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Research Article

RESEARCH ARTICLE

Distinct compositions of free-living, particle-associated and benthic communities of the *Roseobacter* group in the North Sea

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One sentence summary: Roseobacters show distinctive compositions and are able to switch from aerobic to anaerobic utilization of organic sulfur compounds.
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ABSTRACT

The *Roseobacter* group is one of the predominant lineages in the marine environment. While most investigations focus on pelagic roseobacters, the distribution and metabolic potential of benthic representatives is less understood. In this study, the diversity of the *Roseobacter* group was characterized in sediment and water samples along the German/Scandinavian North Sea coast by 16S rRNA gene analysis and cultivation-based methods. Molecular analysis indicated an increasing diversity between communities of the *Roseobacter* group from the sea surface to the seafloor and revealed distinct compositions of free-living and attached fractions. Culture media containing dimethyl sulfide (DMS), dimethyl sulfonium propionate (DMSP) or dimethyl sulfoxide (DMSO) stimulated growth of roseobacters showing highest most probable numbers (MPN) in DMSO-containing dilutions of surface sediments (2.1×10^7 roseobacters cm^{-3}). Twenty roseobacters (12 from sediments) were isolated from DMSP- and DMS-containing cultures. Sequences of the isolates represented 0.04% of all *Bacteria* and 4.7% of all roseobacters in the pyrosequencing dataset from sediments. Growth experiments with the isolate *Shimia* sp. SK013 indicated that benthic roseobacters are able to switch between aerobic and anaerobic utilization of organic sulfur compounds. This response to changing redox conditions might be an adaptation to specific environmental conditions on particles and in sediments.

Keywords: DGGE; pyrosequencing; MPN; enrichment cultures; organic sulfur compounds; *Shimia* sp

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INTRODUCTION

The *Roseobacter* group within the family *Rhodobacteraceae* is a major marine bacterial lineage (Brinkhoff, Giebel and Simon 2008; Pujalte *et al.* 2014) representing a significant part of pelagic and benthic microbial communities (Eilers *et al.* 2001; Selje, Simon and Brinkhoff 2004; Buchan, Gonzalez and Moran 2005). The majority of bacteria affiliated to the *Roseobacter* group (short: roseobacters) were detected in the water column as members of free-living and particle-associated bacterial communities in various marine ecosystems and different geographic regions. They can comprise more than 20% of all bacteria in coastal oceans (Buchan, Gonzalez and Moran 2005) and up to 10% of all cells in tidal-flat sediments (Lenk *et al.* 2012). About 3% of all clones affiliated to the *Roseobacter* group in public 16S rRNA gene libraries originate from marine surface sediments (Mills *et al.* 2003) and can constitute up to 11% in coastal sediments (Gonzales, Kiene and Moran 1999). The fact that marine sediments represent relevant environments for roseobacters is further documented by the observation that ~25% of all species belonging to the *Roseobacter* group with validly published names are of benthic origin (Pujalte *et al.* 2014).

Along with the wide habitat diversity, roseobacters exhibit a broad metabolic versatility (Buchan, Gonzalez and Moran 2005; Wagner-Döbler and Biebl 2006). Most members of the *Roseobacter* group are known to catalyze sulfur transformations (Newton *et al.* 2010; Moran *et al.* 2012). By comparative genomics of 32 isolates belonging to the *Roseobacter* group, Newton *et al.* (2010) revealed that all of them are involved in metabolizing DMSP. Further, it has been shown that DMSP-degrading roseobacters often live in symbiotic relationships with DMSP-producing dinoflagellates (Miller and Belas 2004). Most of the DMSP is degraded by microbial cleavage or demethylation, and a fraction of the volatile degradation product DMS is oxidized by pelagic bacteria (Kiene 1990, Todd *et al.* 2012). As elevated concentrations of DMSP are associated with decaying (and sinking) algal blooms (Pinhassi *et al.* 2005; Wagner-Döbler and Biebl 2006), it is most likely, that this compound is a possible substrate for benthic roseobacters. Interestingly, some of the benthic roseobacters were detected in permanently anoxic sediment layers, indicating an anaerobic metabolism (Inagaki *et al.* 2003; Mouné *et al.* 2003). An adaptation to at least periodic anaerobiosis was detected for *Dinoroseobacter shibae* DFL12^{T} by the analysis of its genome showing the presence of a DMSO-reductase gene and pathways for denitrification and fermentation (Wagner-Döbler *et al.* 2010). For tidal-flat sediments, it was postulated that roseobacters possibly oxidize sulfur compounds in oxic and suboxic sediment layers (Lenk *et al.* 2012). As some roseobacters are capable of utilizing organic sulfur compounds in oxic and anoxic environments (e.g. isolates affiliated to *Sulfitobacter* sp.; Gonzales, Kiene and Moran 1999) or are able to reduce nitrate (e.g. *Leisingera* sp.; Breider *et al.* 2014), they might be able to switch their lifestyle from aerobic to anaerobic metabolism.

The goal of our study was to evaluate the occurrence and diversity of free-living and particle-associated roseobacters in the water column and in both, oxic and anoxic sediments of a coastal sea. We hypothesized that distinct differences exist in the community composition of the *Roseobacter* group from near-surface waters to deeper waters and in the sediment-dwelling microbial communities. Specific DGGE analysis of this group gave a first overview on their distribution patterns. Pyrosequencing-based analysis of 16S rRNA gene amplicons allowed deeper insights into their diversity and relative abundance within the dataset. Additionally, cultivation experiments were carried out with special emphasis on sulfur transformation reactions. Thus, serial dilution cultures were set up to quantify their most probable number (MPN), and to isolate roseobacters that are involved in aerobic DMS and DMSP utilization and/or anaerobic DMSO reduction. Growth experiments with one of the isolates (strain SK013) that is closely related to *Shimia* sp. revealed that this organism is able to shift from DMS oxidation and DMSP degradation under aerobic conditions to anaerobic DMSO and nitrate reduction.

MATERIALS AND METHODS

Sample origin

Water and sediment samples were collected in July 2011 during a cruise with RV 'Heincke' (expedition HE361) to the eastern North Sea (Fig. 1). Six sampling sites were chosen to be analyzed in detail by cultivation, DGGE and pyrosequencing. They represent two shallow sites (water depth: 35 and 26 meters below sea level; mbsl) at the German/Scandinavian coast (station 36: 7°93.57′E, 54°09.02′N and station 5: 7°69.02′E, 56°01.18′N), a shallow (23 mbsl) and a deep site (114 mbsl) in the Skagerrak area (station 8: 8°73.20′E, 57°26.10′N and station 27: 8°35.41′E, 57°36.77′N), as well as two deep sites (181 and 119 mbsl) within the Norwegian trench (station 12: 7°10.00′E, 57°48.87′N and station 16: 3°03.60′E, 59°98.48′N). Intermediate sites (stations 1–3, 6, 7, 10, 11, 13–15) were analyzed by pyrosequencing of water samples from a depth of 3 m, only. These surface samples contained the free-living bacterial fraction and were collected by filtration of several liters of water using 0.2 μm filters after prefiltration at 2.7 μm.

