulfur from sulfide (Wirsen *et al*., 2002). Sievert and colleagues (2007) characterized *Candidatus* Arcobacter sulfidicus, as obligate microaerophilic, opportunistic bacterium that tolerates high sulfide and low oxygen concentrations. They proposed that the formation of rigid filamentous sulfur represents a special adaptation that allows *Arcobacter* populations to aggregate and colonize dynamic environments at oxic-anoxic interfaces.

We hypothesize that the detected *Arcobacter* may originate from sulfidic sedimentary pore water. Recent findings further support the presence of mobile *Epsilonproteobacteria* as elemental sulfur, which was artificially introduced to oxidized surface sediment of Janssand site was rapidly colonized by candidate SOP related to *Sulfurimonas*/*Sulfurovum* spp. (M. Mussman, pers. communication). Thus, the inoculation of sediment into the oxygen-sulfide gradient cultures favored their enrichment over particle attached SOP. In the natural habitat their high motility may allow them to accumulate in the sulfidic pools during low tide when conditions become favorable. Accordingly, their high abundance in the pool provides evidence that the oxidation of sulfide to sulfur is catalyzed by the *Arcobacter* species.

Overall, the pools occur only sporadically along the sand flat and sulfide is generally oxidized before it reaches the sediment surface. Interestingly the absolute cell number of *Arcobacter* spp. in the sulfidic pools was >1000 fold lower than those of gammaproteobacterial, particle-associated SOP in the sediment. Low detection rates have also been observed at two other Wadden Sea sites where *Arcobacter* spp. accounted for 0.1 to 1.6% of all cells in the sediment (Llobet-Brossa *et al*., 1998). Consequently, *Epsilonproteobacteria* most likely play only a minor role in sedimentary sulfur oxidation.

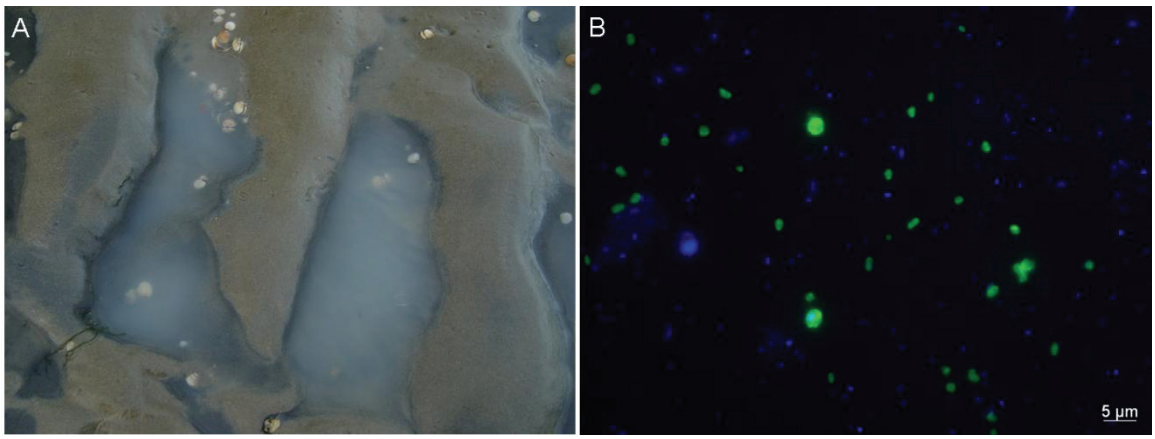

Figure 5 Sulfidic pools with sulfur-oxidizing *Epsilonproteobacteria*.
(A) The whitish coloration results from the oxidation of sulfide to elemental sulfur and polysulfide. (B) Fluorescence *in situ* hybridization revealed substantial proportions of *Arcobacter* spp. (green) which are well known to catalyze this reaction (Sievert *et al*., 2007).

Experimental Procedure

An intertidal pool with visible colloidal sulfur precipitates overlaying the sediment was sampled in April 2005. For DNA extraction four aliquots of 50 ml were filtered on polycarbonate membranes (poresize 0.2 µm, Millipore, Germany) and stored at -20°C. Similarly, 10 ml aliquots were fixed with 1.8%

formaldehyde for 1 h and subsequently filtered for FISH. Amplification of *dsrAB* using primers rDSRA240F and rDSRB808R was performed as described previously.

Oxygen-sulfide gradient cultures were established according to the protocol of (Kamp *et al*., 2006) with the following specification: 8 ml top agar containing artificial seawater supplemented with 100 µM $NaNO_3$ and 2 mM $NaHCO_3$ overlaid 4 ml of sulfidic bottom agar containing 16 mM Na_2S. The calculated flux of sulfide from the bottom agar established a concentration of 1 mM sulfide within the upper cm transition zone of top agar.

In June 2009 0.5 ml sediment from the oxic – anoxic transition zone of 2 – 3 cm depth was sampled and diluted with 200 µl of sterile seawater. After mild sonication on ice (amplitude 10% for 30 sec, pulse 20) 10 µl of sediment slurry were inoculated into the top agar and incubated in the dark at room temperature. After two weeks the zone of visible bacterial biomass accumulation and sulfur precipitation was sampled for subsequent molecular analysis. CARD-FISH and clone library construction for the 16S rRNA and rDSR gene were conducted as described above. Amplification of *dsrA* was performed with the primer pair rDSRA240F and rDSRB403. Thermocycling included an initial denaturation for 4 min 95°C, followed by 30 cycles of 30 sec at 95°C, annealing for 60 sec at 55.5°C and elongation for 3 min at 72°C.

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6. Summarizing Discussion and Concluding Remarks

In this thesis sediment microorganisms that contribute to the cycling of sulfur and carbon in coastal intertidal sediments were characterized by molecular tools. The following section summarizes the major findings of the studies.

6.1. Sulfur Oxidation by the Sediment Microbial Community

During recent years key organisms that contribute to sulfur cyling in prominent sulfidic habitats such as hydrothermal vents or anoxic marine basins have been identified. In contrast, the diversity and ecological role of SOP in highly sulfidic coastal sediments remained poorly characterized. In this study the identity, abundance and activity of non-mat forming SOP inhabiting a coastal intertidal sand flat was investigated. The combination of the full cycle 16S rRNA approach (Amann *et. al*., 1995) and diversity studies of functional genes diagnostic for sulfur oxidation enabled a resolution of the community structure of SOP beyond the level of previous 16S rRNA based studies (Ravenschlag *et al*., 1999; Ravenschlag *et al*., 2001; Bowman *et al*., 2005).

Gamma- and Alphaproteobacteria represent prominent SOP in coastal sediment

Evidence from all clone libraries (Fig.1) suggested an important role of *Gammaproteobacteria*. Generally, PCR bias can introduce an under or overrepresentation of certain phylogenetic groups. Here, the high frequency of *Gammaproteobacteria*-related sequences was also reflected in the high *in situ* abundance of this group detected by FISH and thus supports their relevance for sulfur oxidation. The applied functional genes have been shown to cover a wide range of phylogenetic clades, targeting SOP among the *Alpha-*, *Beta-* and *Gammaproteobacteria* (*aprA*, *dsrAB*, *soxB*), *Epsilonproteobacteria* (*soxB*) and *Chlorobi* (*aprA*, *dsrAB*, *soxB*). However, sequences affiliating with *Beta-* and *Epsilonproteobacteria* have been detected in none of the functional gene libraries. Similarly, sequences affiliating with the *Chlorobi* were absent (*aprA* and *dsrAB* library) or only distantly related to this group (*soxB* library). Particularly, the congruency of the different gene phylogenies supports that members of the *Gamma-* and also *Alphaproteobacteria* are the prominent SOP in Janssand sediment, while SOP of the *Beta-* and *Epsilonproteobacteria* or *Chlorobi* are of minor relevance.

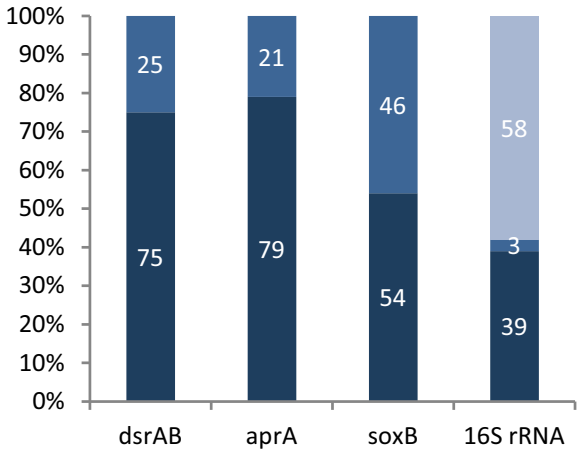

Figure 1 Taxonomic composition of functional (*dsrAB*, *aprA*, *soxB*) and 16S rRNA gene clone libraries.

The charts illustrate the relative proportion of operational taxonomic units [%] affiliating with *Gammaproteobacteria* (▪) and *Alphaproteobacteria* (▪) or other phylogenetic groups (▪).

Congruent evidence from 16S rRNA, *dsrAB* and *aprA* libraries supported the presence of populations related to thiotrophic gammaproteobacterial symbionts of marine invertebrates (Fig.2). In addition, phylogenies of the 16S rRNA and SoxB revealed populations affiliating with the gammaproteobacterial NOR5/OM60 clade (Fig. 2). Their high abundances were of particular interest as these organisms were previously associated with the pelagic habitat. The population densities of NOR5/OM60 clade bacteria that were detected in one milliliter of Janssand sediment are the highest reported so far (Eilers *et al*., 2001; Alonso-Saez *et al*., 2007; Yan *et al*., 2009). The design and application of novel 16S rRNA–targeted oligonucleotide probes enabled detection and quantification of several gammaproteobacterial populations (Fig. 3).

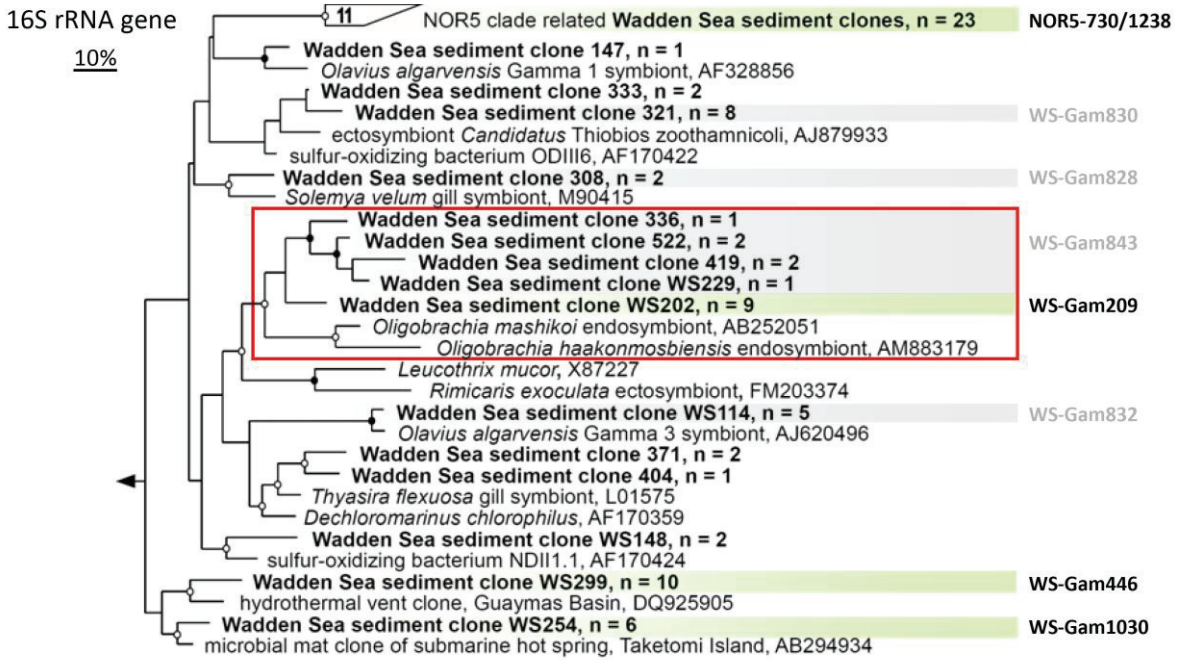

Figure 2 Phylogeny of representative gammaproteobacterial 16S rRNA gene sequences from intertidal sediments of Janssand. Sequences that displayed >97% nucleotide identity were grouped into a single OTU, where 'n' equals the number of sequences per OTU. The tree illustrates sequences that affiliated with the NOR5/OM60 clade, sequences that were related to known sulfur-oxidizing symbionts (e.g. WS-Gam209 targets) and deep-branching sequences (e.g. WS-Gam446 and WS-Gam1030 targets). Those sequences that were targeted by novel, specific oligonucleotide probes are shaded. Grey shading indicates populations that were detected by FISH. Green shading indicates groups that were quantified over the vertical sediment profile. RAxML bootstrap values are indicated for lineages with >70% (●) or >50% (○) support. The bar indicates 10% sequence divergence.

