

ORIGINAL ARTICLE

Three manganese oxide-rich marine sediments harbor similar communities of acetate-oxidizing manganese-reducing bacteria

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Dissimilatory manganese reduction dominates anaerobic carbon oxidation in marine sediments with high manganese oxide concentrations, but the microorganisms responsible for this process are largely unknown. In this study, the acetate-utilizing manganese-reducing microbiota in geographically well-separated, manganese oxide-rich sediments from Gullmar Fjord (Sweden), Skagerrak (Norway) and Ulleung Basin (Korea) were analyzed by 16S rRNA-stable isotope probing (SIP). Manganese reduction was the prevailing terminal electron-accepting process in anoxic incubations of surface sediments, and even the addition of acetate stimulated neither iron nor sulfate reduction. The three geographically distinct sediments harbored surprisingly similar communities of acetate-utilizing manganese-reducing bacteria: 16S rRNA of members of the genera *Colwellia* and *Arcobacter* and of novel genera within the *Oceanospirillaceae* and *Alteromonadales* were detected in heavy RNA-SIP fractions from these three sediments. Most probable number (MPN) analysis yielded up to 10^6 acetate-utilizing manganese-reducing cells cm^{-3} in Gullmar Fjord sediment. A 16S rRNA gene clone library that was established from the highest MPN dilutions was dominated by sequences of *Colwellia* and *Arcobacter* species and members of the *Oceanospirillaceae*, supporting the obtained RNA-SIP results. In conclusion, these findings strongly suggest that (i) acetate-dependent manganese reduction in manganese oxide-rich sediments is catalyzed by members of taxa (*Arcobacter*, *Colwellia* and *Oceanospirillaceae*) previously not known to possess this physiological function, (ii) similar acetate-utilizing manganese reducers thrive in geographically distinct regions and (iii) the identified manganese reducers differ greatly from the extensively explored iron reducers in marine sediments.

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Introduction

Energetically, manganese oxide is a much more favorable electron acceptor than iron oxide or sulfate. Accordingly, manganese-reducing microorganisms are expected to outcompete iron and sulfate reducers for common substrates, and microbial manganese reduction indeed dominates carbon

mineralization over iron and sulfate reduction in some marine sediments (Lovley and Goodwin, 1988; Thamdrup, 2000). Sediments where microbial manganese reduction is important have been found, for example, in the Panama Basin, deep parts of the Skagerrak, the Black Sea and the northern Barents Sea (Canfield *et al.*, 1993a, b; Thamdrup *et al.*, 2000; Vandieken *et al.*, 2006b; Nickel *et al.*, 2008). These are characterized by high manganese oxide concentrations of $25\text{--}185\text{ }\mu\text{mol cm}^{-3}$ to depths of 1 to $>10\text{ cm}$, and manganese reduction accounts for 25–99% of anaerobic carbon oxidation in the upper 10 cm of sediments. These conditions are contrasted by the generally low concentrations ($\leq 20\text{ }\mu\text{mol cm}^{-3}$) and shallow depth distribution ($\leq 2\text{ cm}$) of manganese oxide in most continental marine sediments (Thamdrup, 2000). High concentrations of manganese oxides are often found in the

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deepest parts of fjords or ocean basins, which are suggested to function as traps for manganese that is released from more reducing sediments into the water column and by lateral transport and later re-precipitation accumulates in more oxidized sediments (Thamdrup *et al.*, 1994; Thamdrup, 2000).

The diversity of microbes involved in dissimilatory manganese reduction in aquatic sediments is largely unexplored. Although a large number of microorganisms capable of manganese reduction is known (Lovley *et al.*, 2004), many of these were isolated as iron reducers from environments dominated by iron reduction and were only subsequently shown to reduce manganese oxides in culture. The few species that have been enriched and isolated with manganese oxide include *Shewanella putrefaciens* MR1 from marine sediment and the thermophile *Bacillus infernus* isolated from terrestrial subsurface (Burdige and Nealson, 1985; Myers and Nealson, 1988; Boone *et al.*, 1995). Manganese reduction is rarely tested in the description of new species, so that this capability might be more widespread in existing isolates than is currently known. As detailed studies of manganese-reducing bacteria are missing and most iron reducers are able to reduce manganese oxides as well, we are currently restricted to using these organisms as models for understanding microbial manganese reduction in nature. Considering the large difference in redox potential between manganese and iron oxides (Thamdrup, 2000), however, it seems likely that manganese reduction in the environment is actually carried out by specialized manganese reducers.