Sediment samples and bottom seawater directly above the seafloor were taken by a multi-core sampler (MUC). Aliquots of the samples were transferred to cultivation media or stored at −20°C for further molecular analyses. Other water samples were collected from corresponding locations using 4 l-Niskin bottles mounted on a rosette and equipped with a conductivity-temperature-depth (CTD) probe. These samples were taken from the turbidity maxima at 3 mbsl and deep water layers between 23 and 181 mbsl, respectively. Up to one liter of water (depending on sample turbidity) was first filtered through 5-μm Nuclepore filters (to collect particle-associated bacteria) and subsequently through 0.2-μm Nuclepore filters (to collect free-living bacteria). All filters were stored at −80°C until further processing.

Enrichment cultures

Immediately after sampling, water and sediments from all stations (Table 1) were inoculated in artificial seawater media (Süß *et al.* 2004). The media were slightly modified as Na_2SO_4 was substituted by NaCl. Four media variations were set up by adding (i) DMS (100 μM) and lactate (5 mM), (ii) DMS (500 μM), (iii) DMSP (5 mM) and (iv) DMSO (100 μM) and lactate (5 mM). For each station, water samples from the near surface and bottom seawater, as well as sediment samples from the seafloor (0–1 cm below sea floor; cmbsf) and deeper, presumably anoxic layers (4–11 cmbsf) were incubated in serial dilutions of the four media described above. All preparations for anoxic incubations were performed within an anoxic chamber. The dilutions were set-up stepwise in three parallels from 10^{-2} to 10^{-7} in polypropylene 96-deepwell plates (Beckman, Fullerton, CA). Every well was filled with 900 μl medium, to which 100 μl of inoculum was added. Two middle rows of the plates served as sample-free controls. The plates containing media (i)—(iii) were incubated under oxic conditions

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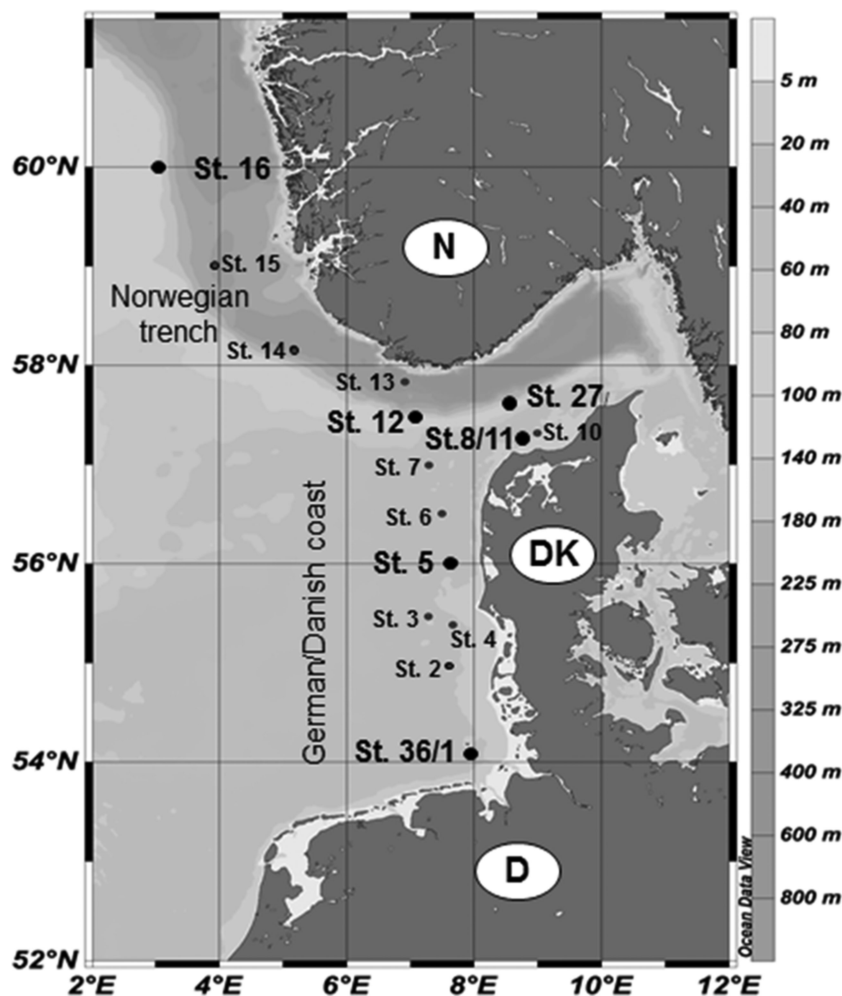

Figure 1. Track of the RV Heincke cruise HE361 (June 2011). Samples were taken along the German (D)/Danish (DK) coast and the Norwegian (N) trench. Six stations (bold) were analyzed in detail by cultivation and molecular analysis. Near-surface water samples from the other stations (small font) were used for comparison of pyrosequencing data, only. The map was created with Ocean Data View (Schlitzer, R., Ocean Data View, http://odv.awi-bremerhaven.de, 2004).

and those containing medium (iv) were incubated anoxically for six months in the dark at 4°C as described in Süß *et al.* (2004). Due to technical problems during sampling, samples from station 36 and 27 could not be inoculated in the anoxic medium.

Analysis of MPN series and isolation of bacteria

Growth was tested according to Martens-Habbena and Sass (2006). The cells within each well were stained with SYBR Green I (Molecular Probes, Eugene, OR) and growth was detected by using a microtiter plate reader (FLUOstar OPTIMA bMG, Offenburg, Germany). MPN counts were calculated as described by de Man (1977) and corrected for the values obtained from sample-free dilution series. Dilutions indicating growth were further subjected to DNA extraction and PCR to determine specific MPN values for the *Roseobacter* group. Furthermore, grown dilution cultures served as isolation source for indigenous bacteria. Aliquots (100 μl) from the MPN wells that were tested positive for growth of roseobacters were spread on agar plates or transferred to deep-agar dilutions containing the respective media. The agar plates and tubes were incubated in the dark at 15°C under oxic and anoxic conditions for more than two and six months, respectively. Colonies from the agar plates were picked and further subcultured at least five times to obtain pure cultures. Colonies from the anoxic agar tubes were picked and are under process to further subcultivations.