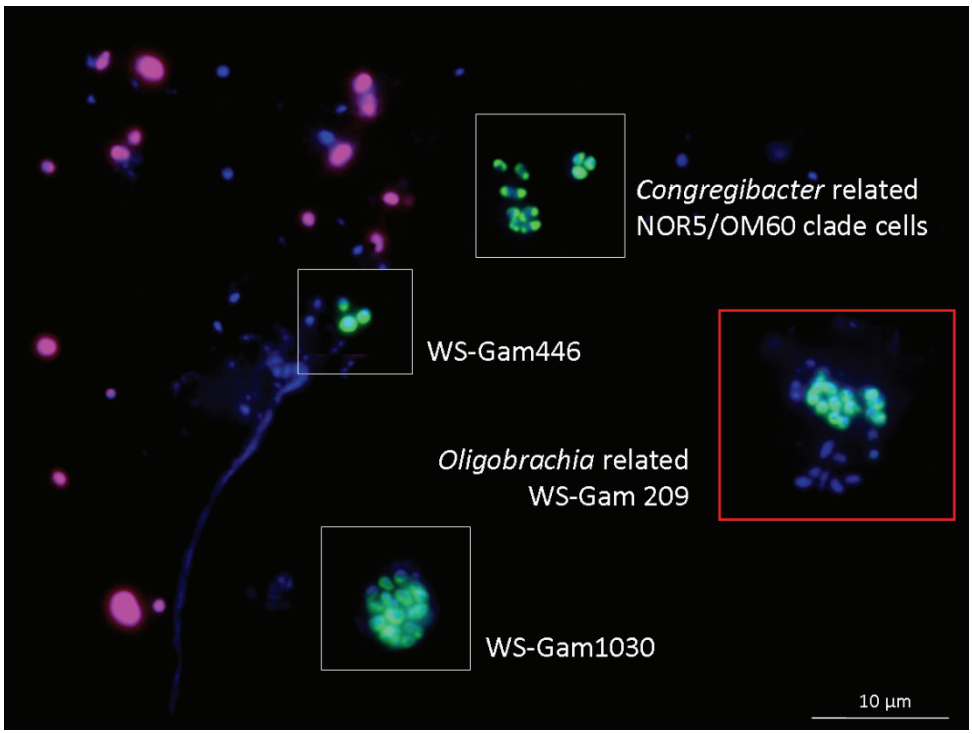

Figure 3 Epifluorescence microscopy images of the sediment microbial community.
The application of novel oligonucleotide probes enabled *in situ* detection and quantification of distinct gammaproteobacterial populations. For the WS-Gam209 population several lines of evidence suggest a sulfur-oxidizing metabolism. Blue, DNA staining of all microbial cells; magenta, *Gammaproteobacteria* hybridized to the general class specific probe GAM42a; green, novel gammaproteobacterial populations hybridized to the specific probes

The comprehensive sequence based diversity study further implicated *Alphaproteobacteria* in sedimentary sulfur cycling (Fig. 1). Here, analysis of different functional genes proved to be of high value. Although the sequencing effort was extensive, alphaproteobacterial sequences were strongly underrepresented in the 16S rRNA gene libraries. Accordingly, they provided little support for a role of this class in sulfur cycling. In contrast, the *dsrAB* and *aprA* libraries comprised substantial proportions of *Alphaproteobacteria*-related sequences. Most of them could not be assigned to any known SOP. The limited resolution of functional gene phylogenies confirms that the majority of environmental SOP has not been identified yet. Complementary analysis of the *soxB* diversity revealed numerous phylotypes that affiliated with the marine *Roseobacter* clade. However, the limited number of 16S rRNA sequences in the clone libraries from bulk sedimentary DNA strongly hampered the identification of candidate sulfur-oxidizing populations on the 16S rRNA level. We therefore conducted fluorescence activated cell sorting of hybridized *Roseobacter* clade bacteria (RCB*)* followed by PCR based amplification of the 16S rRNA gene. This methodological approach significantly increased the number of RCB associated 16S sequences in the library. The obtained 16S sequences provide future targets for *de novo* probe design and *in situ* quantification of distinct RCB populations.

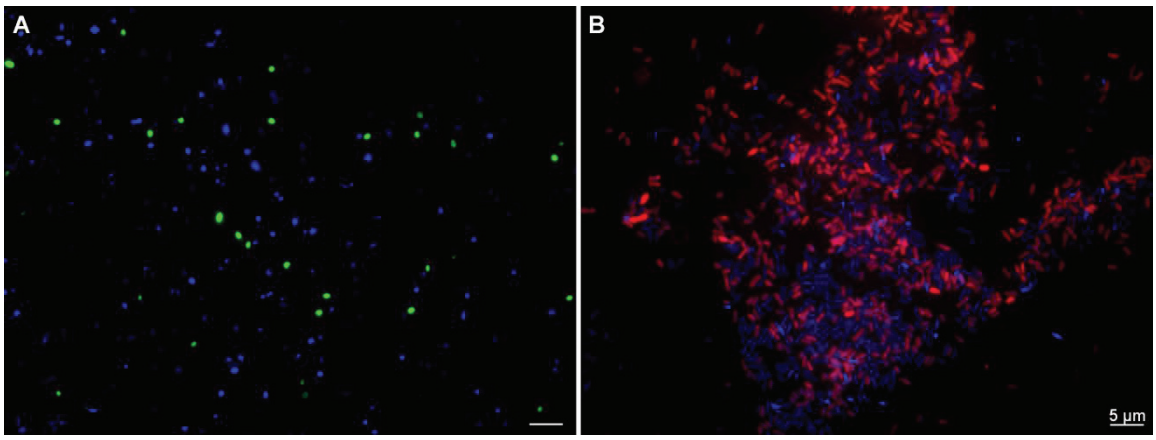

Figure 4 Epifluorescence microscopy images of sedimentary RCB.
(A) RCB (green) detected at Janssand site and (B) RCB (red) in the sulfidic enrichment culture of Koenigshafen. DNA staining of additional microbial cells (blue)

Novel sulfur-oxidizing populations - identity, abundance and activity

One aim of the study was to combine sequence and activity based methods to resolve the community structure of SOP in detail. The high diversity of *Gammaproteobacteria*-related sequences in all libraries, the partially congruent tree topologies, the high *in situ* abundances and the detection of CO_2 incorporation by single gammaproteobacterial cells provided strong circumstantial evidence for the prevalence of different sulfur-oxidizing populations.

We succeeded to characterize the identity, abundance and activity of a population that was most closely related to the sulfur-oxidizing endosymbiont of the tubeworms *Oligobrachia mashikoi* and *Oligobrachia haakonmosbiensis* (Fig. 2, Fig. 3). This population, termed WS-Gam209 group, comprised between 3 to 4 % of the total prokaryotic community and accounted for more than 10% of all *Gammaproteobacteria*.

In addition, metagenomic analysis, targeted cultivation and geneFISH identified previously unknown *dsrAB*-possessing RCB (Fig. 4). We currently lack any data on their *in situ* abundance. Initial attempts to quantify these organisms in Janssand sediments using geneFISH were hampered by a high signal to noise ratio (Fig. 5, C. Moraru and S. Lenk, unpublished data).

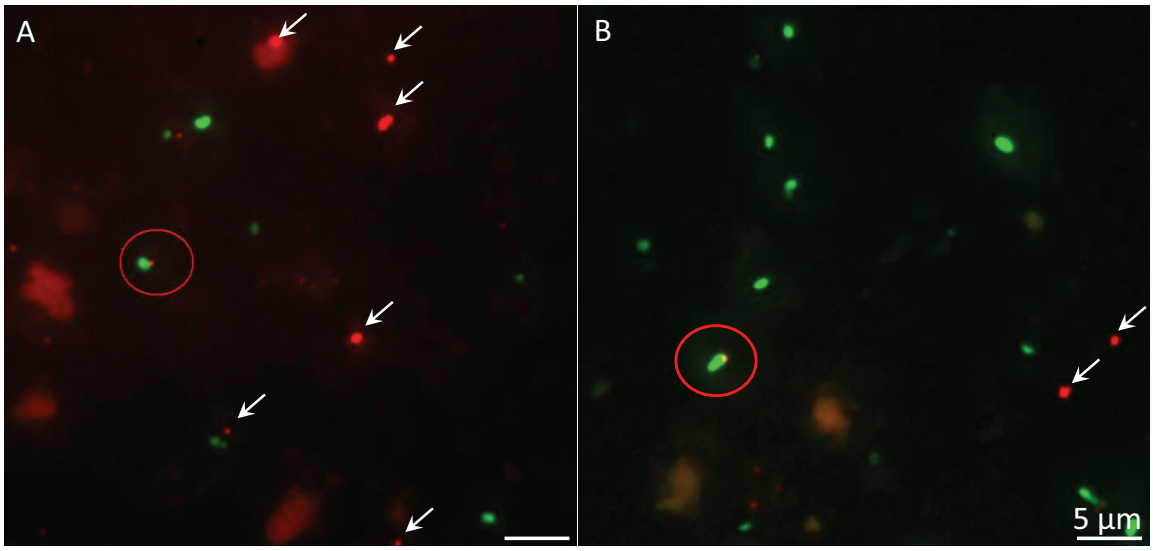

Figure 5 GeneFISH on Janssand sediment.
(A) The *dsrA*-targeting polynucleotide probe (red) matching the *dsrA* encoded on Wadden Sea sediment fosmid WS101A12 was hybridized to RCB (green) stained by the 16S rRNA targeting probe ROS537. Numerous gene signals did not overlay with target cells (arrows). (B) Hybridization of the negative control polynucleotide probe NonPoly350 to the same sample indicated unspecific signals. Similar results were obtained after hybridization of the *dsrA*-targeting and negative control probe to *Alphaproteobacteria* (Alf 968) and *Bacteria* (probe EUB I-III).

In contrast, the targeted enrichment yielded valuable insights into the identity and activity of this novel *dsrAB*-possessing RCB. The organisms are most closely related to a nitrate reducing, benzoate degrading strain that was previously isolated from anoxic waters of the Black Sea (Zengler, 1999). Consistent with its close relatedness results from cultivation provided evidence for benzoate utilization by the enriched RCB. In addition, the *in situ* incorporation of this compound by RCB of Janssand sediment could be demonstrated using MAR-FISH (Zerjatke, 2009). While aromatic compound degradation has been suggested to be a widespread capability of RCB (Buchan *et al*., 2000) further investigation are necessary to quantify its utilization by sedimentary populations under aerobic and anaerobic conditions.

We can currently not infer whether the enriched organisms are able to oxidize inorganic sulfur compounds. Their growth in sulfide amended mineral medium and the co-occurrence with putative sulfur-oxidizing *Espilonproteobacteria* suggests a utilization during lithoheterotrophic growth on acetate as it has been shown for several RCB isolates from sulfidic habitats (Sorokin, 1995; Teske *et al*., 2000; Sorokin *et al*., 2005). However, the enriched RCB might have grown organoheterotrophic on acetate alone or metabolic exudates and decaying microbial biomass. Thus, further growth experiments have to demonstrate their lithoheterotrophic growth capabilities on sulfide, thiosulfate and sulfur. That growth was particularly supported under aerobic conditions with organic sulfur compounds is consistent with the fact that RCB generally inhabit fully aerated surface waters where they degrade DMS and DMSP (Moran *et al*., 2003). However, among the validly described RCB only few species, including *Sagittula stellata*, *Sulfitobacter pontiacus* and *Silicibacter pomeroyi* have been demonstrated to oxidize both organic and inorganic sulfur compounds (Gonzalez *et al*., 1997; Gonzalez *et al*., 2003). While in these bacteria the Sox multienzyme complex is responsible for the utilization of inorganic sulfur compounds, results from this study identified the rDSR pathway as additional sulfur oxidation pathway in marine RCB.