To our knowledge, only one previous study from the Black Sea has combined the analysis of activity and identity of manganese reducers in sediments (Thamdrup *et al.*, 2000). Interestingly, most probable number (MPN) counts of manganese reducers were three orders of magnitude higher than MPN counts of iron reducers, suggesting that different groups of microorganisms conveyed the two processes. *Arcobacter*- and *Pelobacter*-related species were identified as manganese reducers by 16S rRNA gene clone library surveys of the highest MPN dilutions that showed growth. Whereas *Pelobacter* belongs to the order *Desulfuromonadales* that contains many characterized iron- and manganese-reducers, *Arcobacter* had not been previously recognized to be capable of reducing metal oxides.

Stable isotope probing (SIP) of nucleic acids is a cultivation-independent method that is widely applied to link utilization of an isotope-labeled (for example, ^{13}C) substrate to the phylogenetic identity of the responsible organisms. Substrate labeled with ^{13}C is fed to, for example, sediment incubations so that the RNA and DNA of microorganisms turning over this substrate become labeled with ^{13}C and the organisms can be identified subsequently by molecular methods (Whiteley *et al.*, 2006; Whiteley *et al.*, 2007; Pester and Loy, 2010). Particularly in SIP

incubations with only one dominating electron-accepting process, selective isotopic enrichment of nucleic acids strongly suggests that the microorganisms harboring these heavy nucleic acids are the ones that catalyze this process. With this technique, members of the order *Desulfuromonadales* were identified to couple dissimilatory iron reduction to the oxidation of ^{13}C -acetate in a uranium-contaminated aquifer and rice field soil (Chang *et al.*, 2005; Hori *et al.*, 2010). Other bacteria, which became isotopically labeled by metabolizing ^{13}C -labeled substrates under iron-reducing conditions, belonged to members of the class *Betaproteobacteria* and Gram-positive *Peptococcaceae* that had not before been linked to iron reduction (Kunapuli *et al.*, 2007; Hori *et al.*, 2010; Pilloni *et al.*, 2011).

In this study, RNA-SIP was applied for the first time to identify manganese-reducing bacteria in three manganese oxide-rich marine sediments from Swedish, Norwegian and Korean waters where the dominance of microbial manganese reduction in carbon oxidation was previously shown or suggested (Canfield *et al.*, 1993a, b; Engström *et al.*, 2005; Hyun *et al.*, 2010). As acetate is an important electron donor for microbial iron and sulfate reduction in marine sediments (Sørensen *et al.*, 1981; Parkes *et al.*, 1989; Fukui *et al.*, 1997; Finke *et al.*, 2007), we chose (^{13}C -labeled) acetate as the substrate for RNA-SIP studies and MPN counts of manganese reducers.

Materials and methods

Study sites

Manganese oxide-rich sediment was sampled at three locations: the Gullmar Fjord on the west coast of Sweden (58°19.35N 011°32.75E; Engström *et al.*, 2005), the Norwegian Trough in Skagerrak between Denmark and Norway (58°14.1N 09°32.1E; Canfield *et al.*, 1993a, b) and the Ulleung Basin in the East Sea off South Korea (36°59.81N 130°59.75E; Hyun *et al.*, 2010). Sampling is detailed in Supplementary Methods. Manganese oxides of surface sediment from the Skagerrak site have been shown to consist of pure Mn(IV) oxide (Canfield *et al.*, 1993b; Thamdrup and Dalsgaard, 2000) whereas in Gullmar Fjord it was estimated to be 2/3 Mn(IV) and 1/3 Mn(II) (Goldberg *et al.*, 2012).

Sediment incubations

Incubations were started either immediately after sampling (Ulleung Basin and Skagerrak) or for Gullmar Fjord sediment, 12 days after storing the intact cores with the overlying water being aerated. For the incubations, sediments from 0–5 cm depth in Gullmar Fjord and Skagerrak and from 0–2.5 cm in Ulleung Basin were pooled from several sediment cores, mixed with anoxic site water (3:1 sediment: site water w/w for Gullmar Fjord and Skagerrak,

and 1:1 for Ulleung Basin) and homogenized. Three sediment slurries were prepared for each field site and transferred under N_2 into glass bottles that were closed with thick butyl stoppers, flushed with N_2 and incubated anoxically at 6 °C in the dark for 13 days (Gullmar Fjord and Ulleung Basin) or 8 days (Skagerrak). For each experiment, two of the slurries were amended every day (except for day 5, 11 and 12 for the Gullmar Fjord incubations) with $\sim 170 \mu M$ (final concentration) of either unlabeled (^{12}C -) or (U - ^{13}C)-labeled acetate. The third slurry was incubated as a control without acetate addition. During the incubations, slurries were subsampled for pore-water and solid phase analyses, RNA/DNA extractions and sulfate reduction rate measurements just before each addition of acetate (described in detail in Supplementary Methods). After subsampling, the headspace was flushed with N_2 .