Growth media and culture conditions for *Shimia* sp. SK013

Shimia sp. SK013 was tested for utilization of organic sulfur compounds under oxic and anoxic conditions. Picked colonies from agar plates served as inoculum for further tests. *Shimia* sp. SK013 was incubated in artificial sea water media with slight modifications as described above. Under oxic conditions, DMS (500 μM) and DMSP (5 mM) were used as a sole carbon sources. DMSO (30 mM) and lactate (5 mM) were used for anoxic incubations. Additionally, nitrate utilization was tested using $NaNO_3$ (30 mM) and lactate (5 mM) under anoxic conditions. Growth was measured spectrophotometrically (Biochrom Libra S12, Cambridge, UK) by absorption at a wavelength of 438 nm. To confirm substrate specific growth, cultures were subcultivated in fresh test tubes containing the original media with the substrates to be tested. All cultures were incubated at 20°C.

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DNA extractions

For sediments, the DNA was extracted from 0.25 g of the samples, using the MO BIO Power Soil DNA Isolation Kit (Carlsbad, California), following the manufacturer's instructions. PCR-grade water was used to elute the purified DNA of the spin columns for downstream applications. The DNA of filtered water and particles was isolated after bead beating, phenol–chloroform extraction, and isopropanol precipitation as described previously (Stahl *et al.* 1988; MacGregor *et al.* 1997). Treatment by sodium dodecyl sulfate (SDS, 1.75% of a 25% solution) was applied instead of lysozyme (Stevens, Brinkhoff and Simon 2005), precipitation was done with 30 μl sodium acetate and 750 μl isopropanol at −20°C overnight, and 50 μl of PCR-grade water (Eppendorf, Hamburg, Germany) was used for dissolving the DNA at 4°C overnight.

For cultivated samples, a freeze–thaw extraction protocol was used. From each well of the dilution series that was tested positively for growth, 100 μl were transferred into 1.5-ml reaction tubes (Eppendorf, Hamburg, Germany). The tubes were repeatedly heated to 60°C for 5 min, and then frozen in liquid nitrogen for 1 min for four cycles. The cell lysate was stored at −20°C and used for further molecular analysis.

Amplification of 16S rRNA genes from environmental samples and enrichment cultures

PCR was used to amplify 16S rRNA gene fragments for DGGE analysis. For calculating the *Roseobacter*-group specific MPN from the dilution series, DNA from microtiter wells that showed growth was amplified by using a general *Bacteria*-specific PCR according to Wilms *et al.* (2006) to exclude inhibitory effects. To specifically target the *Roseobacter* group in cultures and in environmental samples, the GC-clamp containing primer pair RoseoGC536f/GrB735r (Rink *et al.* 2007) was used. The PCR reaction mixture (50 μl) contained 0.2 mM dNTP's, 1.5 mM $MgCl_2$, 0.2 mM of each primer, 1 × Red *Taq* Buffer (Sigma, Munich, Germany), 0.2 ng μl^{-1} BSA, 2 U Red *Taq* DNA polymerase (Sigma, Munich, Germany) and 2–4 μl DNA template (2-6 ng μl^{-1}). The PCR was performed in a thermal cycler (Mastercycler, Eppendorf, Hamburg, Germany) for 10 cycles under the following conditions: 94°C for 4 min, 94°C for 30 s, 65°C for 1 min, 72°C for 1 min. After that, the samples were run for another 33 cycles with an annealing temperature of 63°C, followed by a final elongation step at 72°C for 10 min. After amplification, the PCR products were visualized on a 1.5% (w/v) agarose gel.

DGGE analysis

DGGE was carried out as described by Süß *et al.* (2004) using an INGENYphorU-2 system (Ingeny, Leiden, the Netherlands). The amplicons (ca. 200 bp) obtained by the *Roseobacter*-group specific PCR were mixed with loading buffer (40% [w/v] glycerol, 60% [w/v] 1× Tris-acetate-EDTA [TAE], bromphenol blue). PCR products were loaded onto polyacrylamide gels (6% w/v) stored in 1 × TAE (40 mmol l^{-1} Tris, 20 mmol l^{-1} acetate, 1 mmol l^{-1} EDTA), with a denaturing gradient from 50% to 70% (100% denaturant correspond to 7 mol l^{-1} urea and 40% formamide). DGGE-gels were run at a constant voltage of 100 V and at a temperature of 60°C for 20 h. After electrophoresis, gels were stained for 2 h with 1 × SYBRGold (Molecular Probes, Leiden, Netherlands) and destained in distilled water for 20 min in distilled water prior to UV transillumination.

Cluster analysis of DGGE community patterns

The software package GelComparII, version 6.5 (Applied Maths, St-Martens-Latem, Belgium) was used for cluster analysis according to Wilms *et al.* (2006). Since all lanes of a DGGE gel contained a characteristic degree of smear, a background subtraction was performed to make different lanes comparable. Therefore, a background scale of 20% was applied in the software package. The densitometric curves were compared using the Pearson coefficient (Pearson 1926). A position tolerance optimization was performed to fit the curves to the best possible match. Dendrograms were generated using the UPGMA method (Sogin and Gunderson 1987).

Sequence analysis of DGGE bands and pure cultures

To identify the phylogenetic affiliation of pure cultures, genomic DNA was recovered from picked colonies. The cell pellet was suspended in 100 μl of PCR-grade water (Eppendorf, Hamburg, Germany) and treated by the freeze and thaw procedure as described above. Two μl of the final extract were added to 48 μl of PCR mixture. Nearly full-length bacterial 16S rRNA gene sequences were amplified using the *Bacteria*-specific primers 8f/1492r according to Overmann and Tuschak (1997). For sequence analysis of DGGE bands, those were excised, transferred to 50 μl of PCR-water and incubated over night at 4°C to elute the DNA. 2 μl of the eluted DNA was taken for reamplification by using the *Roseobacter*-group specific primer pair as described above without the GC-clamp. The reamplification comprised 26 PCR cycles (annealing temperature 55°C). All PCR products were purified using the QIAquick PCR purification Kit (Qiagen GmbH, Hilden, Germany) and were commercially sequenced (GATC, Cologne, Germany). The partial 16S rRNA sequences were compared to those in GenBank using the BLAST function (Altschul *et al.* 1997). All partial 16S rRNA gene sequences of DGGE bands and isolates have been deposited in the EMBL database under accession numbers HG423215 – HG423283.