Additional sulfur-oxidizing populations

We identified numerous unknown *dsrAB*-possessing *Alphaproteobacteria* that affiliated distantly with the RCB-derived *dsrAB* phylotypes. The DsrAB encoded on fosmid WS085G8 grouped with a cluster of PCR-derived sequences but displayed comparatively low sequence identity (74%) to the DsrAB of fosmid WS101A12. Accordingly, fosmid WS085G08 could not be assigned to any RCB representative using TaxSOM (Weber *et al*., 2010). Currently we can not infer the identity of these organisms.

Diversity and ecological relevance of dsrAB-carrying SOP

In 2009, Loy and colleagues published a first, comprehensive phylogenetic tree of DsrAB. It presented all currently known *dsrAB*-carrying SOP. In addition, a number of environmental sequences were presented. While Loy and colleagues (2009) found a comparable low diversity in alkaline lake sediment and oceanic open ocean waters, our study revealed a highly diverse community of *dsrAB*-possessing SOP in coastal sediment. Three major findings of our study substantially contributed to the resolution of the DsrAB phylogeny.

In a first *dsrAB*-targeted approach, we could successfully amplify the gene from the sulfur-oxidizing endosymbiont of *Oligobrachia haakonmosbiensis*. This allowed us to relate sediment derived *dsrAB* sequences to the thiotrophic symbiont and to link these sequences to the abundant WS-Gam209 population. In a second *dsrAB*-targeted approach we recovered a novel *dsrAB* phylotype from a genome fragment of an uncultured *Roseobacter*-clade bacterium. While in a third *dsrAB*-targeted approach we detected a closely related phylotype in a RCB containing enrichment culture. Both phylotypes improve the phylogenetic resolution of *dsrAB*-carrying *Alphaproteobacteria* and expand the group of known *dsAB*-carrying SOP to members of the ubiquitously distributed, ecological relevant marine *Roseobacter* clade.

Whether the rDSR complex of the diverse Janssand populations' functions in the oxidation of intermediary formed sulfur globules and whether it enables the respective SOP to utilize environmental available elemental sulfur remains a subject for future studies. Results from our metagenome analysis support a dioxygenase-dependent utilization of external elemental sulfur under aerobic conditions as described for aerobic acidophilic *Gammaproteobacteria* (Rohwerder and Sand, 2003). Our cultivation based *in vitro* experiments add some evidence for the oxygen-dependence of this reaction as the *dsr*-possessing RCB of the enrichment culture showed no pronounced growth on sulfur under anaerobic conditions.

Overall, the high diversity and additional quantitative PCR data (Lenk, 2006) support the ecological relevance of *dsrAB* possessing *Alpha*- and *Gammaproteobacteria* for sedimentary sulfur oxidation. The detected *dsrB* copy numbers of up to 10^7 cells ml^{-1} are in a similar order of magnitude as some of the novel gammaproteobacterial populations. For comparison, in sulfidic Namibian shelf waters a group of "only" 10^5 cells ml^{-1} was most likely catalyzing the detoxification of hydrogen sulfide (Lavik *et al*., 2009).

Biological sulfur oxidation potential of sedimentary SOP

Recent findings on the above mentioned Namibian shelf waters added to our view on the ecological role of chemotrophic SOP (Lavik *et al*., 2009). In sediments of the Janssand intertidal sand flat sulfide was so far hypothesized to be chemically oxidized by iron (Al-Raei *et al*., 2009; Jansen *et al*., 2009).

Complementary, the investigations of this study provide evidence that the re-oxidation of reduced inorganic sulfur compounds results from the concerted action of chemolithoautotrophic and chemolithoheterotrophic populations. These findings are consistent with cultivation studies that isolated representatives of both physiologic groups from coastal sites sediments (Brinkhoff *et al*., 1998; Podgorsek and Imhoff, 1999).

We found that autotrophic *Gammaproteobacteria* account for 3×10^8 cells ml sediment^{-1} (April 2005) and collected various evidence for a sulfur-oxidizing physiology of these organisms. Accordingly, the flux calculations demonstrated that the detected population densities could account for an entire biological sulfide removal and that the inferred cell specific sulfide oxidation rates are well comparable to microbial oxidation rates of chemotrophic SOP identified in Namibian shelf waters (Lavik *et al*., 2009).

In addition, sulfur-oxidizing RCB were detected. Currently detailed information on the abundance of these mutually lithoheterotrophic SOP I is lacking as data from quantitative qPCR of the *soxB* gene are not available. To date, several cultured *dsr* and *soxB* possessing *Alphaproteobacteria,* among them *Magnetospirillum* spp. (Geelhoed *et al*., 2010) and species in the genera *Sulfitobacter* and *Citreicella* (Sorokin, 2003; Sorokin *et al*., 2005) have been shown to display increased heterotrophic growth yields on acetate with reduced sulfur compounds. Accordingly, the proportion of 25 % acetate incorporating RCB detected by our MAR-FISH analysis could serve as proxy for the number of thiotrophic RCB. Their total abundance would amount to 3×10^7 cells ml^{-1} (April 2005) which is in the upper range of MPN based counts that suggested 10^5 to 10^8 acetate assimilating thiosulfate oxidizing bacteria ml^{-1} of coastal sediment (Podgorsek and Imhoff, 1999). In summary, our quantitative findings indicate a significant role of microbial sulfide oxidation in organic rich coastal sediments.

Different strategies of SOP in contrasting habitats of Janssand site

We observed contrasting microbial community compositions in the sediment and intertidal pool (Fig. 6). Consistent results from FISH, 16S rRNA and DsrAB gene analysis suggest that separated guilds of grain attached, biofilm forming vs. motile, pore-water inhabiting bacteria flourish in the contrasting habitats of Janssand sediment compartment. Grain-attached SOP comprise members of the *Gammaproteobacteria* and RCB. They reside immobilized in the transition zone of oxygen, nitrate and sulfide where they account for high proportions of the sediment microbial community. In this intermediate zone extracellular electron transport processes were recently detected (Nielsen *et al*., 2010). The bioelectric currents couple sulfide oxidation in several centimetres' depth to oxygen reduction at the sediment surface likely via bacterial nanowires. The grain-attached SOP detected in this study might provide a matrix for such currents.

In contrast, low abundant *Epsilonproteobacteria* related to *Arcobacter* spp. flourish in the porewater. They can respond to changing availability of oxygen and sulfide during inundation and exposure of the flat by directed chemotactic movement (Sievert et al., 2007). Similarly, filamentous nitrate storing *Beggiatoa* spp. glide in the suboxic zone of many sheltered coastal sediments between the interface of oxygen and sulfide (Jørgensen, 2010a). At Janssand site so far no *Beggiatoa* were detected. While they are generally noticed when growing as mats on sediment surfaces, recent findings suggest that scattered

filaments, which account for less than 0.01% of all cells, are hidden in the uppermost few centimetres and widespread in coastal sediments (Jørgensen *et al*., 2010b).

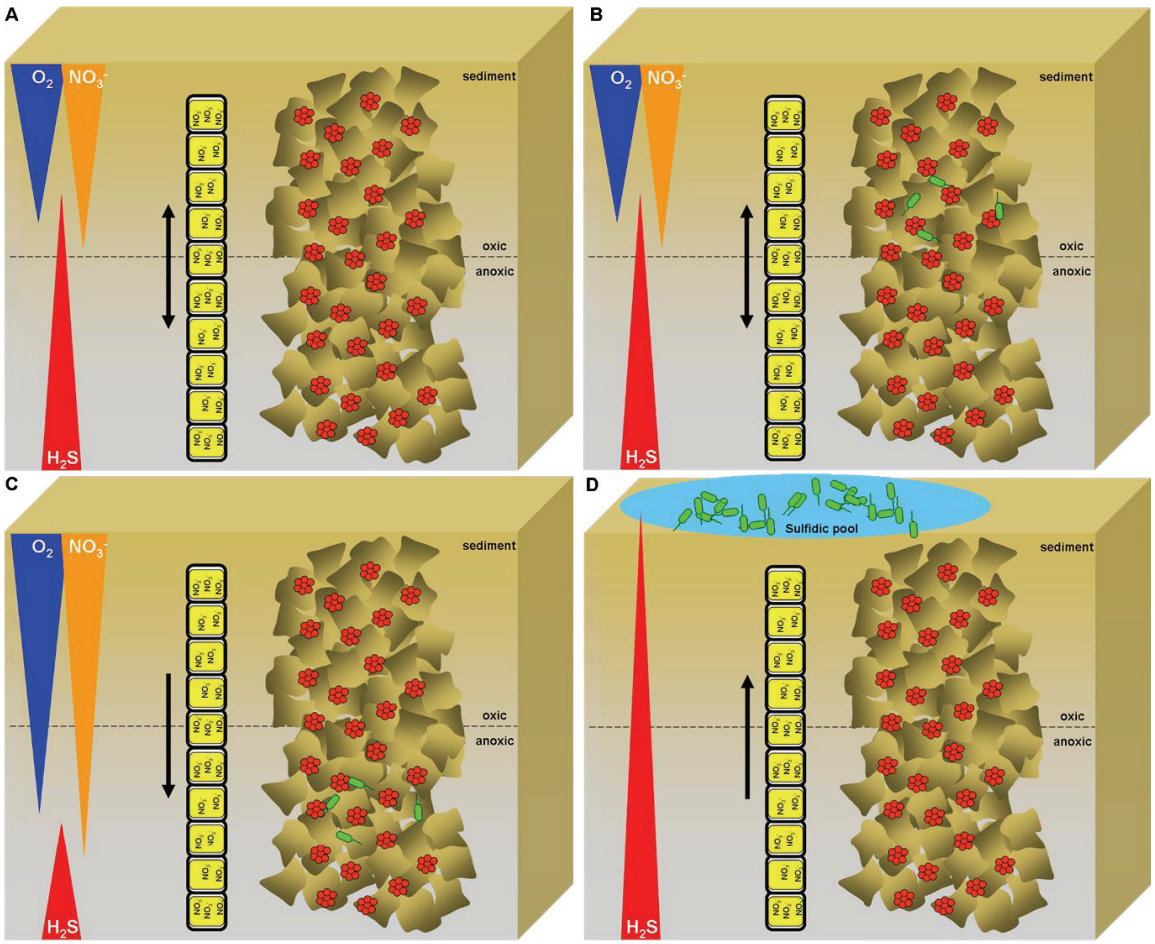

Figure 6 Strategies of SOP at sediment surfaces.
Two different functional guilds inhabit the sediment compartment of Janssand site. (**A**) Grain-attached bacteria (red) persist as biofilm-like structures around particles. (**B**) Motile SOP (green) inhabit the porewater. (**C**) During high tide oxygen penetrates deeper into the sediment. Mobile SOP can respond to fluctuating environmental conditions by directed movement towards i.e. deeper sediment layers. (**D**) In contrast, exposure of the sandflat during low tide restricts oxygen penetration. At the low water line sulfidic porewater seeps out from the sediment into pools where *Arcobacter* species temporarily accumulate. In addition, *Beggiatoa* species (yellow) can inhabit the oxic-anoxic transition zone of coastal sediments. Courtesy of M. Mußmann

6.2. Carbon Uptake by the Sediment Microbial Community

Over the last decade our knowledge on the ecophysiology of uncultured microorganisms was significantly broadened. Modern molecular technologies allowed scientists to probe deeper into the identity and activity of single cells. Here, MAR-FISH and nanoSIMS were applied to trace the fate of CO_2 and acetate in a coastal sediment microbial community (Fig. 7).