MPN counts and DNA extraction

MPN counts of manganese reducers were conducted with Gullmar Fjord sediment with six different electron donors and manganese oxides as electron acceptor (for details see Supplementary Methods). Hungate tubes were incubated for 18 months at 15 °C and activity was indicated by the complete disappearance of brown manganese oxide. From the highest active dilution of acetate-oxidizing cultures, DNA was extracted, amplified and cloned as described in Supplementary Methods.

RNA/DNA extraction and density gradient centrifugation

RNA and DNA were co-extracted from ~ 1 g frozen sediment ($-80^\circ C$) from different sampling days (day 0, 2 and 6 for Gullmar Fjord; day 0 and 7 for Ulleung Basin; and day 0 and 6 for Skagerrak sediments) of the three treatments with the Power-Soil Total RNA Isolation Kit in combination with the DNA Elution Accessory Kit (MoBio, Carlsbad, CA, USA). The RNA was further purified with a Qiagen (Hilden, Germany) AllPrep DNA/RNA Mini Kit and quantified with Ribogreen RNA-quantification kit (Invitrogen, Eugene, OR, USA). Portions of 530 ng total RNA were separated by density gradient ultracentrifugation and the gradients were fractionated and further processed as described by Whiteley *et al.* (2007). The concentration of RNA in each fraction was determined using Ribogreen-based fluorimetry (Invitrogen). Terminal restriction fragment length polymorphism (T-RFLP) analysis, cloning and phylogenetic analyses of 16S rRNA genes are detailed in Supplementary Methods.

Nucleic acid sequences

The sequence data have been submitted to the GenBank database under accession Nos. JN621327-JN621401 (Gullmar Fjord, day 0), JN621402-JN621479 (Ulleung Basin, day 0), JN621480-JN621560

(Skagerrak, day 0), JN621561-JN621649 (Gullmar Fjord, heavy SIP fraction), JN621650-JN621713 (Ulleung Basin, heavy SIP fraction), JN621714-JN621756 (Skagerrak, heavy SIP fraction) and JN621757-JN621780 (MPN counts, Gullmar Fjord).

Results and discussion

Microbial manganese reduction during sediment incubations

Sediments from Gullmar Fjord and Ulleung Basin were chosen because of their high concentration of manganese oxide (Engström *et al.*, 2005; Hyun *et al.*, 2010), which has been found to favor microbial manganese reduction at other locations (Canfield *et al.*, 1993a, b; Thamdrup, 2000; Thamdrup *et al.*, 2000; Vandieken *et al.*, 2006b; Nickel *et al.*, 2008). For the Skagerrak station, dissimilatory manganese reduction was repeatedly observed to be the principal anaerobic electron-accepting process in the upper 10 cm of sediment (Canfield *et al.*, 1993a, b; Thamdrup and Dalsgaard, 2000).

For each of the three sediments, parallel incubations with labeled and unlabeled acetate showed very similar developments with respect to the measured biogeochemical parameters during the 8–13 days of incubation (Figures 1, 2 and 3). Dissolved inorganic carbon (DIC) concentrations corrected for $CaCO_3$ precipitation increased in most slurries except for the control incubations without acetate addition from Ulleung Basin and Skagerrak, where DIC concentrations decreased or only increased initially (Figure 1). The precipitation of $CaCO_3$, a characteristic of sediment incubations dominated by manganese reduction (Thamdrup *et al.*, 2000), was observed in most slurries (data not shown). The decrease in DIC concentration after the $CaCO_3$ correction in Ulleung Basin and Skagerrak controls was likely due to the precipitation of $MnCO_3$, which cannot be quantified because of simultaneous production of Mn^{2+} by manganese reduction (Thamdrup *et al.*, 2000). Thus, carbon oxidation rates might be underestimated (Thamdrup *et al.*, 2000).