Pyrosequencing of 16S rRNA genes

All 27 water, particle and sediment samples were investigated by pyrosequencing based 16S rRNA gene analysis. The V3-V5 region of the environmental DNA (50 ng μl^{-1}) was amplified according to Schneider *et al.* (2013). The primers used for amplification contained the Roche 454 pyrosequencing adaptors (underlined), the key (italic), one unique MID per sample and a template specific sequence: Forward primer V3for-MID-137-151: 5′-CCA TCT CAT CCC TGC GTG TCT CCG AC-TCAG-MID (137-151)-TAC GGR AGG CAG CAG-3′ and reverse primer V5rev: 5′-CCT ATC CCC TGT GTG CCT TGG CAG TC-*TCAG*-CCG TCA ATT CMT TTG AGT-3′. The PCR reactions were set up according to the manufacturer's instructions of the Phusion PCR Master Mix Kit (Thermo Scientific, Bremen, Germany). The reaction mixture (50 μl) contained 15.5 μl of PCR H_2O, 25 μl of 2 × Phusion Master Mix with HF Buffer, 2 μl of BSA, 1.5 μl of 100% DMSO, 2.5 μl of primers and 1 μl of template. The PCR was performed in a thermal cycler (Mastercycler, Eppendorf, Hamburg, Germany) under the following conditions: 98°C for 4 min, 26 cycles with 98°C for 30 seconds, 60°C for one min, 72°C for one min followed by a final elongation step at 72°C for 5 min. After amplification, the complete PCR mixture (50 μl) was loaded on 1% (w/v) agarose gels the bands were excised and the DNA was extracted using peqGOLD Gel Extraction Kit (PEQLAB Biotechnologie GmbH, Erlangen, Germany) according to the manufacturer's

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instructions. The concentration and purity of the DNA samples were determined spectrophotometrically (Nanodrop, Thermo Scientific, Wilmington, USA). Purified DNA (5 ngμl^{-1}) was sequenced using a Roche GS-FLX++ 454 pyrosequencer (454 Life Sciences, Branford, USA) at the Institute for Microbiology und Genetics (Göttingen, Germany). Raw data of all 27 samples generated in this study were deposited in the NCBI Short Read Archive under accession *SRA096062*. Data from the 12 near-surface samples (3 mbsl) were deposited under accession number *SRA082674*.

Processing and analysis of pyrosequencing-derived 16S rRNA gene datasets

After raw data extraction, reads shorter than 300 bp, with an average quality value below 25, possessing long homopolymer stretches (>8 bp), or primer mismatches (>3) were removed. Subsequently, sequences were denoised employing Acacia (v1.53) (Bragg *et al.* 2012). Remaining primer sequences were truncated employing cutadapt (Martin 2011). Chimeric sequences were removed using UCHIME (denovo and reference mode) and with the most recent SILVA SSU119NR database reference dataset (DeSantis *et al.* 2006; Edgar *et al.* 2011). Processed sequences of all samples were combined, sorted by decreasing length, and clustered employing the UCLUST algorithm (Edgar 2010). Sequences were clustered in operational taxonomic units (OTUs) at 1%, 3% and 20% genetic dissimilarity according to Simon *et al.* (2009). OTUs at 3 and 20% sequence divergence represent species and phylum level, respectively (Schloss and Handelsman 2005). Phylogenetic composition was determined using the QIIME assign˙taxonmy.py script (Caporaso *et al.* 2010). A consensus sequence was determined for each OTU using USEARCH and classified by BLAST alignment against the Silva SSURef 119 NR database (Pruesse *et al.* 2007). Sequences were classified with respect to the silva taxonomy of their best hit. Rarefaction curves, Shannon indices (Shannon 2001) and Chao1 indices (Chao and Bunge 2002) were calculated as described by Wemheuer *et al.* (2014). In addition, the maximal number of OTUs (n_{max}) was estimated for each sample using Michaelis–Menten-fit. To compare bacterial community structures across all samples based on phylogenetic or count-based distance metrics, an ordination plot (nonmetric multidimensional scaling, NMDS) was calculated based on weighted-unifrac distances in R (v 3.1.0) using the vegan package (Oksanen *et al.* 2011). The distance matrix was generated in QIIME. For this purpose, a phylogenetic tree was calculated with PyNAST using representative OTU sequences. The coefficient of determination (R^2) was calculated using the envfit function in vegan.

RESULTS

Free-living and particle-associated roseobacters show distinct diversity patterns

Sediment and water samples from the German/Scandinavian North Sea coast (Fig. 1) were analyzed by DGGE using *Roseobacter*-group specific primers to reveal differences in community composition of free-living, particle-associated and benthic communities. Cluster analysis of DGGE patterns revealed specific differences between free-living and attached roseobacters (Fig. 2). Interestingly, particle-associated communities of the