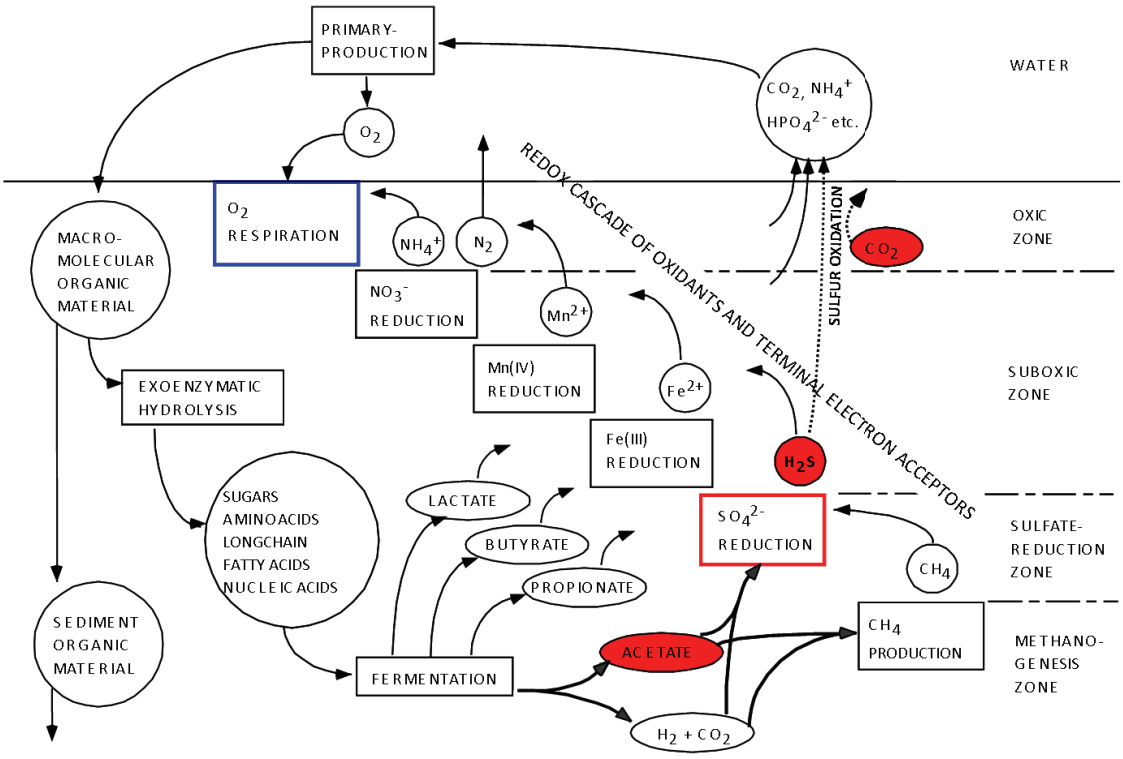

Figure 7 Organic matter degradation in marine sediments.
The initial step in organic matter degradation is extracellular enzymatic hydrolysis of macromolecular compounds. Subsequently oligomers and monomers are fermented to volatile fatty acids (acetate, propionate, butyrate and lactate) and H_2. These products are terminally oxidized to CO_2 and water using different electron acceptors. Sulfate reduction is the most important terminal oxidation process in marine shelf sediments leading to production of toxic H_2S. Our study on the Janssand intertidal sand flat revealed the highest uptake of acetate at the oxygenated sediment surface. Here, CO_2 fixation is likely coupled to inorganic sulfur compound oxidation. In contrast, we could hardly detect acetate incorporation by sulfate reducing prokaryotes in different sediment depths. Figure modified from Jørgensen (2000)

Gammaproteobacteria dominate dark CO_2 fixation and acetate uptake

MAR-FISH analysis provided evidence that the coastal sediment of Janssand site bears a rich community of autotrophic prokaryotes that fix inorganic carbon (Fig. 8 and 9). As found for SOP from pelagic redox clines (Grote *et al*., 2008; Jost *et al*., 2008; Glaubitz *et al*., 2009) the observed CO_2 incorporation by single cells provided strong evidence for an autotrophic metabolism coupled to sulfur-oxidizing activity of prevailing *Gammaproteobacteria*. MAR-FISH thus extends sequence based findings of 16S rRNA and functional gene analysis. Uptake was highest in the slurries and at the surface of the core, which is consistent with the fact that CO_2 fixation is coupled to the oxygen dependent oxidation of reduced sulfur compounds (Thomsen and Kristensen, 1997; Nielsen *et al*., 2010).

The findings complement earlier studies that investigated the role of bacterial chemoautotrophs in coastal sediments (Kepkay and Novitsky, 1980) by providing quantitative data on their *in situ* abundance and phylogenetic affiliation. The identified chemoautotrophs feed a microbial foodweb apart from infiltrated organic matter and benthic microalgal communities (Billerbeck *et al.*, 2007) and thus promote primary production in marine sediments.

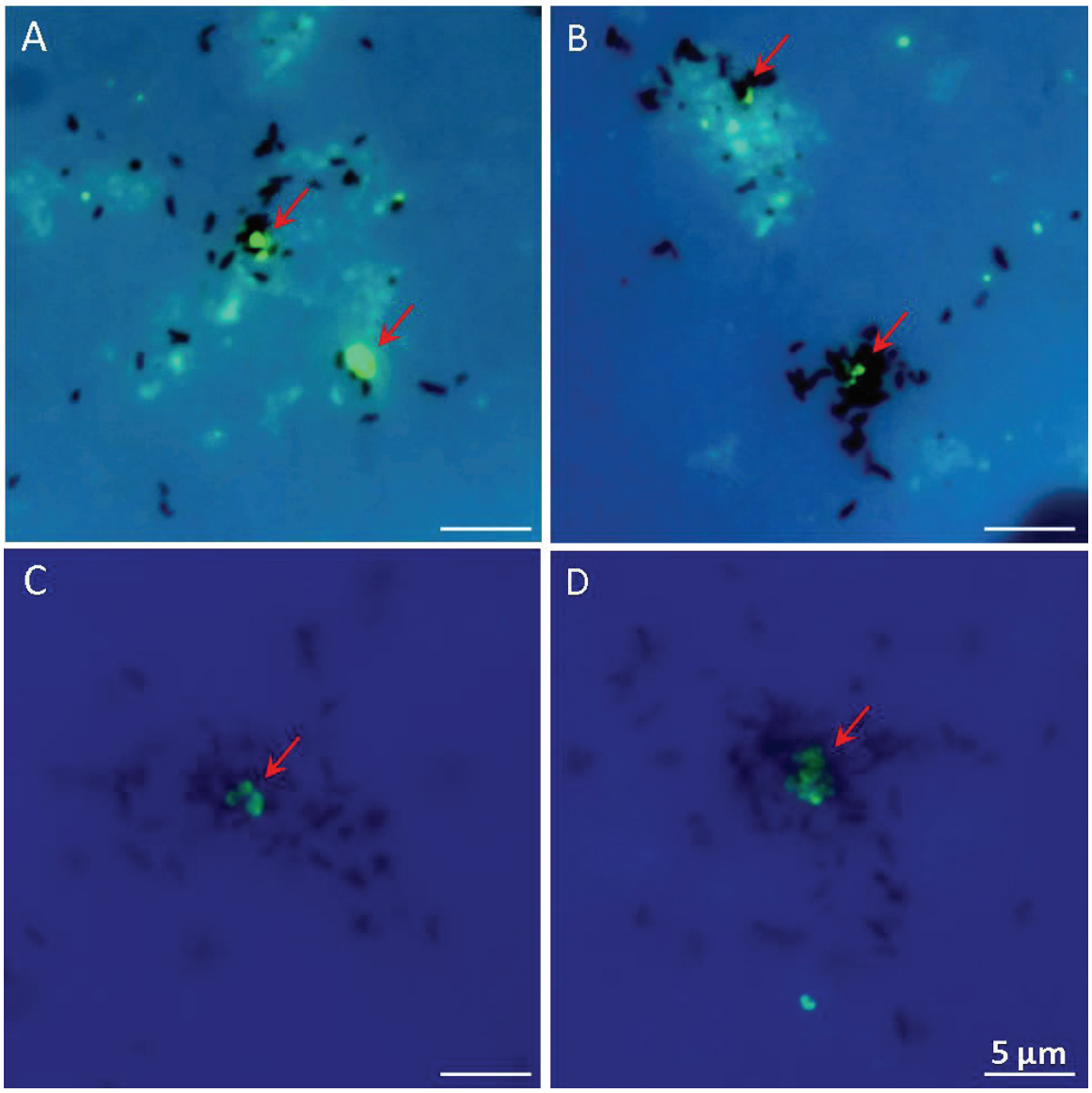

Figure 8 Epifluorescence microscopy images of substrate assimilating cells.
Microautoradiography combined with FISH visualizes *Gammaproteobacteria* (green) that assimilated (A, B) $^{14}CO_2$ or (C, D) ^{14}C-acetate. The precipitation of silver grains (dark granules) associated with single cells and cell aggregates indicated substrate uptake. Due to efficient ultrasonic treatment cell aggregates were only occasional detected. In future studies high-resolution nanoSIMS analysis may visualize different activity patterns of individual cells clustered in aggregates.

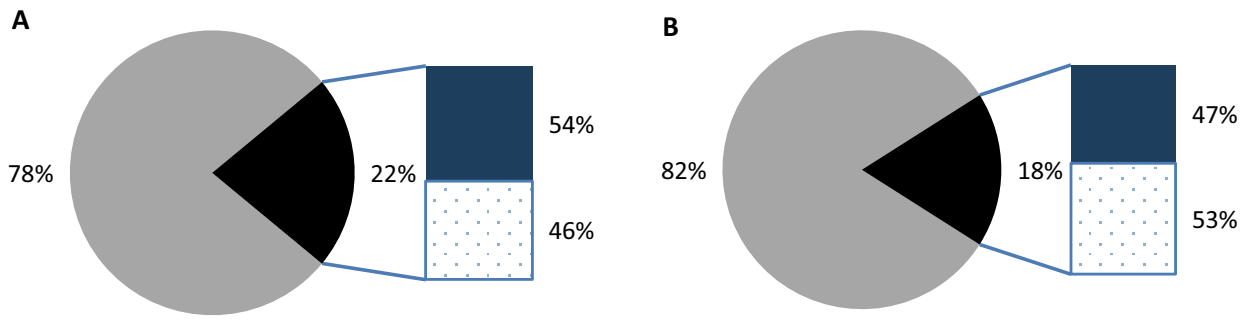

Figure 9 Incorporation of inorganic and organic carbon by the sediment microbial community.
The relative abundances of (A) $^{14}CO_2$ or (B) ^{14}C-acetate incorporating microbial cells (▪) and *Gammaproteobacteria* (▪) were assessed by MAR-FISH. The proportion of cells that did not incorporate the substrate (▪) and the proportion of substrate incorporating cells other than *Gammaproteobacteria* (▫) are indicated. Surface sediment sampled in June 2009 was incubated in slurries under oxic conditions.

In addition, the assimilation of organic carbon (Fig. 8 and 9) was quantified. Acetate serves as a carbon source for several giant sulfur oxidizers (Nelson and Castenholz, 1981; Schulz and Schulz, 2005; Hogslund *et al*., 2009) and *Thiobacilli* and *Thiomicrospira* species (Kuenen and Veldkamp, 1973; Wood and Kelly, 1989) and can be linked to sulfide and thiosulfate oxidation (Hagen and Nelson, 1996; Schulz and de Beer, 2002; Otte *et al*., 1999; Nielsen *et al*., 2000). Here, we can not directly link *in situ* acetate uptake to sulfur-oxidizing activity of the labeled *Gammaproteobacteria*. However, some of the acetate utilizing cells might represent lithoheterotrophic or mixotrophic populations. As organic compounds are readily available in the surface sediment of Janssand, it is likely that prevailing SOP use both sulfide and acetate.

Similarly, RCB contribute to turnover of acetate. The observed assimilation under oxic conditions is consistent with their high *in situ* abundance in the surface layers of Janssand site and an aerobic lifestyle. In deeper permanently anoxic sediment layers RCB accounted for lower proportions of the microbial community. We hypothesize prevailing cells to be of low activity or inactive. Here, our cultivation experiments yielded evidences for a facultative anaerobic metabolism of sedimentary RCB. Both, metagenomic information and cultivation experiments indicated the use of dimethylsulfoxide and nitrate as alternative electron acceptors. In addition, Koepke (2007) and Sass and colleagues (2009) isolated facultative anaerobic RCB from nearby tidal flats that grew anaerobic with acetate as electron donor and trimethylamine-N-oxide (TMAO) and dimethyl sulfoxide (DMSO) as terminal electron acceptor. These findings add evidence for an adaptation of sedimentary RCB to anoxic conditions. Alonso and Pernthaler (2005) who observed glucose uptake of pelagic RCB under anaerobic conditions proposed that respective physiology represents a special adaptation to temporally anoxia which aggregate-associated RCB face upon burial in sediments. Similarly, the facultative anaerobic lifestyle of sedimentary RCB likely facilitates a response to temporal oxygen limitation in the tidal surface sediment. Whether it enables life under permanently anoxic conditions in deeper sediment layers needs be explored by further experiments.