Manganese oxide concentrations ranged from 38 to $95 \mu mol cm^{-3}$ (Table 1), which is high enough to expect manganese reduction to dominate anaerobic carbon oxidation completely (Thamdrup, 2000). Manganese reduction was indicated directly by the accumulation of dissolved Mn^{2+} during the incubations from Gullmar Fjord and Ulleung Basin, and accumulation rates increased substantially with acetate amendment, suggesting that the process was limited by organic carbon (Figure 2). The rate of Mn^{2+} accumulation strongly underestimates the rate of manganese reduction in manganese oxide-rich sediments owing to the strong adsorption of Mn^{2+} to manganese and iron oxide surfaces as well as potential precipitation of $MnCO_3$ (Canfield *et al.*, 1993a, Thamdrup *et al.*, 2000). Such strong adsorption likely explains the lag phase in Mn^{2+}

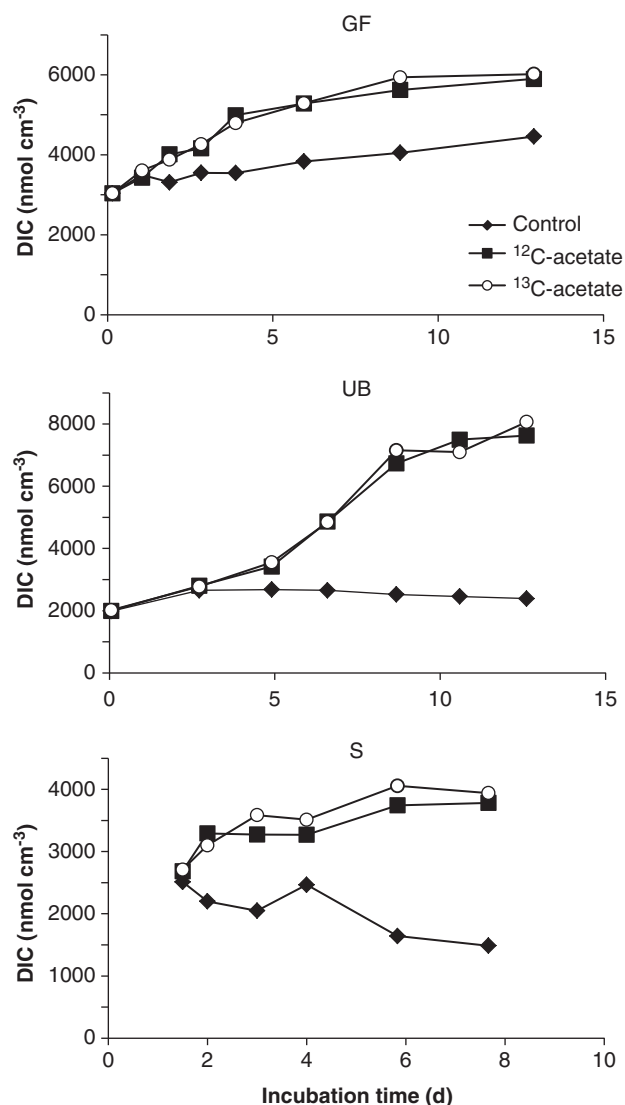


Figure 1 DIC concentration corrected for CaCO₃ precipitation during incubations of Gullmar Fjord (GF), Ulleung Basin (UB) and Skagerrak (S) sediments.

accumulation in the Ulleung Basin experiment as well as the total lack of accumulation in the Skagerrak sediment, which had the highest manganese oxide content. Very low rates of Mn²⁺ accumulation were previously reported for the most manganese oxide-rich part of this sediment (Thamdrup and Dalsgaard, 2000).

Fe²⁺ and sulfide concentrations in the porewater stayed below the detection limit throughout the incubations with and without acetate amendment (sulfide was not measured for Skagerrak incubations) and sulfate reduction rates were either very low ($\leq 3.1 \text{ nmol cm}^{-3} \text{ d}^{-1}$) or below detection throughout all the incubations of Gullmar Fjord and Ulleung Basin sediments. Sulfate reduction was not measured in the Skagerrak sediment but the process has previously been shown to be unimportant there (Canfield *et al.*, 1993a, b; Thamdrup and Dalsgaard, 2000).