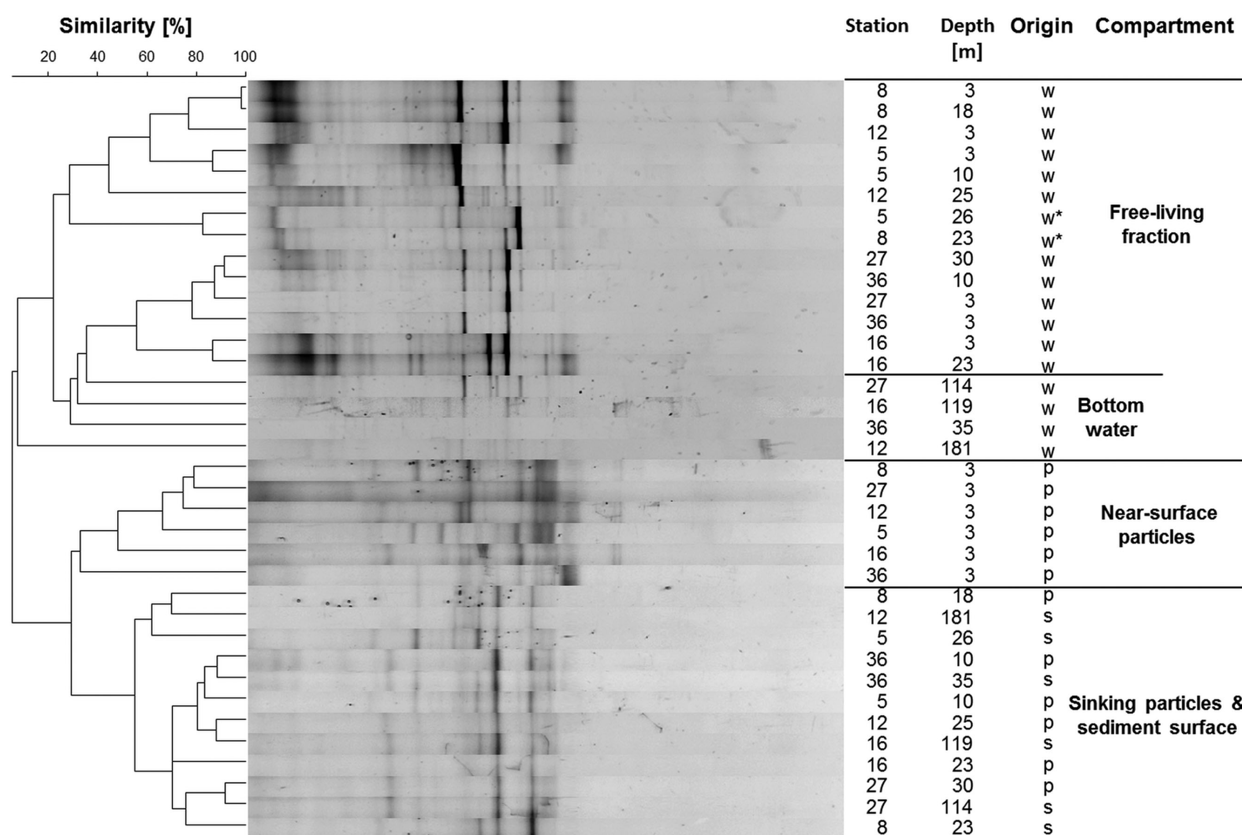

Figure 2. Cluster analysis of *Roseobacter*-group specific DGGE patterns. The densitometric curves of the DGGE community profiles were obtained by applying GelComparII and compared using the Pearson coefficient. The dendrogram was generated using the UPGMA method. Sampling sites, water depth in meters below sea level (mbsl) and origin (*w* = water, *p* = particles, *s* = sediments) are indicated. * = two bottom water samples (St. 5 and 8) clustering with other deep and surface waters.

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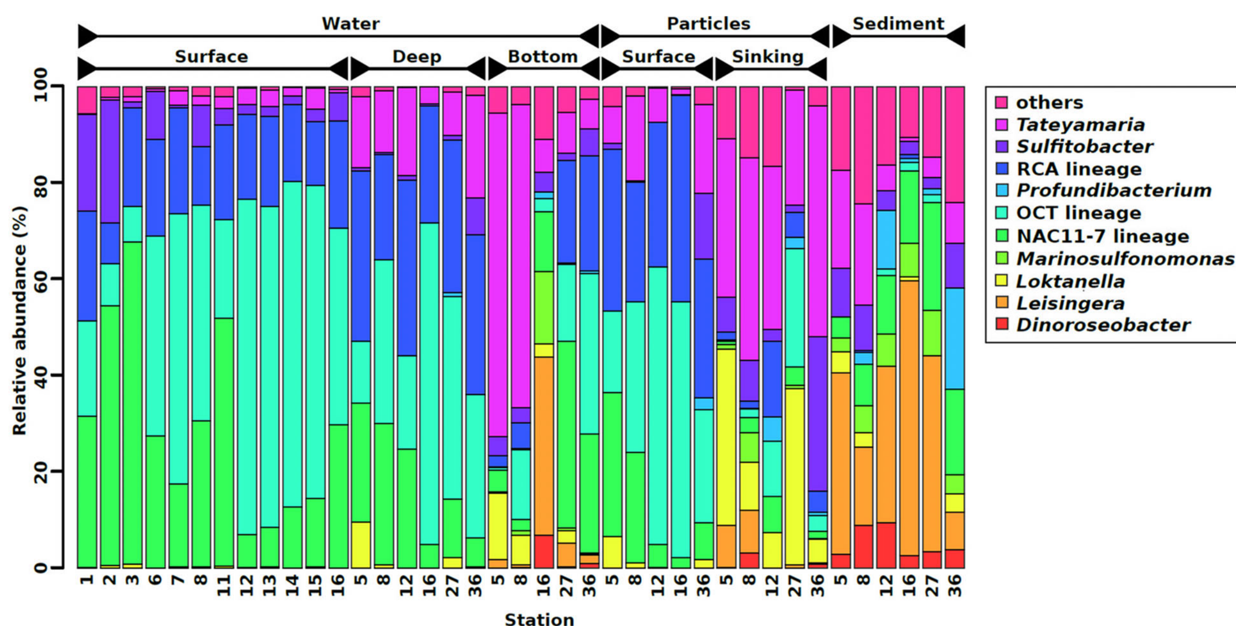

Figure 3. Amplicon-based diversity patterns of the *Roseobacter* group within the different free-living and particle-associated fractions. The sequences that were affiliated to the roseobacters (75.000 reads) were assigned to OTUs on a 99% sequence similarity level. The samples are named by sampling site (1–27).

Roseobacter group from the upper water column (3 mbsl, near-surface particles) clustered separately, while the community composition of sinking particles from waters at the bottom of the mixed layer (10–30 m) showed high similarities to that of the sediment surface. Sequencing of representative DGGE bands indicated the presence of specific members of the *Roseobacter* group within the different pelagic and benthic compartments (Figs S1 and S2; Tables S1 and S2, Supporting Information). Some bacteria such as relatives of *Sulfitobacter donghicola* were part of all free-living and particle-associated communities. Bacteria affiliated to *Nereida ignava* occurred in the entire water column down to the seafloor, whereas relatives of *Phaeobacter caeruleus* only occurred in shallow waters. Sequences related to *Thalassobius mediterraneus* were detected exclusively in bottom waters. The communities on the near-surface particles (3 mbsl) were most notably different from the other samples. Still some bacteria occurred on all particles, e.g. affiliates of *S. dubius*, whereas others were exclusively present in either the sediment such as relatives of *Roseovarius crassostreae* or in the shallow water samples like *S. pontiacus* affiliates.

As the DGGE analysis gave a limited phylogenetic resolution (amplicon size: ~200 bp), pyrosequencing of 16S rRNA gene amplicons spanning the V3-V5 region was performed on the same material. A total of 388 415 high-quality 16S rRNA gene sequences were thereby obtained. The coverage was 58.3, 68.2 and 91.7% at 1, 3 and 20% genetic distance, respectively. Thus, the majority of the bacterial diversity was recovered with the surveying effort which is also in accordance with calculated rarefaction curves (data not shown). About 75 000 sequences were affiliated to members of the *Roseobacter* group with an average of ~17% roseobacters per sample. In the samples of the free-living fractions, ~23% of all sequences were affiliated to roseobacters, whereas on particles and in sediments about 18% and 2% were related to this group, respectively. To display diversity patterns for this phylogenetic group in the different compartments, the sequences were assigned to OTUs at a 99% sequence similarity level. In general, the diversity of members of the *Roseobacter* group within sediments and on sinking particles was much higher than that of water samples and near-surface particles (Fig. 3). Diversity patterns indicated increasing numbers of yet uncultured roseobacters from the sea surface to the seafloor. Water samples were dominated by members of subclusters within the *Roseobacter* group: NAC11-7, the *Roseobacter* Clade Affiliated (RCA) cluster; and the OCT lineage comprising the cluster NAC1-2, NAC11-6 and CHAB-I-5. As already identified by DGGE analysis, sinking particles and sediments were dominated by *Loktanella* and *Leisingera* species, respectively and also harbored a variety of other roseobacters including *Sulfitobacter* species. One exception for the general separation of free-living and particle-associated roseobacters was the bottom water sample from station 16 in the Norwegian trench. The respective community composition was highly similar to that of the underlying sediment surface indicating a probable mixing during sample recovery which was not visible in the DGGE cluster analysis.