Uptake of additional organic substrates

The consumption of particular dissolved organic matter fractions in the sea has been the subject of intense MAR-FISH investigations on marine bacterioplakton communities. However, nearly nothing is known about assimilation patterns of different substrates by sediment microbial communities, nor has the relative contribution of various phylogenetic groups to substrate uptake been investigated so far. Accordingly, surface sediment sampled in 2009 was incubated with various organic substrates, including the sugars glucose and n-acetylglucosamine, the amino acid leucine and the organosulfonate taurine. Between 10 and 20% of the total microbial community incorporated the different substrates (Fig. 10). Consistent with previous incubations (Zerjatke, 2009) numerous cells incorporated glucose under oxic and anoxic conditions. Preliminary analysis revealed that members of the *Gammaproteobacteria* accounted for substantial proportions of substrate-assimilating cells for all carbon compounds (Fig. 11). Initial results indicate that RCB and *Bacteroidetes* assimilate glucose and N-acetylglucosamin. Leucine, a commonly used tracer of bacterial protein synthesis was chosen as a substrate for dissolved free amino acid incorporation. Similar to acetate incorporation patterns, *Gammaproteobacteria* and RCB accounted for the highest fractions of leucine-active cells. In contrast, only very few *Deltaproteobacteria* and cells of *Desulfobacteraceae* assimilated the substrate (<2% of total probe targets). *Bacteroidetes* and *Planctomycetales* did not incorporate leucine. The findings are consistent with investigation on pelagic RCB that have been found to account for substantial proportions of glucose and leucine incorporating cells in coastal North Sea waters {Alonso, 2006 #3747; Alonso, 2006 #4566}. Similarly, pelagic *Gammaproteobacteria* and *Bacteroidetes* contributed to glucose turnover {Alonso, 2006 #4566}. Further analysis has to assess community bulk incorporation rates for applied substrates and the fraction and identity of substrate-incorporating cells in the different phylogenetic groups.

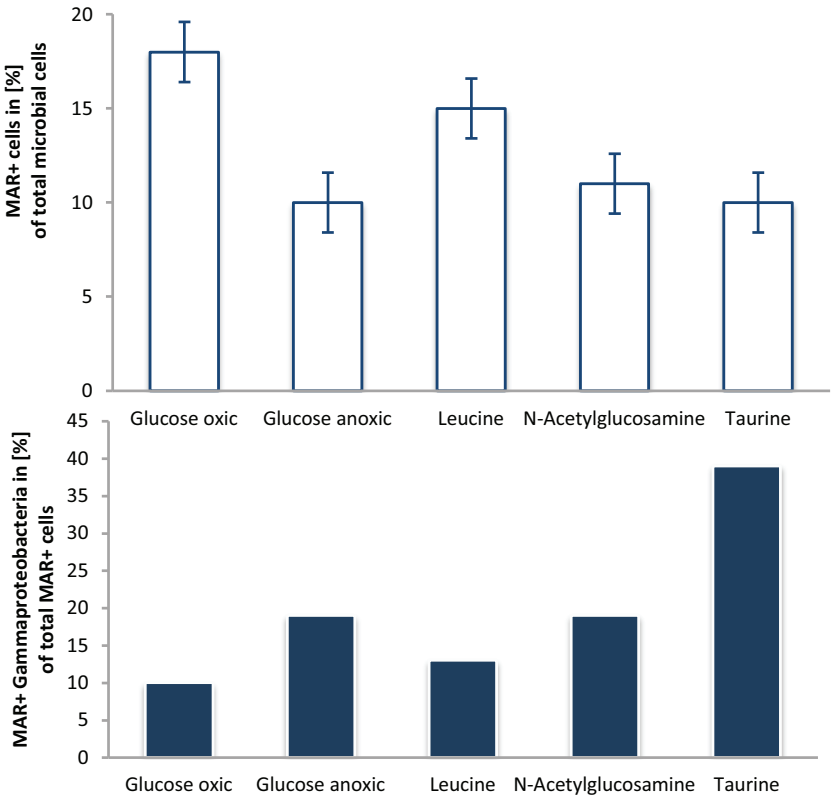

Figure 10 Assimilation of additional organic substrates by the microbial community.

The relative proportion of cells that incorporated the substrates is given in % of total microbial cells. Generally sediment (0-1 cm) was incubated oxically. For glucose, sediment (10-11 cm depth) was additionally incubated anoxically. Bars illustrate mean values of duplicate incubations. Exposure times to microautoradio-graphic emulsion: glucose, leucine, taurine 10 days; N-acetylglucosamine 4 days

Figure 11 Assimilation of additional organic substrates by Gamma-proteobacteria.

The relative proportion of substrate assimilating *Gammaproteobacteria* is given in % of total substrate assimilating cells (preliminary data from one parallel of two replicates).

Gammaproteobacteria as abundant and active sediment group

In summary, *Gammaproteobacteria* accounted for the highest proportion of substrate assimilating cells among prevailing sediment bacteria (Fig. 9 and 11). In addition, they constituted the largest clone fractions within all 16S libraries and represented the numerically dominant group *in situ* throughout the study period.

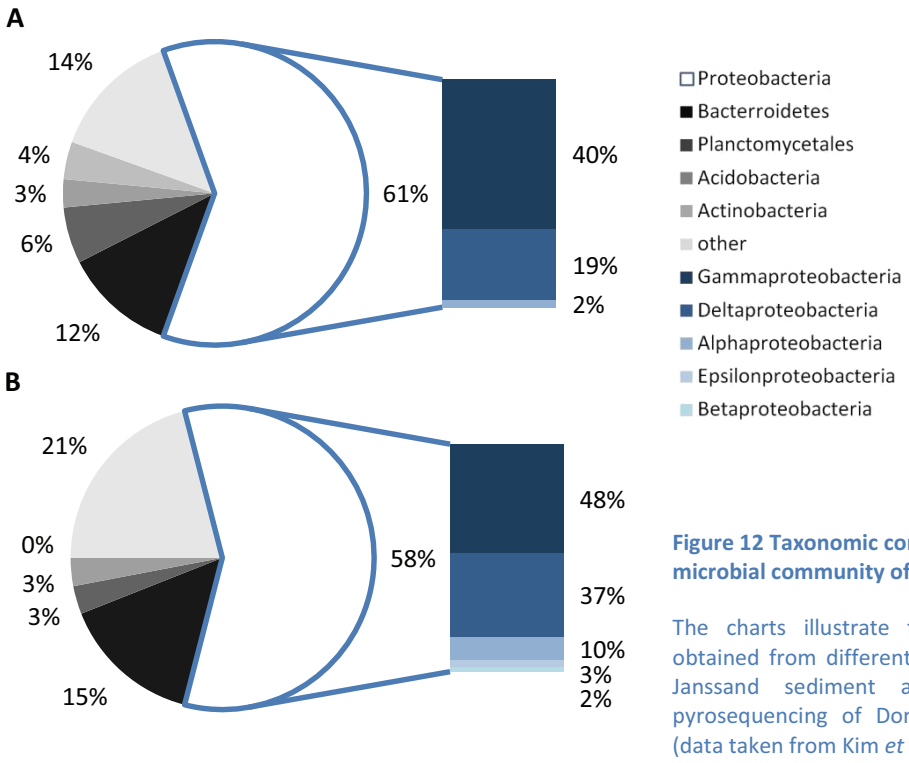

Figure 12 Taxonomic composition of the sediment microbial community of geographically distant tidal sites.

The charts illustrate the (**A**) affiliation of sequences obtained from different 16S rRNA gene clone libraries of Janssand sediment and (**B**) those obtained from pyrosequencing of Dongmak tidal flat sediment, Korea (data taken from Kim *et al*., 2008).

Large fractions of *Gammaproteobacteria*-related clones (Fig. 12) have been repeatedly observed for coastal (Kim *et al*., 2004; Polymenakou *et al*., 2005; Hong *et al*., 2006; Bi-Wei *et al*., 2009), aquaculture (Asami *et al*., 2005) and polar sediment (Bowman *et al*., 2005; Li *et al*., 2009; Teske *et al*., 2011) as well as permeable shelf (Hunter *et al*., 2006), subseafloor (Santelli *et al*., 2008) and hydrothermal sites (López-García *et al*., 2003). Similarly, high FISH counts were reported for the investigated (Ishii *et al*., 2004) and related North Sea sites (Llobet-Brossa *et al*., 1998; Musat *et al*., 2006) or different sediments worldwide (Ravenschlag *et al*., 2001; Perner *et al*., 2007; Schauer *et al*., 2010). However, few studies have defined specific gammaproteobacterial populations (Ravenschlag *et al*., 2001) and the metabolic potential of its versatile members has been rarely studied by molecular tools. Edlund and colleagues (2008) found major portions of *Gammaproteobacteria* in 16S rRNA gene libraries constructed from bromodeoxyuridine (BrdU)-labeled DNA and from cDNA obtained by reverse transcription polymerase chain reaction (rt-PCR) of RNA and thus provided evidence for their *in situ* growth and activity in Baltic Sea sediments. Teske and colleagues (2011) studied the 16S rRNA gene diversity and extracellular enzyme activities of heterotrophic sediment communities in surface and subsurface sediment. They suggested *Gammaproteobacteria* to contribute to extracellular enzymatic hydrolysis of polysaccharides and algae extracts (Teske *et al*., 2011) but lacked a direct link to the identity of substrate hydrolyzing microbes. Additional SIP based studies repeatedly recovered gammproteobacterial sequences from coastal

sediment demonstrating metabolic activity by *in situ* substrate incorporation of phytodetritus (Gihring *et al*., 2009) and low molecular weight compounds (Miyatake *et al*., 2009; Webster *et al*., 2010).

Overall, our MAR-FISH experiments expand previous findings as they provide first evidence for a numerical relevance of the active *Gammaproteobacteria* under oxic and anoxic conditions in different sediment layers of a coastal site. Given their global distribution our findings support a central role in sulfur and carbon cycling in marine sediments worldwide.

MAR-FISH concluding remarks

Application of the MAR-FISH method provided first insights into the *in situ* abundance of autotrophic and heterotrophic *Gammaproteobacteria*. However, we could hardly resolve CO_2-fixation and acetate incorporation to the level of distinct gammaproteobacterial populations. Bacteria of the heterotrophic NOR5/OM60 clade, which did not take up acetate, might prefer other carbon compounds. However, preliminary attempts also failed to detect incorporation of alternative substrates such as glucose (Zerjatke, 2009). In case of the WS-Gam446 population the lack of substrate uptake might be addressed to their low *in situ* abundance (<1% of all cells). Assuming that on average only 20-30% of hybridized target cells are labeled, as found for the CO_2-incoporating WS-Gam209 group, the proportion of WS-Gam446 cells with associated silver grain precipitates detected by manual analysis would not allow a reliable quantification. Here, automated high-throughput microscopy holds a promising improvement for elaborative manual analysis of sediment bacteria (Zeder *et al*., 2009; Zeder and Pernthaler, 2009). The current preparation of Janssand samples will allow a fully automated enumeration of MAR and FISH signals in the near future (M. Zeder, personal communication). In addition, the application of density gradient centrifugation prior to MAR-FISH can significantly increase the cell density on membrane filters and facilitate a more efficient automated microscopic analysis.

The specific probes applied here cover approximately one third of all *Gammaproteobacteria*. Accordingly the proportion of cells not targeted by the probes might account for the observed uptake. During the course of the study the entire gammaproteobacterial community composition could not be resolved. The high overall diversity and the insufficient sequence coverage along with the limited set of applied probes hampered further resolution. Similar findings for sulfate reducing *Deltaproteobacteria* have been reported (Mussmann *et al*., 2005). Thus, an array of low abundant species likely contributes to the observed substrate uptake and accounts for the overall gammaproteobacterial diversity *in situ*. Adequate resolution of the gammaproteobacterial community structure by FISH might be achieved by application of extended probe sets and modified counting protocols (Gomez-Pereira *et al*., 2010), which allow the reliable detection and quantification of scarce species.

6.3. Future Perspectives

The sequence and isotope based analysis of single sediment populations represents a major methodological advance of this thesis. Although SIP has brought substantial progress to single-cell microbial ecology of sediment microbial communities during recent years, the analysis by modern imaging-technologies that enable identification and enumeration of substrate incorporating microbes still remains a weak point. Here, MAR-FISH and nanoSIMS were successfully applied to marine sediments. Previously, these methods had only been used to follow substrate uptake by

bacterioplankton communities. Particularly, for MAR-FISH adequate cell detachment by ultrasonic treatment and optimized dispersal by dilution minimized particle background and allowed the assignment of silver grain precipitates to individual cells. Additional application of MAR-FISH to samples from the Argentina continental margin (J.Sawicka, unpublished data) revealed first insights into substrate incorporation patterns of microbial communities that are indigenous to muddy shelf sediments from several hundred meters depths. The method is thus ideally suited for future exploration of the activity of uncultured sediment microbes from diverse habitats.