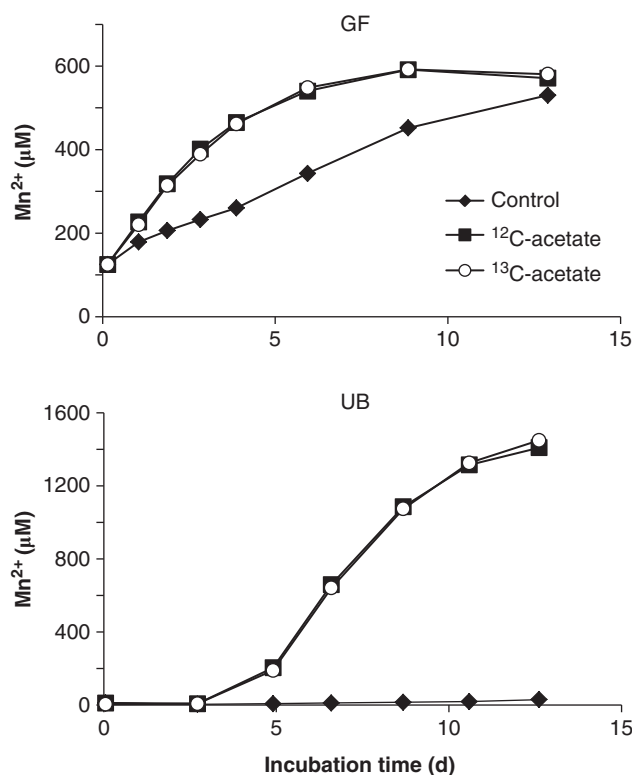


Figure 2 Porewater Mn²⁺ concentrations during incubations of GF and UB sediments. Mn²⁺ concentrations in all three S incubations stayed $< 5 \mu\text{M}$.

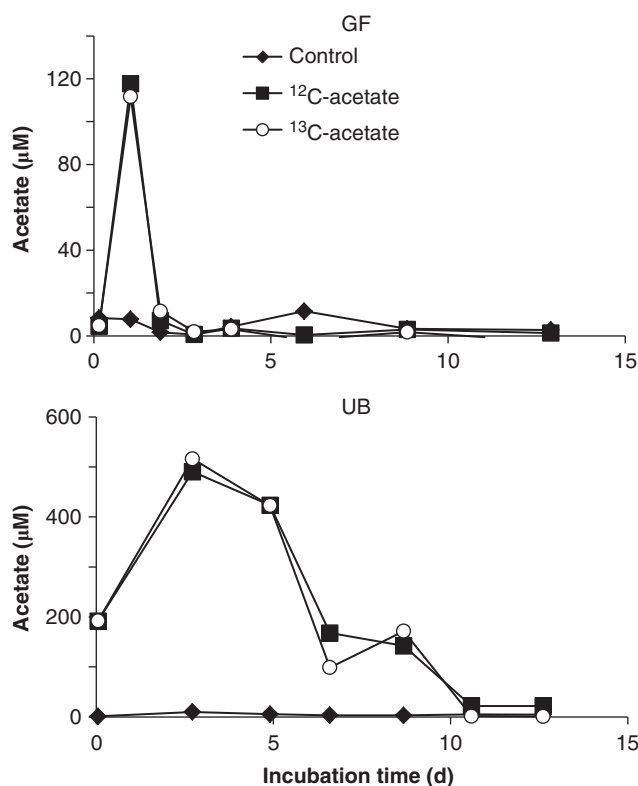


Figure 3 Acetate concentrations during incubations of the GF and UB sediments. Acetate concentrations during the S sediment incubations were not measured.

Table 1 Manganese and iron concentrations ($\mu\text{mol cm}^{-3}$) at the beginning of the incubations

Sediment	Total Mn	Total Fe	Fe(III)
Gullmar Fjord	37.9	49.7	45.8
Ulleung Basin	40.2	17.8	15.0
Skagerrak	94.5	53.2	52.9

As discussed in earlier studies (Canfield *et al.*, 1993a, b; Thamdrup, 2000), reduction of iron or sulfate could be masked by rapid reoxidation with manganese oxide, but manganese-reducing bacteria are expected to outcompete iron and sulfate reducers when manganese oxide is abundant (Lovley and Goodwin, 1988), and previous experiments with molybdate inhibiting sulfate reduction and ferrozine as a scavenger for Fe^{2+} showed no sign of such reactions (Canfield *et al.*, 1993b). Supporting these findings, our RNA-SIP results identified no known taxa of sulfate-reducing bacteria (see below, Table 2). Thus, based on our results and previous studies, we conclude that manganese reduction was the dominating electron-accepting process during the incubations of the three sediments from Gullmar Fjord, Ulleung Basin and Skagerrak, with minimal contribution of sulfate reduction to anaerobic carbon oxidation (<2% for Gullmar Fjord and Ulleung Basin incubations; assuming a stoichiometry of 2:1 for DIC produced to sulfate reduced (Thamdrup and Canfield, 1996)).