Data from the pyrosequencing analysis of the different samples was used to generate an NMDS plot based on the composition of phylotypes related to the *Roseobacter* group. An additional set of water samples from a depth of 3 mbsl that were recovered during the same sampling campaign (small black dots in Fig. 1) was integrated in this calculation. The diversity of the community compositions is displayed in distinct cluster formations (Fig. 4). Samples from sediments and sinking particles form two distinct clusters clearly separated from the other compartments. The samples from bottom waters are distributed among deep water samples, sinking particles and sediments. The observed variance in the NMDS plot with an R^2 of 67.8% for the community structures of the *Roseobacter* group is explained by the sample origin which was already indicated by the diversity plot shown in Fig. 3. This general trend is in accordance with the clustering of DGGE community profiles. However, the clear separation between most bottom water samples and the rest of the free-living bacterial fraction, as seen in the DGGE clusters is not as visible in the NMDS plot.

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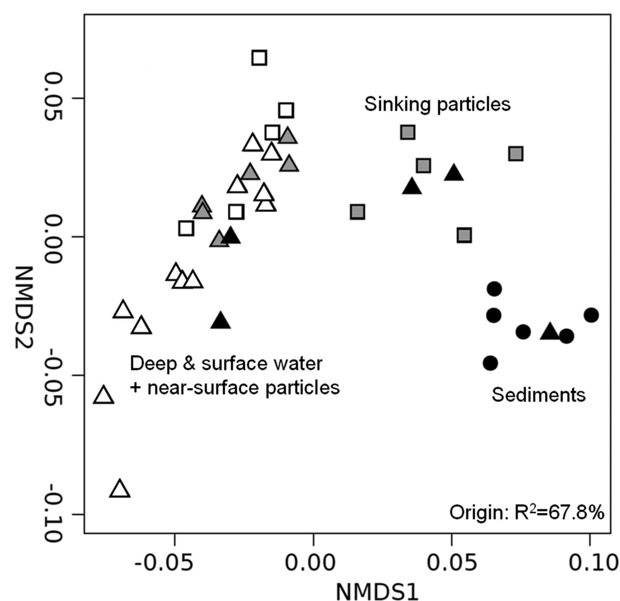

Figure 4. Ordination plot based on nonmetric multidimensional scaling (NMDS). Community compositions of the *Roseobacter* group are arranged due to phylogenetic distance metrics. Water samples are displayed as triangles (white = surface, gray = deep and black = bottom), particles are displayed as squares (white = near-surface, gray = sinking), sediments are shown as black circles. The coefficient of determination (R2) is given.

Amendment with methylated sulfur compounds stimulated the enrichment of roseobacters

Apart from using cultivation-independent methods, a subset of samples was inoculated in serial dilution cultures to quantify the amount of roseobacters that utilize organic sulfur compounds. Their growth was detected by specific PCR in enrichments amended with DMS (with and without lactate), DMSP or DMSO incubated with water samples from 3 mbsl and bottom seawater, as well as with sediments from the upper centimeter of the seafloor and deeper, presumably anoxic layers between 4 and 11 centimeter below seafloor (cmbsf). The results were used to estimate most probable numbers of DMS, DMSP and DMSO-utilizing roseobacters for the different environmental samples that are able to grow under the given conditions (Table 1). Further, all dilution series were screened for the diversity of roseobacters and used for their isolation. Interestingly, the anoxic enrichments with DMSO as electron acceptor showed the highest MPN with 1.1×10^8 bacterial cells cm^{-3} for the sediment surface of station 16 and up to 2.1×10^7 cells cm^{-3} that are affiliated to the *Roseobacter* group (station 12, seafloor). All dilution series containing DMSP and half of the series containing DMS as sole carbon source stimulated growth of benthic roseobacters.

Abundant new sediment-associated isolates of the *Roseobacter* group

A total of 263 isolates were obtained from the oxic enrichments. From these strains, 45 showed a positive result by PCR-screening using *Roseobacter*-group specific primers and were subsequently

Table 1. MPN analysis of pelagic and benthic North Sea samples. Oxic media were amended with DMS and DMSP as sole carbon sources or DMS and lactate. Anoxic media contained lactate as carbon source and DMSO as electron acceptor.

			DMS+lactate (10^3 cells ml^{-1})		DMS (10^3 cells ml^{-1})		DMSP (10^3 cells ml^{-1})		DMS0+lactate (10^3 cells ml^{-1})	
Stations	Origin	Depth	BAC	ROS	BAC	ROS	BAC	ROS	BAC	ROS
36	Surface	3 mbsl	14	9	9	9	0.4	0	–	–
	Bottom	35 mbsl	300	0	0.4	0.4	0.15	0	–	–
	Seafloor	0–1 cmbsf	150	40	9	9	0.9	0.9	–	–
	Deep*	4–5 cmbsf	9	4	0.9	0.4	0.023	0.023	–	–
5	Surface	3 mbsl	4	0.4	0	0	0	0	2000	1100
	Bottom	26 mbsl	2.3	0.4	0.9	0	4	0	700	700
	Seafloor	0–1 cmbsf	1.5	1.1	4.3	4.3	0.4	0.4	7500	7500
	Deep*	5–6 cmbsf	4	4	1.5	1.5	40	0	2000	2000
8	Surface	3 mbsl	0.4	0.4	300	0	0	0	2000	1100
	Bottom	23 mbsl	0.4	0.4	0.4	0	0	0	15	7
	Seafloor	0–1 cmbsf	70	70	9	9	0.023	0.023	15000	2800
	Deep*	5–6 cmbsf	4	4	0.9	0.9	0.023	0.023	230	230
27	Surface	30 mbsl	7	7	0.4	0	3	0	–	–
	Bottom	114 mbsl	2.3	2.3	0.9	0.9	30	0	–	–
	Seafloor	0 cmbsf	200	200	70	70	0.023	0.023	–	–
	Deep*	10–11 cmbsf	700	700	0.4	0.4	0.9	0.9	–	–
12	Surface	25 mbsl	0	0	0.9	0.9	0	0	300	300
	Bottom	181 mbsl	0	0	0.023	0.023	0	0	15	15
	Seafloor	0–1 cmbsf	40	40	0.43	0.15	0.23	0.23	21000	21000
	Deep*	5–6 cmbsf	40	40	2.4	0.43	0.023	0.023	1200	750
16	Surface	23 mbsl	40	0	0.4	0	0	0	0	0
	Bottom	119 mbsl	0.4	0.4	400	0	0	0	4	4
	Seafloor	0–1 cmbsf	40	40	90	90	0.023	0.023	110000	300
	Deep*	5–6 cmbsf	4	4	30	30	4	0	11000	30

*All samples except those from the deeper sediments were analyzed by pyrosequencing.
Surface = samples from 3 mbsl; Bottom = bottom waters above the seafloor; Seafloor = sediment surface; Deep = deeper, presumably anoxic sediments.
BAC = bacteria, ROS = roseobacters.