Magneto-FISH of sedimentary Gammaproteobacteria

To access the specific Wadden Sea sediment microbial populations for phylogenetic and physiological characterization we also applied magneto-FISH. The method was recently introduced by Pernthaler and colleagues (2008) and represents an alternative to flow cytometry. It offers the possibility to purify microorganisms directly from the sediment without elaborative density-gradient centrifugation and simplifies processing of radioactive labeled samples. Magneto-FISH is based on the specific attachment of fluorescently stained target cells to paramagnetic beads coated with fluorochrome-targeting antibody and allows the separation of any probe hybridized target population from sediment particles and non-hybridized cells.

CARD-FISH with probe GAM42a was performed to specifically stain *Gammaproteobacteria* (Fig. 13A). The sediment-cell-suspension was hybridized in solution, sonicated and subjected to bead capture as previously published (Pernthaler *et al*., 2008). Only few gammaproteobacterial cells were recovered after separation of beads from sediment. In addition, the captured cell fraction contained numerous non-target cells (Fig.13 B-D). Accordingly, we did not proceed with further analysis of the captured cells by 16S rRNA gene sequencing, MAR-FISH or nanoSIMS. Longer incubation times and repeated separation procedures might increase the efficiency of capture and purity of target cells in future applications.

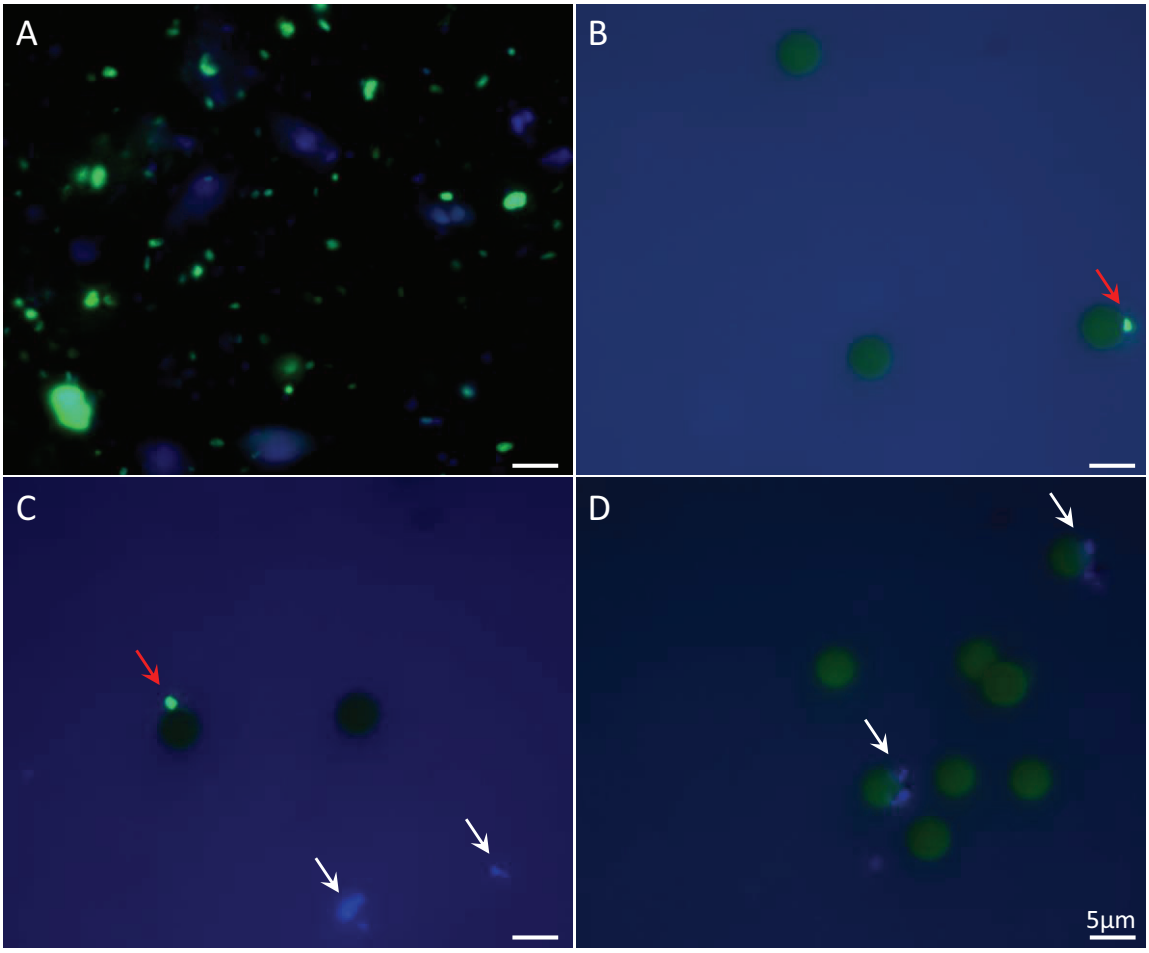

Figure 13 Magneto-FISH of sedimentary Gammaproteobacteria.
(A) Epifluorescence microscopy images of sediment-cell-suspension after FISH prior to paramagnetic bead capture. Target cells are stained in green (fluorescin-conjugated tyramide) and non-target cells are stained in blue (DAPI). (B-D) Collected cell fraction after bead capture containing few *Gammproteobacteria* (red arrows) and numerous non-target cells (white arrows).

Fluorescence activated cell sorting of sediment bacteria and its perspectives

The development of a workflow which combines the separation of cells from sediment particles, their targeted hybridization and targeted sorting constitutes a valuable methodological approach (Fig. 14). The combination of density gradient centrifugation with Flow Cytometry-based sorting of fluorescently labeled target cells enables phylogenetic and ecophysiological studies of otherwise “inaccessible” sediment bacteria (Kalyuzhnaya *et al*., 2006; Amalfitano and Fazi, 2009). For the Janssand intertidal sandflat it provided first insights into the phylogenetic diversity of specific target populations (RCB, NOR5/OM60 clade, Fig. 15 and 16). In addition, it proved to be ideally suited for a fast and background free analysis of sediment bacteria by nanoSIMS (Fig. 17). Current bottlenecks of the methods include a careful assessment of cell loss during density gradient centrifugation. Although purified cell fractions have been shown to be representative of the original sample (Fazi et al., 2005), cell recovery needs to be optimized for different sediments by, i.e. adaptation of centrifugation time and efficient sediment-cell detachment. The proper detachment of cells from solid sediment particles and the effective separation of bacterial aggregates are also preconditions for high purity fluorescence activated cell sorting. Here,

the 16S rRNA gene library generated from the sorted RCB contained numerous sequences not affiliating with the *Roseobacter* clade, which might have resulted from the co-sorting of, though few, unlabeled non-target cells.

In summary, the results presented here contribute a fundament for further "nano scale views" on sediment microbial communities. For future studies, the amplification of functional genes, MDA and geneFISH of specific target populations constitute promising downstream applications in the advancing field of single cell science.

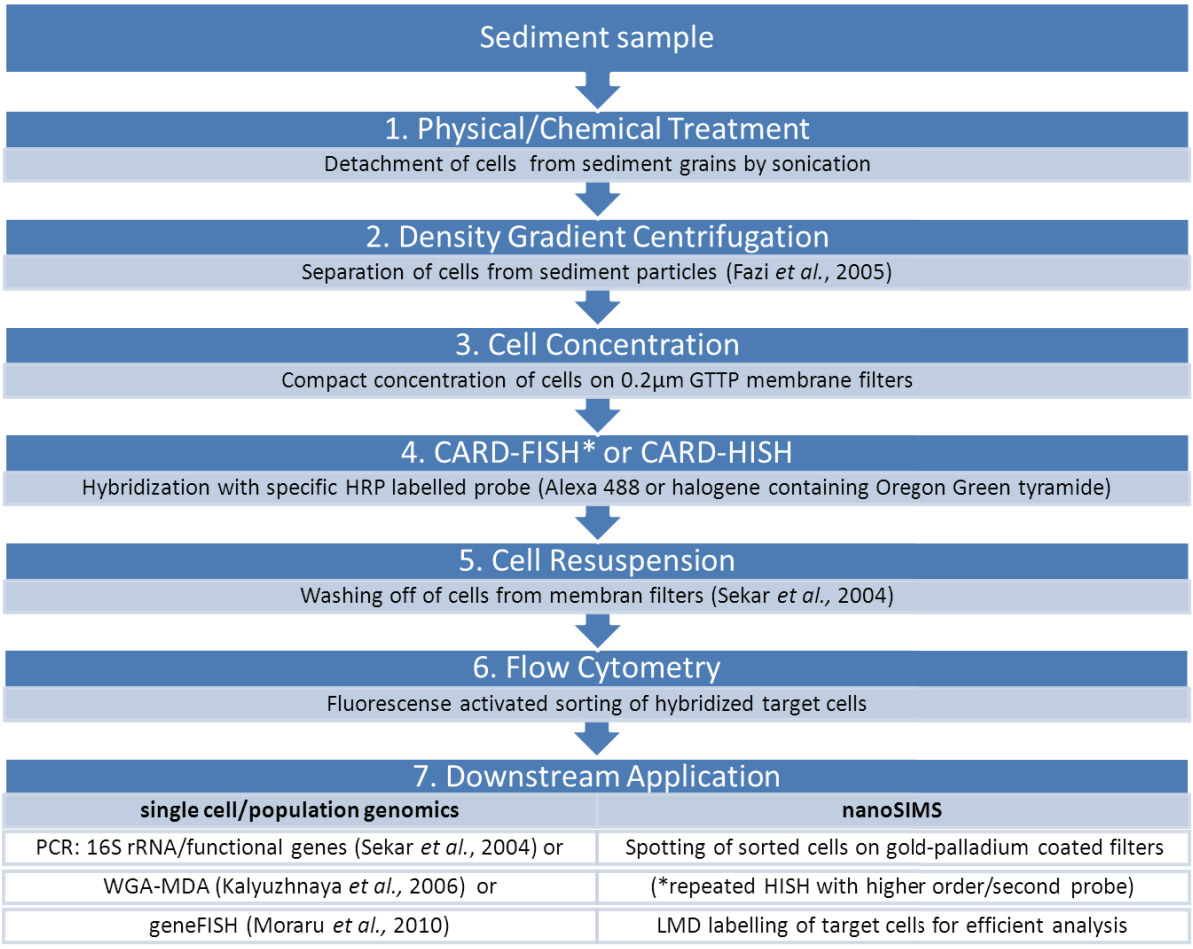

Figure 14 Methodological approach used to purify sediment bacteria for nanoSIMS analysis.
Alternative downstream applications are also shown (Sekar et al., 2004; Kalyuzhnaya et al., 2006; Moraru et al., 2010).