Acetate oxidation during the sediment incubations

Control incubations of Gullmar Fjord and Ulleung Basin sediments that were not amended with acetate showed acetate concentrations <12 μM throughout the duration of the experiments (Figure 3) (not measured for Skagerrak incubations), which is at the lower end of reported concentrations for marine sediments (Sansone and Martens, 1982; Fukui *et al.*, 1997; Wellsbury *et al.*, 2002; Finke *et al.*, 2007; Heuer *et al.*, 2009). Acetate has been shown previously to be the most important volatile fatty acid in terms of concentration and turnover, and identified as important substrate for anaerobic terminal electron-accepting processes in marine sediments (Sørensen *et al.*, 1981; Parkes *et al.*, 1989; Fukui *et al.*, 1997; Finke *et al.*, 2007). The addition of 170 μM acetate per day to our incubations exceeded these low concentrations of acetate in the control without acetate and stimulated its mineralization (Figures 1 and 3). Transient accumulation of acetate at the beginning of the acetate-amended incubations showed that the microbial community in Gullmar Fjord and Ulleung Basin sediments (not tested for Skagerrak) needed time to adjust to elevated acetate concentrations (Figure 3). However, already after the second addition, the microbial community from Gullmar Fjord was able to oxidize the added acetate and bring the concentration to the level of the control within 1 day. In the Ulleung

Basin incubations, transient acetate accumulation to a maximum of 500 μM lasted longer but started to decrease again at day 3 with concentrations similar to the control being reached after 11 days (Figure 3).

In all three sediments, DIC production was significantly stimulated by the addition of acetate compared with the controls without acetate addition (Figure 1). For Gullmar Fjord sediment, the total anaerobic carbon oxidation rates in the incubations with acetate amendment were threefold higher than in the control (Figure 1). The potential DIC production from the oxidation of all added acetate accounted for 108%, 74% and 73%, respectively, of the measured increase in DIC production for Gullmar Fjord, Ulleung Basin and Skagerrak. Thus, acetate oxidation must have dominated DIC production in the incubations with acetate amendment. Addition of acetate should relieve the competition between manganese and sulfate reducers at least in Ulleung Basin sediments, where acetate accumulated in the pore water (Figure 3) (Achtnich *et al.*, 1995). Thus, the absence of sulfate reduction must be explained by a small population of acetate-oxidizing sulfate reducers in surface sediments or by inhibition by unknown factors.

Bacterial community composition in the three manganese oxide-rich sediments

Bacterial 16S rRNA gene clone libraries of the geographically distinct sediments at day 0 revealed a diverse benthic community for all three sediments (Supplementary Table 1, Supplementary Figure 1). Already at the approximated family level ($\geq 90\%$ 16S rRNA gene sequence similarity), the three surface sediment bacterial communities were highly dissimilar (0.77–0.82 Bray-Curtis dissimilarity, Supplementary Table 2). This was corroborated by T-RFLP fingerprinting analyses with little overlap between major terminal restriction fragments (T-RFs), especially between the Ulleung Basin and the geographically closer situated Gullmar Fjord and Skagerrak sediments (Supplementary Figure 2). Combined analysis of the clone libraries and T-RFLP profiles indicated that the only major ubiquitously found microbial taxa in all three sediments belonged to the *Sinobacteraceae* and/or *Chromatiaceae* (based on a T-RF calling accuracy of ± 1 base pair) (Supplementary Table 1, Supplementary Figure 2), with both families belonging to the *Gammaproteobacteria*. Major similarities in community composition became only apparent at higher taxonomic levels and comprised *Alpha*-, *Gamma*- and *Deltaproteobacteria*, as well as *Planctomycetes* and *Acidobacteria* (Supplementary Figure 1).

Acetate-oxidizing bacteria identified by SIP of 16S rRNA

The bacterial community composition did not seem to change markedly throughout the incubations from any of the stations, as no major changes in

Table 2 Phylogenetic affiliation and relative abundance of bacterial 16S rRNA sequences obtained from clone libraries of heavy SIP fractions of the Gullmar Fjord (GF), Ulleung Basin (UB) and Skagerrak (S) sediments as well as from most probable number (MPN) counts of GF sediment