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Table 2. Origin and closest type strains of isolates affiliated to the *Roseobacter* group.

Strain affiliation	Station	Water Depth (mbsl)	Sediment depth (cmbsf)	Origin	Medium	Closest cultured relative	Max Ident. (%)
SK003/38	36	3		Surface	DMS+Lactate	*S. dubius*	99
SK025	36	3		Surface	DMS+Lactate	*Sulfitobacter sp.*	99
SK024/29	36	35		Bottom	DMS	*Loktanella salsilacus*	99
SK009/10	36	35	0–1	Seafloor	DMS+Lactate	*Roseobacter* sp.	99
SK031/32	36	35	4–5	Deep*	DMS	*Huaishuia halophile*	100
SK040	5	26	5–6	Deep*	DMSP	*P. arcticus*	99
SK042	5	26	5–6	Deep*	DMSP	*P. inhibens*	97
SK002/23	8	23		Bottom	DMS+Lactate	*Shimia haliotis*	99
SK012	8	23	0–1	Seafloor	DMS+Lactate	*R. litoralis*	99
SK013	27	114	0–1	Seafloor	DMS+Lactate	*Shimia haliotis*	99
SK015/21	12	181	0–1	Seafloor	DMS+Lactate	*Pseudoruegeria* sp.	96
SK033	12	181	0–1	Seafloor	DMS	*Rhodobacterales bacterium*	99
SK011	16	119		Bottom	DMS+Lactate	*Roseobacter* sp.	98

*Deep: sediments from deeper, presumably anoxic layers.

subcultured on agar plates containing their respective media. After 16S rRNA gene sequencing and BLAST analysis, 20 strains turned out to be affiliated to the *Roseobacter* group (Table 2). Twelve of these strains derived from sediment samples including 6 strains from media containing DMS + lactate.

Genetic signatures of two isolates from the oxic enrichments (SK010 and SK012) were detected in the pyrosequencing-derived 16S rRNA gene dataset. Both strains were isolated from the seafloor of site 12 (Norwegian trench). Sequences related to strains SK010 (affiliated to *Roseobacter* sp. R22) and SK012 (affiliated to *Roseobacter litoralis*) constituted on average 0.04% of all bacterial communities in the pyrosequence datasets and 0.2% of the communities that can be affiliated to the *Roseobacter* group. They were most abundant in the sediment datasets of station 5 (7.2%) and 36 (3.9%), respectively. In general, the proportions of sequences of SK010 and SK012 were much higher in the sediment datasets (4.7%), and they were found in four and five out of six sediment datasets, respectively. Thus, they seem to be widespread members of benthic bacterial communities.

Initial anoxic enrichments which showed a *Roseobacter*-group specific PCR signal were subcultured in deep-agar dilutions and additionally analyzed for their diversity by DGGE. While no roseobacters were among the first 84 picked colonies of the anoxic enrichments after several months of incubation, sequencing of DGGE bands revealed growth of various roseobacters in the different dilution steps. For instance, bands of the original sample from the deeper sediments of station 16 were affiliated to species of the genera *Roseobacter*, *Shimia*, *Sulfitobacter*, *Phaeobacter*, *Nereida* and *Dinoroseobacter* (Fig. 5). While a strain affiliated to *N. ignava* did not grow in the dilution cultures, the *Phaeobacter* strain was specifically enriched in dilutions of 10^{-2} and 10^{-3}. Additional bands that were affiliated to another *Phaeobacter* and a *Sulfitobacter* species showed up in the 10^{-3} dilution step. The last two dilution steps (10^{-4} and 10^{-5}) showed faint bands affiliated to *D. shibae* but were dominated by relatives of *Roseobacter* group and *Shimia* species.

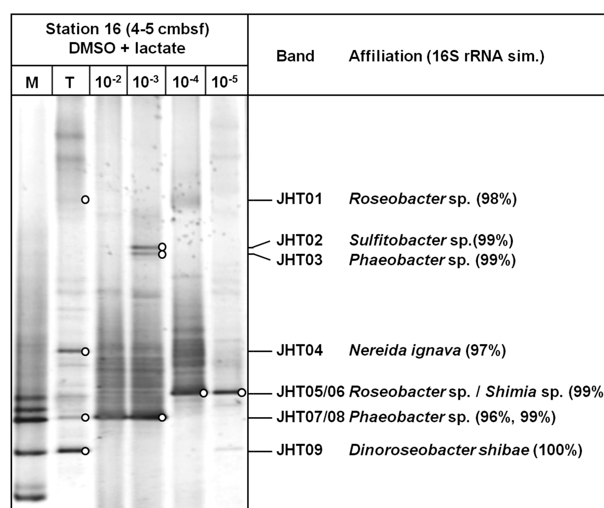

Figure 5. DGGE analysis of one anoxic dilution series from the deeper sediment (4–5 cmbsf) of station 16. PCR amplicons (~200 bp) were generated by using *Roseobacter*-group specific primers. Representative DGGE bands (white circles) were excised and sequenced. The next relatives in GenBank are indicated. M = marker, T = original sediment.

Shimia sp. SK013 grows aerobically and anaerobically on organic sulfur compounds

One of our isolates, *Shimia* sp. SK013, was used to test our hypothesis that some roseobacters are able to switch their lifestyle from aerobic to anaerobic metabolism. Therefore, the strain was used for testing growth on different organic sulfur compounds under both, oxic and anoxic conditions. *Shimia* sp. SK013 was isolated from serial dilution cultures (dilution step 10^{-5}) amended with DMS and lactate inoculated with surface sediments of station 27. Additionally, sequences affiliated to *Shimia* sp. were detected in high dilution steps (10^{-5}) of anoxic enrichments (containing DMSO as electron acceptor) from deep layers (4–5 cmbsf) of station 16.

The growth experiments to test whether *Shimia* sp. SK013 can also grow without lactate as substrate revealed that the strain can use DMS and also DMSP as sole carbon sources under oxic conditions. Even though the cultures only grew to an optical density of up to 0.13, continuous growth was observed even after subcultivation using the respective media. Anoxic cultivation with DMSO as electron acceptor also revealed relatively weak growth of *Shimia* sp. SK13 (OD_{438} = 0.12) after 10 days of incubation with decreasing values to OD_{438} = 0.07 at day 20. A slight increase in growth was observed after a pulse of 10 mM DMSO from days 20 to 30 resulting in an OD_{438} of 0.1. A similar growth behavior was observed after a second pulse of DMSO. The same

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result was obtained in cultures amended with nitrate (3 × 10 mM) showing optical densities of ∼0.1.