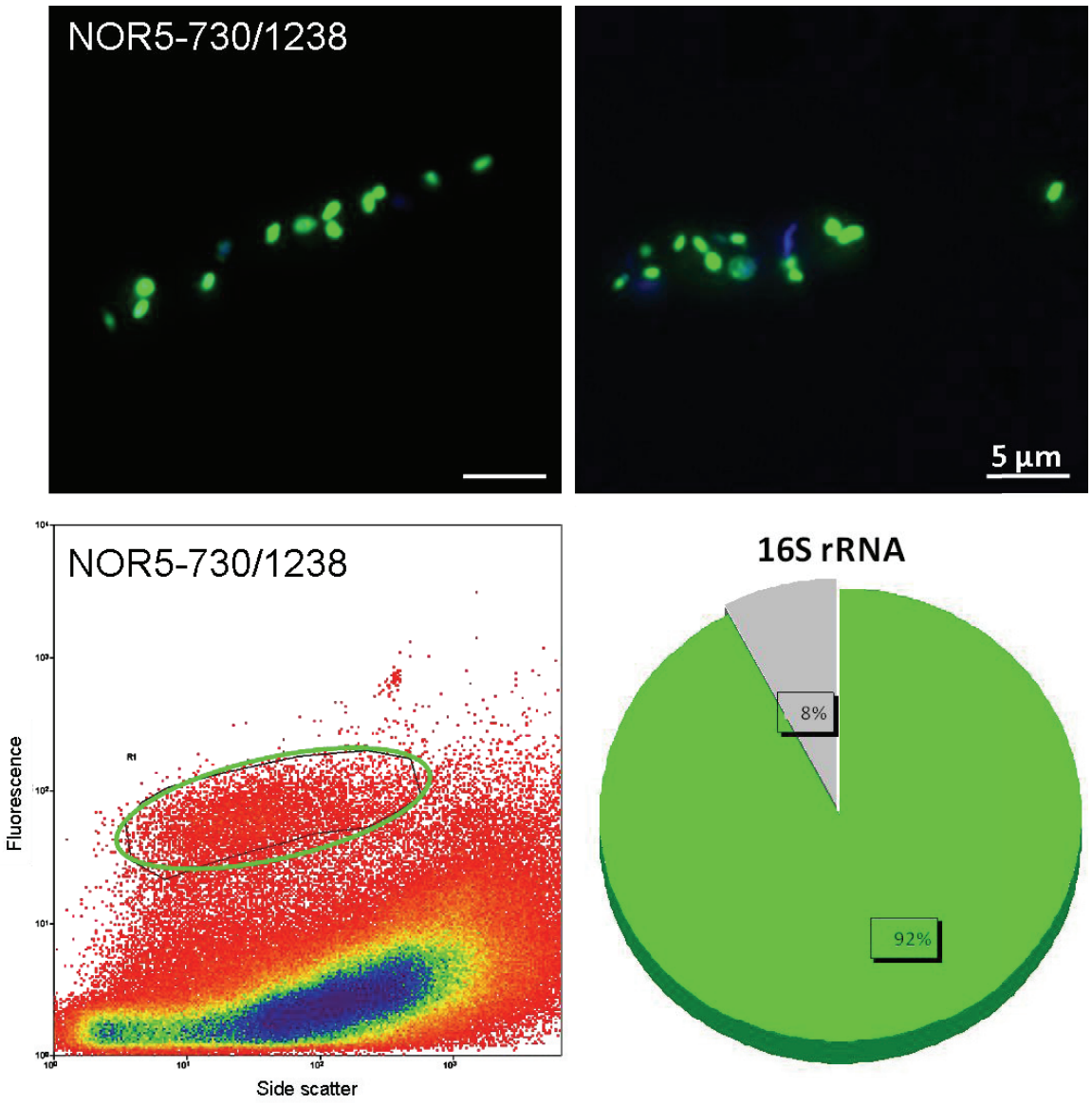

Figure 15 Fluorescence activated cell sorting of NOR5/OM60 clade bacteria.
Illustrated are epifluorescence microscope images of sediment bacteria hybridized to probe NOR5-730 and NOR5-1238 (green, probe; blue, DAPI) after fluorescence activated cell sorting, the targeted cell fraction (green circle) as identified by fluorescence and side angle light scatter in a bivariant dot plot diagram and the composition of the 16S rRNA gene library obtained from 37.000 sorted target cells. The proportion of sequences associated with the NOR5/OM60 clade is shown in green.

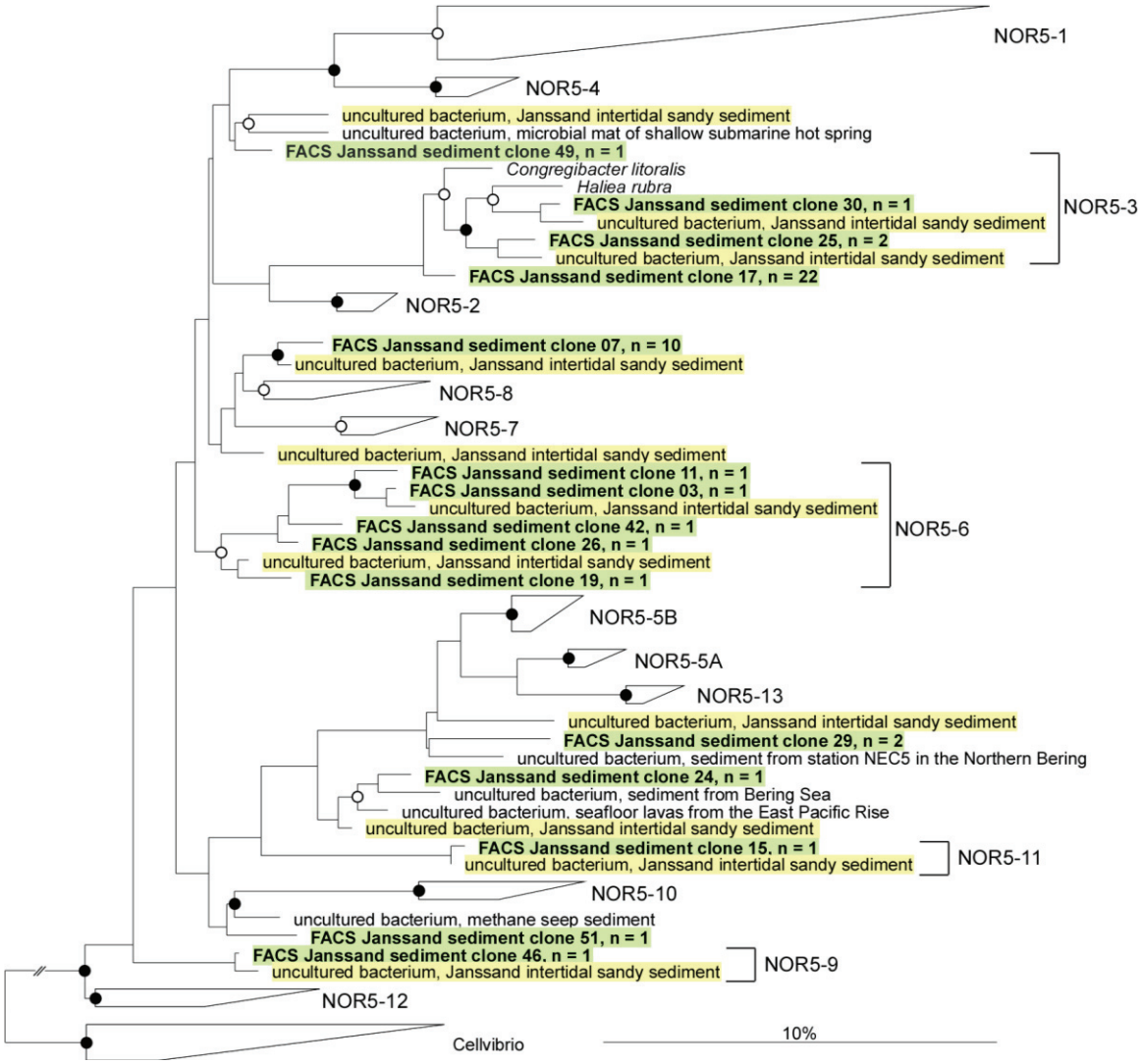

Figure 16 Phylogeny of the 16S rRNA of sedimentary NOR5/OM60 clade bacteria.
The tree illustrates sequences that were obtained after fluorescence activated sorting of cells hybridized to probe NOR5-730 and NOR5-1238 (■). Sequences that displayed >97% nucleotide identity were grouped into a single OTU, where 'n' equals the number of sequences per OTU. Most OTUs affiliated with sequences previously obtained from bulk DNA of Janssand sediment (■). The NOR5/OM60 subclades are indicated according to Yan and colleagues (2009). RAxML bootstrap values are shown for lineages >70% (●) and >50% (○) bootstrap support. The bar indicates 10% sequence divergence.

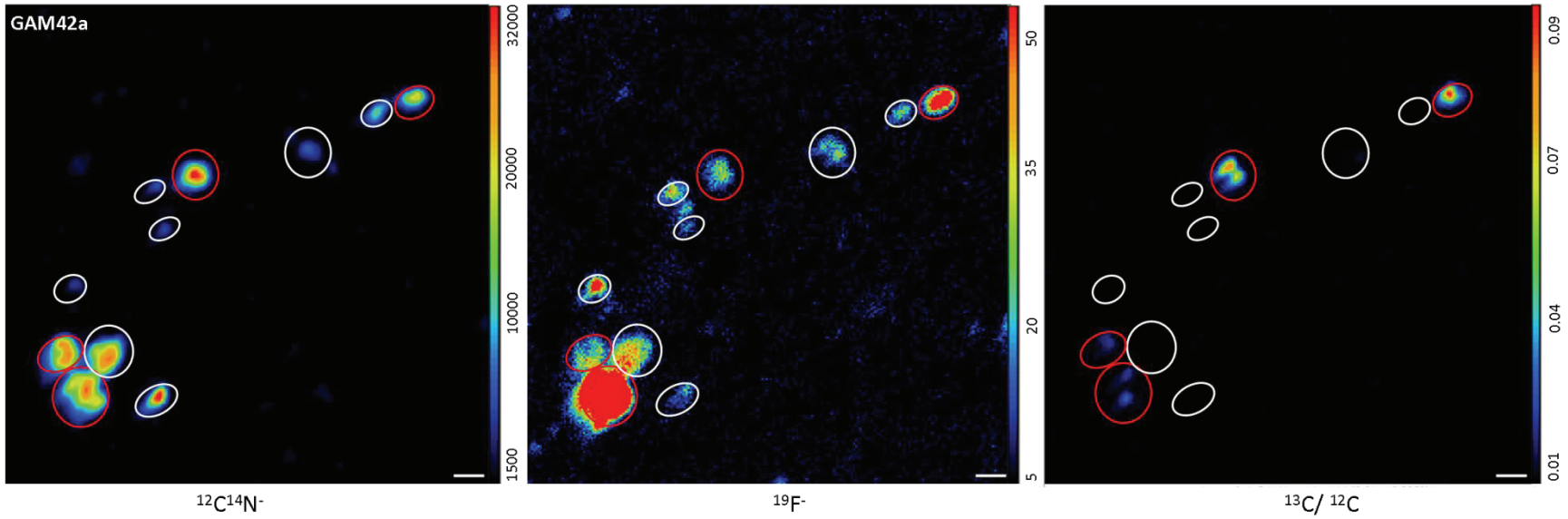

Figure 17 ^{13}C-acetate incorporation by individual cells of *Gammaproteobacteria* (GAM42a) revealed by nanoSIMS after fluorescence activated cell sorting.
Circels indicate single cells of *Gammaproteobacteria* identified by the natural abundances of ^{12}C-carbon and ^{14}N-nitrogen (^{12}C ^{14}N-) and the abundance of fluorine (^{19}F-) after HISH with a HRP-conjugated oligonucleotide probe and CARD with a fluorine containing tyramide. The ^{13}C/^{12}C ratios reveal cells that were enriched in ^{13}C -acetate (red circles). The scale bar corresponds to 1µm.

Outlook

Future studies need to further infer identity and diversity of substrate incorporating microbes. For this purpose a combination of SIP, full cycle 16S rRNA approach and MAR-FISH constitutes a powerful methodological approach (Ginige *et al*., 2005) as it could overcome the weaknesses of applying each method separately. In case of the MAR-FISH technique substrate uptake by diverse microorganisms can only be monitored at the level of available oligonucleotide probes, which constitutes a clear deficiency. Here, sequences that are obtained from the ^{13}C fraction of SIP incubations could yield additional targets for the design of novel oligonucleotide probes. Subsequent FISH will than reveal the abundance of respective target organisms and microautoradiography or nanoSIMS can visualize substrate uptake. Previous studies successfully applied microcalorimetry and SIP to trace major conumers of glucose (Kleindienst, 2008) and algae derived biomass (J. Graue, unpublished data) in the sediment of Janssand site (Kleindienst, 2008). The combined approach of SIP and MAR-FISH could elucidate whether a number of rare populations contribute to substrate utilization or whether few populations could monopolize substrate turnover.

Flow cytometry based sorting with subsequent multiple displacement amplification of genomic DNA from 500 target cells (M.Mußmann, pers. communication) is now feasible and thus provides genomic access to a variety of populations. The approach could help to improve phylogenetic resolution of the different functional gene phylogenies, which are biased towards the well studied symbiotic species. In addition, it will allow resolving the population structure on the level of 16S rRNA by examining the diversity within individual clusters of related species (Gray *et al*., 1999b; Achtman and Wagner, 2008). Accordingly, gammaproteobacterial cells targeted by the novel probes (including those that displayed abundances <1 % of all cells) could be sorted and simultaneously screened for different functional genes of sulfur oxidation (Dissimilatory sulfite reductase, *dsrAB*; Sulfate thiohydrolse, *soxB*) and carbon metabolism (RubisCO, *cbb*). After assignment of functional genes to the 16S rRNA based identity of the sorted cells, populations of interest might be further analyzed for CO_2 and/or acetate incorporation using nanoSIMS. Alternatively, the sequencing of entire population metagenomes (Woyke *et al*., 2006) will allow a more comprehensive reconstruction of their metabolic capabilities. To date, invertebrate symbionts still represent some of the best characterized uncultured members of *Gammaproteobacteria* as their genetic inventory and sulfur-oxidizing physiology has been extensively studied (Stewart *et al*., 2005 Dubilier *et al*., 2008). A dissection of the complex sediment microbial assemblages by the above mentioned applications will reduce diversity to defined target organisms of interest and provide more comprehensive insights into the nature of single populations or even species that thrive in coastal marine sediments.