Phylogenetic affiliation	OTU no.	Clone frequency (%) of				T-RFs (bp)	
		Heavy SIP fractions			MPN counts	MspI	XapI
		GF	UB	S			
<i>Proteobacteria</i>							
Unclassified		1.2	0	0	0	(452)	(623)
<i>Alphaproteobacteria</i>							
<i>Rhodospirillales</i>							
<i>Rhodospirillaceae</i>							
Unclassified		0	1.5	0	0	(68)	(627)
<i>Gammaproteobacteria</i>							
<i>Alteromonadales</i>							
Unclassified	1	2.3	10.8	17.4	0	498, 499 , (609)	669, 670
<i>Colwelliaceae</i>							
<i>Colwellia</i>	2	25.6	27.7	0	0	(475), 495 , 497, (508)	(646), 666 , 668, (679)
<i>Colwellia</i>	3	0	6.2	2.2	0	495 , 497, (607)	666 , 668
<i>Colwellia</i>	4	0	4.6	0	0	499	670
<i>Colwellia</i>	5	5.8	0	4.3	0	497	668
<i>Colwellia</i>	6	0	0	0	16.7	475	646
<i>Pseudoalteromonadaceae</i>							
<i>Pseudoalteromonas</i>		0	0	2.2	0	(157)	(94)
<i>Shewanellaceae</i>							
<i>Shewanella</i>	7	0	0	0	8.3	495, 497	666, 668
<i>Alteromonadaceae</i>							
Unclassified		1.2	0	0	0	(490)	661
<i>Oceanospirillales</i>							
<i>Oceanospirillaceae</i>							
Unclassified		0	1.5	0	0	(206)	(669)
Unclassified	8	22.1	10.8	39.1	8.3	(494), 495 , 499, (514)	152 , (666, 686)
Unclassified	9	0	0	15.2	0	495 , 497, 499	152 , 154
Unclassified	10	1.2	0	0	20.8	495	666
Unclassified	11	2.3	0	0	0	495	120
<i>Oleiphilaceae</i>							
Unclassified		0	0	0	4.2	496	666
<i>Sedimenticola</i>	12	4.7	0	0	0	150	668
<i>Deltaproteobacteria</i>							
<i>Desulfobacterales</i>							
<i>Desulfobulbaceae</i>							
<i>Desulfobacterium</i>		0	0	0	4.2	165	669
<i>Desulfuromonadales</i>							
Unclassified	13	0	0	0	20.8	508, 510	366, 368
Unclassified		0	1.5	0	0	510	368
<i>Geobacteraceae</i>							
Unclassified	14	1.2	3.1	2.2	0	165	368
Unclassified	15	1.2	3.1	0	0	163	366
<i>Geopsychrobacter</i>	16	0	3.1	0	0	165	354
<i>Epsilonproteobacteria</i>							
<i>Campylobacterales</i>							
<i>Campylobacteraceae</i>							
<i>Arcobacter</i>	17	0	12.3	4.3	0	473, 475	644, 646
<i>Arcobacter</i>	18	0	7.7	2.2	0	475	646
<i>Arcobacter</i>	19	15.1	0	0	0	475	646
<i>Arcobacter</i>	20	8.1	1.5	2.2	16.7	(473), 475	(644), 646
<i>Arcobacter</i>	21	7.0	4.6	2.2	0	475	646
<i>Bacterioidetes</i>							
Unclassified		1.2	0	0	0	(98)	(175)
Candidate Division BD1-5							
Unclassified	22	0	0	6.5	0	169	191
Good's coverage (%)		82	77	74	67		
Total number of clones		86	65	46	24		

Phylogenetic information is resolved to the approximate genus level ($\geq 95\%$ 16S rRNA sequence identity). Multiple mentioning of the same taxon represent sequences $>95\%$ sequence similarity. Lengths of T-RFs are given for restriction digests with the enzymes *MspI* and *XapI*. Multiple T-RFs represent different restriction sites of different sequences (determined in silico from clone sequences). T-RFs in parenthesis were only recognized for one sequence and T-RFs in bold can be found in T-RFLP profiles of heavy SIP fractions (Figure 4, Supplementary Figure 4). The phylogenetic position of OTUs is illustrated in Figure 5. Clone frequencies of 1.2%, 1.5%, 2.2% and 4.2% represent one clone sequence for GF, UB, S and MPN counts clone libraries, respectively.