DISCUSSION

As main outcome of our study, both, DGGE analysis and amplicon-based sequencing of 16S rRNA genes indicated a broad overlap in community composition of the *Roseobacter* group detected on sinking particles and the sediment surface. In general, communities with an attached lifestyle were more diverse and clearly distinct from the free-living fraction. A variety of roseobacters was enriched and isolated from all sampling sites and compartments with media containing different organic sulfur compounds. The ability of *Shimia* sp. SK013 to utilize organic sulfur compounds under both, oxic and anoxic conditions indicates their metabolic capability to switch between both lifestyles.

Habitat specific characteristics trigger the separation of pelagic and benthic roseobacters

Specific differences between free-living, particle-associated and benthic bacterial fractions were previously identified by Stevens, Brinkhoff and Simon (2005) from an intertidal region of the German Wadden Sea. The authors identified particles as an intermediate habitat that is characterized by bacterial transformation processes occurring in both, the water column and at the sediment surface. Also, Rink *et al.* (2007, 2011) detected higher diversity of particle-associated roseobacters relative to the free-living communities. However, the present study is the first to examine specific differences in the composition of the *Roseobacter* group among the free-living, particle- and sediment-associated bacterial communities using a combination of next-generation sequencing and cultivation.

The low diversity of roseobacters we have detected within surface water samples is in accordance with previous investigations on the water column and on phytoplankton blooms (West *et al.* 2008; Teeling *et al.* 2012). Even though there is a striking difference between the free-living and the attached lifestyle, an overlap in microbial diversity between both compartments is visible. The release of algal exudates triggers growth of highly specialized microbial communities on the phytoplankton-born particles (Grossart *et al.* 2005). As near-surface particles are composed of fresh algal material, the associated communities are mainly degrading their exudates (Cole 1982). It was shown that roseobacters exhibit mutualistic interactions with planktonic algae and dinoflagellates (Gonzalez *et al.* 2000; Wagner-Döbler and Biebl 2006, Onda, Azanza and Lluisma 2015). In contrast, sinking particles in deeper layers are constituted of decaying organic matter (Cowie and Hedges 1992, Wakeham *et al.* 1997), and thus harbor different microbial communities (Schweitzer *et al.* 2001; Zhu *et al.* 2013). In general, environmental conditions such as high nutrient concentrations and a complex organic matter composition are comparable to those found at the sediment surface. Thus, both compartments exhibit similar microbial community patterns (Novitsky 1990; Llobet-Brossa, Rossello-Mora and Amann 1998). The recalcitrant organic matter found on sinking particles and the sediment surface stimulates highly diverse, slow-growing microbial communities with various metabolic capacities. The variety of available ecological niches and interactions between different microbial community members is reflected in the high proportion of roseobacters that are assigned as 'uncultured'. This type of environment is hard to be mimicked under laboratory conditions, which results in a lack of respective isolates.

Both molecular investigations are complementary and help to guide the cultivation of *Roseobacter*-group affiliated bacteria

The separation of *Roseobacter*-group community structures with a free-living and an attached lifestyle detected by both molecular methods was much clearer in the DGGE analysis. However, the primers used in this study are specific for roseobacters but also target a limited number of *Rhodobacteraceae* and other *Alphaproteobacteria*. This is also in accordance with the molecular screening of our isolates that revealed the presence of some non-*roseobacter Rhodobacteraceae* in the culture collection. Additionally, the primers generate a PCR product with a length of ∼200 bp, which only gives a low phylogenetic resolution. These limitations can be minimized by amplicon-based sequencing of 16S rRNA genes using universal primers to digitally extract information on certain groups from the whole dataset. However, screening of enrichment cultures by DGGE can generally lead to the isolation of strains even if their colonies are hidden under a broad diversity of other colonies within the subcultures. Our study is an example that displays the advantage of dilution to extinction of fast-growing, but less abundant species along serial dilution cultures. As roseobacters were not the majority within the entire microbial community, many colonies of other microorganisms were present in the anoxic agar tubes shielding the enriched roseobacters and hindered their isolation. Without DGGE screening of the enrichments, growth of these strains would not have been detected.

Aerobic and anaerobic utilization of organic sulfur compounds

DMSP is an osmolyte that is produced by marine macro- and microalgae (Yoch 2002). This compound is released from lysed, dead or grazed algae and subsequently degraded by various bacteria. Elevated concentrations of DMSP are associated to algal blooms that in turn attract high numbers of roseobacters (Gonzalez *et al.* 2000), which were among the first bacteria isolated from DMSP-containing media (Wagner-Döbler and Biebl 2006). Sedimentation of algal material might lead to an enrichment of DMSP-consuming roseobacters at the seafloor. In our enrichments, growth of roseobacters was mainly stimulated in sediment samples when DMSP or DMS were provided as sole carbon sources. When DMSP is demethylated, the degradation product DMS might be oxidized to DMSO. The high numbers of isolates affiliated to the *Roseobacter* group that we have obtained from DMS-amended media indicate their role in this degradation process. On sinking particles and especially in sediments, oxic and anoxic microniches are present in close proximity. If aerobically produced DMSO diffuses into anoxic regions, this compound can be used as electron acceptor. In our enrichments, we found high numbers of roseobacters reducing DMSO in water and sediment samples. This indicates the presence of facultative anaerobic roseobacters in both, oxic and anoxic compartments. The isolation of *Shimia* sp. SK013 from oxic sediments and the detection of *Shimia* sp. in enrichments from presumably anoxic sediment layers in dilutions of 10^{-5} indicate that at least 10^5 cells of *Shimia* sp. were present per cm^3 of sediment at two different sampling stations. This might only account for 0.1%–0.01% of benthic microbial communities. Compared to the

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average cell density in water samples of ~10^6, it is quite a high number.

The ability of *Shimia* sp. SK013 to switch from aerobic degradation of organic sulfur compounds to anaerobic respiration was verified by whole genome sequencing (Acc. No. IMG2608642164) revealing the presence of genes for a DMSP-lyase, a DMSP-demethylase as well as genes for nitrate, nitrite and DMSO reduction. Other members of the *Roseobacter* group e.g. *Leisingera* species or *Sulfitobacter* species are as well-known to perform aerobic and anaerobic respiration such as nitrate reduction (Breider *et al.* 2014) or both, nitrate and DMSO reduction (Gonzales, Kiene and Moran 1999, Ivanova *et al.* 2004). The fact that relatives of *Sulfitobacter* species were isolated from aerobic cultures and detected to grow in anaerobic enrichments also points to their ability to switch between the two lifestyles. While experiments under laboratory conditions not necessarily reflect those in the environment, our examples indicate that switching lifestyles might be a common feature of benthic roseobacters explaining their presence in oxic and anoxic habitats.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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