A comparable approach might combine MAR-FISH or HISH-SIMS with laser microscope dissection. After excision of isotopically-labeled cells and subsequent MDA a screening for 16S rRNA (Gloess *et al*., 2008) and functional marker genes will allow the phylogenetic and functional characterization of individual cells involved in substrate uptake

Flow cytometric sorting and liquid scintillation counting of radioactively labeled, fluorescently stained populations (Zubkov *et al*., 2002) combined with MAR will reflect substrate assimilation rates of single

cells better than the average incorporation rates inferred from the total number of MAR-positive cells. In this way the relative contribution of the WS-GAM209 population to overall dark CO_2 fixation could be further investigated. Similarly, flow cytometric sorting provides a powerful tool for the targeted enrichment and subsequent MAR-FISH analysis of rare Wadden Sea sediment populations. For nanoSIMS, the density of target cells spotted on the membrane filters needs to be increased to speed up analysis, to increase the number of analyzed target cells and allow scans of higher resolution. This might currently be achieved by sorting a higher number of target cells.

Double-labeling experiments with ^{2}H- or ^{14}C-acetate and ^{13}C-bicarbonate could reveal substrate incorporation patterns of autotrophic, heterotrophic and mixotrophic populations. Tracer experiments with ^{35}S-labeled sulfate could be applied to identify and quantify elemental sulfur S^0 utilizing/accumulating bacteria (Gray *et al*., 1999a). Here, future studies might focus on the conspicuous pools and characterize SOP that oxidize sulfide to elemental sulfur and those that ultimately oxidize elemental sulfur to sulfate. In this respect it would be of interest to follow the accumulation of *Arcobacter* species and the formation of zero-valent sulfur species over time during ebb tide. Wether the pool inhabiting *Epsilonproteobacteria* originate from sedimentary pore water that drains into the pools should be further investigated by selective pore water sampling.

Simultaneous detection of S^0-accumulating and ^{13}C-acetate incorporating bacteria might enable quantification of chemolithoheterotrophic SOP, in particular RCB. Quantitative PCR of the *dsrAB* or *soxB* and *soxCD* gene could provide additional estimates on the abundance of sulfur-oxidizing RCB and the general role of the SOX multi-enzyme pathway in coastal sediment.

To quantify the contribution of sediment microbial communities to carbon mineralization MAR-FISH experiments should trace assimilation patterns of various organic substrates in the different compartments of the Janssand sand flat. Here, MAR-FISH can enable novel insighs into the activity of subsurface microbial communities that reside in several meters depth, which might provide a model system for deep-biosphere studies (Wilms *et al*., 2006, Engelen and Cypionka, 2009). Another application could investigate the effect of elevated sulfide concentrations and low redox potential on heterotrophic microbial activity in reduced anoxic sediment surfaces (‘black spots’) as compared to oxidized surface sediments (Freitag *et al*., 2003; Middelburg and Levin, 2009). In addition, the leucine incorporation method could be used to assess prokaryotic heterotrophic production (Danovaro, 2009). Complementary to recent studies that investigated phytodetritus degradation (Gihring *et al*., 2009; Teske *et al*., 2011) the incorporation of isotopically labeled algae biomass by *Gammaproteobacteria*, *Planctomycetes* and *Bacteroidetes* could be detected.

In addition to culture-independent investigations, targeted cultivation approaches for autotrophic and heterotrophic SOP are needed (C. Dona, S. Lenk, unpublished data). Those can provide representative isolates that are desired for testing generated hypotheses under defined laboratory conditions. Here, the obtained RCB isolate AK199a/b (S.Lenk, S. Hahnke, unpublished data) already represents a valuable target organism. Additional genomic analysis will reveal whether it possesses a metabolic island of sulfur oxidation.

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Weber, M. (*submitted*) Practical Application of Self-Organizing Maps to interrelate biodiversity and functional data in NGS-based environmental metagenomics *submitted*.

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Additional Contributions

Co-Authored Manuscipts

Yan, S., Fuchs, B.M., Lenk, S., Harder, J., Wulf, J., Jiao, N.-Z., and Amann, R. (2009) Biogeography and phylogeny of the NOR5/OM60 clade of *Gammaproteobacteria*. *Syst Appl Microbiol* **32**: 124-139.

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Thesis supervision

Zerjatke, K. (2009) Mikroautoradiographie und Genexpression von Bakterien im Oberflächensediment der Gezeitenzones des Wattenmeeres. *Diploma Thesis, Fachbereich für Biologie und Chemie*, Universität Bremen, Bremen

Appendix A: Sediment MAR-FISH

Incubations

- prepare prefixed controls: resuspend 0.5 ml of fresh sediment sample in 1 ml of 96% ethanol or in 1 ml of 4 % formaldehyde (aqueous solution in sterile seawater), incubate 1 h at room temperature, wash of ethanol/formaldehyde
- transfer 1 ml of sediment sample and mix with 1 ml of sterile filtered seawater/porewater (i.e. for incubations in sterile glass vials), include 2-3 replicates per sediment sample and prefixed controls
- add substrate (20-100µM acetate) and isotope tracer (1, 2-^{14}C-acetate, 1-5 µCi) to the slurry
- add formaldehyde to stop incubation (1-2 % final conc.), mix thoroughly, fix at 4°C overnight
- wash samples by 3 steps of centrifugation (14.000rpm for 5-10 min) and subsequent addition of 1 ml of 1 × PBS, store in PBS: Ethanol (2:3) at -20°C

Ultrasonic treatment

- SonoPlus ultrasonic probe (Bandelin Electronic, Berlin, Germany)
- sonicate 7 × 30 sec on ice (amplitude 30%, pulse 20), include × 30 s break intervals break interval

Bulk CO_2 fixation

- Vortex sonicated sediment slurry
- pipette 10 µl of the slurry supernatant and transfer to scintillation cocktail (Ultima Gold XR, Packard)
- assess the amount of incorporated radioactivity by a liquid scintillation counter (Tri-Carb 2900 Packard, Perkin Elmer, USA)

Filtration

- dilute 10 µl of sediment slurry in 90 µl of PBS and resuspend 10-15 µl of dilution in 5 ml PBS
- filter on 0.2 µm GTTP filters, Ø 25 mm, apply gentle vacuum of 200 mbar
- stain filter section with DAPI and inspect proper cell detachment and dispersion under an epifluorescence microscope, ideally 20 – 30 cells per microscopic field, adjust dilution
  alternatively: for muddy sediment: dilute 100 µl of sediment slurry with 500µl PBS prior to sonication, then sonicate 1-3 × 30 sec on ice, filter 20-50 µl of supernatant, inspect and adjust

MAR-FISH

- CARD-FISH is performed according to the protocol of Ishii and colleagues (2004)
- MAR is performed according to the protocol of Alonso and Pernthaler (2005)

Alonso, C., and Pernthaler, J. (2005) Incorporation of glucose under anoxic conditions by bacterioplankton from coastal North Sea surface waters. *Appl Environ Microbiol* **71**: 1709-1716.

Ishii, K., Mussmann, M., MacGregor, B.J., and Amann, R. (2004) An improved fluorescence in situ hybridization protocol for the identification of Bacteria and Archaea in marine sediments. *FEMS Microb Ecol* **50**: 203-212.

Appendix B: Workflow for Purification of Cells from Sediment

Used for Janssand sandy sediment, Sylt muddy sediment, Xiamen (China) muddy sediment

- work on ice

Cell detachment

- sediment sample (0.4 -0.5 ml) in PBS:EtOH, centrifuge, remove PBS:EtOH displace with 1 × PBS (autoclaved and fresh!), resuspend sediment
- sonicate sediment sample for cell detachment; sandy sediment: Lenk et al. (2011), optimize protocol
- transfer sonicated sample to 15 ml falcon tube, add to 10 ml with PBS
- (for muddy sediment sonication might proceed here and not prior)
- vortex for few seconds , wait for heavy sediment particles to settle (subsample i.e. 100 µl, filter, FISH, count abundance of target population or count on parallel sample a priori)
- disperse slurry in 1 ml portion into 2 ml Eppendorf vials (makes ~10 vials final)

Density gradient centrifugation (after Fazi *et al*., 2005)

- use syringe with long needle to slowly place 1ml Histodenz solution (60% w/v in PBS, density 1.3) under the sediment slurry, phases should not mix
- centrifuge in a table top centrifuge at 14,000g for 90 min at 4°C
- collect carefully the supernatant with cell layer above Histodenz ~1ml
- vortex the collected supernatants, disperse subsamples (2–8 ml, depends on required cell density) in sufficient PBS, filter on membran filters (i.e. 0.2 µm, 25 or 47 mm GTTP filters for FISH)
- for subsequent cell sorting a maximum volume of collected supernatant should be filtered to achieve high cell density

Remarks

- proper cell detachment from particles is a prerequisite, physical detachment by ultrasonic treatment works very well (can be combined with chemical solutions)
- cell loss should be optimized (e.g. adapt centrifugation time)
- assess cell loss after density gradient centrifugation

Fazi, S., Amalfitano, S., Pernthaler, J., and Puddu, A. (2005) Bacterial communities associated with benthic organic matter in headwater stream microhabitats. *Environ Microbiol* **7**: 1633-1640.

Lenk, S., Arnds, J., Zerjatke, K., Musat, N., Amann, R., and Mussmann, M. (2011) Novel groups of *Gammaproteobacteria* catalyze sulfur oxidation and carbon fixation in a coastal, intertidal sediment. *Environ Microbiol* **13**: 758-774

Appendix C: Workflow for FACS of Cells from Sediment

Used for *Gammaproteobacteria*, *Roseobacter,* NOR5/OM60 clade

Purification of cells from sediment

- see protocoll

Target cell hybridization (CARD-FISH) on membrane filters

- FISH with probe of interest at 46°C (hybridization temperature) overnight, CARD with a fluorescin labeled or halogene labled tyramide (i.e. Alexa 488 or OregonGreen) depending on downstream application
- no agarose embedding of filters, no lysozyme permeabilization
- run a parallel hybridization with NON-EUB338 as negative control in FACS!
- afterwards: check for successfully stained target cells (specificity, brightness)

Cell Resuspension (after Sekar *et al*., 2004)

- place filters with hybridized target cells in 2 ml Eppendorf tube (1-2 filters/tube)
- add 1.5 ml of 150 mM NaCl containing 0.05% Tween 80
- pre-incubation: incubate horizontally at 37°C for 30 min at a shaker at 250 rpm
- Resuspension: vortex the Eppendorf tube for 15 min at 2500 rpm (glue vials with strong Tesafilm to the vortex adapter)
- remove filters with tweezers, subsample (i.e. 10 –50 µl) of cell solution on filter or glas slide to assess the number of target cells
- inspect filters for remaining target cells to evaluate resuspension efficiency

Sorting

- stain cell suspension with DAPI (1 µg ml^{-1}) if needed
- FACS sorting based on Fluorescence and SSC (and DAPI)
- inspect purity of sorted cell fraction: counterstain with DAPI and/or hybridize with additional probe and adequate tyramide (e.g. Oregon Green-labeled ^{19}F tyramide for nanoSIMS) depending on downstream analysis

Recommendations

- work in parallels, i.e. from 0.5 ml of Janssand sediment ~5 filters are processed
- process a NON-EUB probe on parallel sample as background control for FACS, start with adequate sample volume epending on the abundance of target cells

Sekar, R., Fuchs, B.M., Amann, R., and Pernthaler, J. (2004) Flow sorting of marine bacterioplankton after fluorescence in situ hybridization. *Appl Environ Microbiol* **70**: 6210-6219.

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Selbständigkeitserklärung

Gemäß §6 (5) Nr. 1 - 3 der Promotionsordnung erkläre ich, die Arbeit mit dem Titel:

„Molecular ecology of key organisms in sulfur and carbon cycling in marine sediments“ ohne unerlaubte fremde Hilfe angefertigt zu haben. Es wurden keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet. Die den verwendeten Werken wörtlich oder inhaltlich entnommenen Stellen habe ich als solche gekennzeichnet.

Darüber hinaus erkläre ich, dass es sich bei den von mir eingereichten Arbeiten um identische Exemplare handelt.

Sabine Lenk Bremen, März 2011