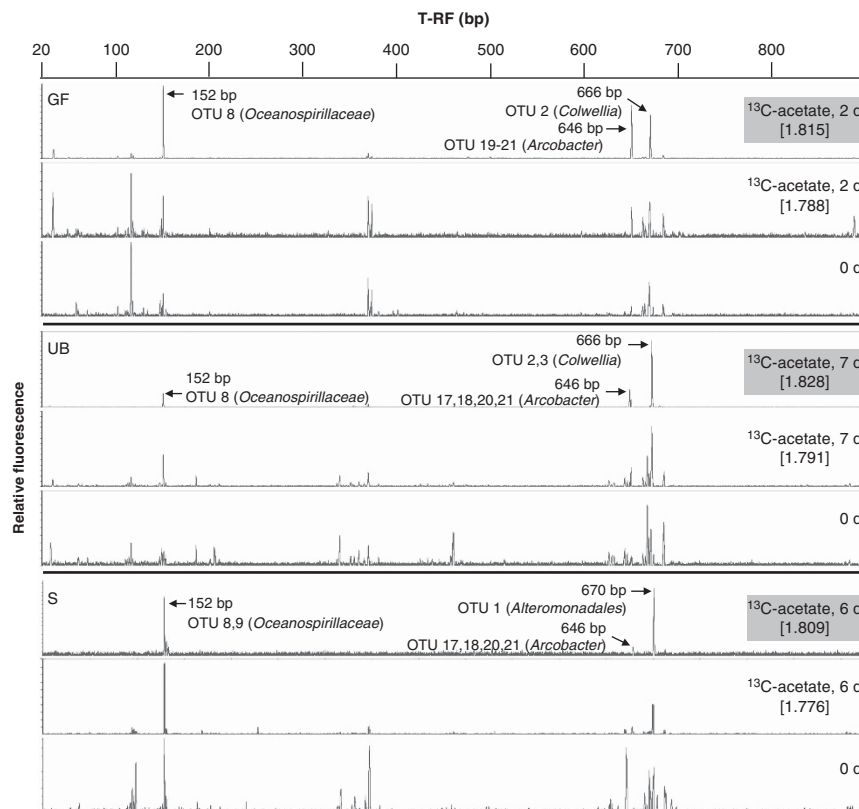


Figure 4 T-RFLP analysis (*XapI*) of 16S rRNA-complimentary DNA amplicons obtained from one selected heavy and light RNA fraction of incubations with ^{13}C -acetate of GF, UB and S sediments in comparison with the original sediment at day 0. Representing 16S rRNA-complimentary DNA clones of major T-RFs can be found in Table 2 and a differential T-RFLP analysis (*MspI*) of heavy and light fractions in Supplementary Figure 4. Numbers in parentheses represent the density of fractions in g ml^{-1} , heavy fractions ($\geq 1.80 \text{ mg l}^{-1}$) are marked by gray background.

community fingerprints were detected in 16S rRNA gene-based T-RFLP analyses (Supplementary Figure 2). Minor increases in T-RF abundances were observed for T-RFs at 475 base pairs for Gullmar Fjord and at 495 base pairs for Skagerrak in acetate-amended incubations. As T-RFLP patterns of controls without acetate did not change, this might point to growth initiation of represented bacteria triggered by the addition of acetate.

Isopycnic RNA centrifugation of selected time points within the first 7 days of incubation revealed significant amounts of ^{13}C -labeled RNA in heavy SIP-fractions of ^{13}C -acetate incubations ($> 1.80 \text{ g ml}^{-1}$, Lueders *et al.*, 2004), which was corroborated by comparison with heavy fractions of the incubations with ^{12}C -acetate and without acetate (Supplementary Figure 3). Successful labeling was also supported by a general absence of reverse transcriptase-PCR products of bacterial 16S rRNA from the heavy SIP-fractions of ^{12}C -acetate and the non-amended control incubations after 19–22 PCR cycles (Supplementary Figure 4 and data not shown, respectively).

Bacterial 16S rRNA resolved within the centrifugation gradients were initially analyzed by T-RFLP fingerprinting, using the restriction enzyme *MspI*.

In all three sediments, T-RFLP profiles obtained from heavy RNA-SIP fractions of the ^{13}C -acetate incubations showed a much lower diversity compared with light fractions and the original bacterial community at the beginning of the incubations (Supplementary Figure 4). This clearly indicates that only a few bacterial groups oxidized the added ^{13}C -acetate and concomitantly incorporated ^{13}C into their RNA. In the ^{13}C -acetate incubations, T-RFLP profiles (*MspI* digest) of the heavy SIP fractions were dominated by two to three major T-RFs for all three sediments (Supplementary Figure 4); and a differential T-RFLP analysis with the restriction enzyme *XapI* indicated label incorporation by bacteria represented by three different T-RFs for each sediment (Figure 4).

Clone libraries of reversely transcribed bacterial 16S rRNA from heavy fractions of ^{13}C -acetate incubations were dominated by four groups for all three sediments (Table 2). In combination with the T-RFLP analyses, these groups were regarded as active acetate-oxidizing manganese reducers and were affiliated to the genera *Colwellia* (*Colwelliaceae*, *Gammaproteobacteria*, operational taxonomic unit (OTU) 2-5) and *Arcobacter* (*Campylobacteraceae*, *Epsilonproteobacteria*, OTU 17-21) as well as to new genera within the *Oceanospirillaceae*

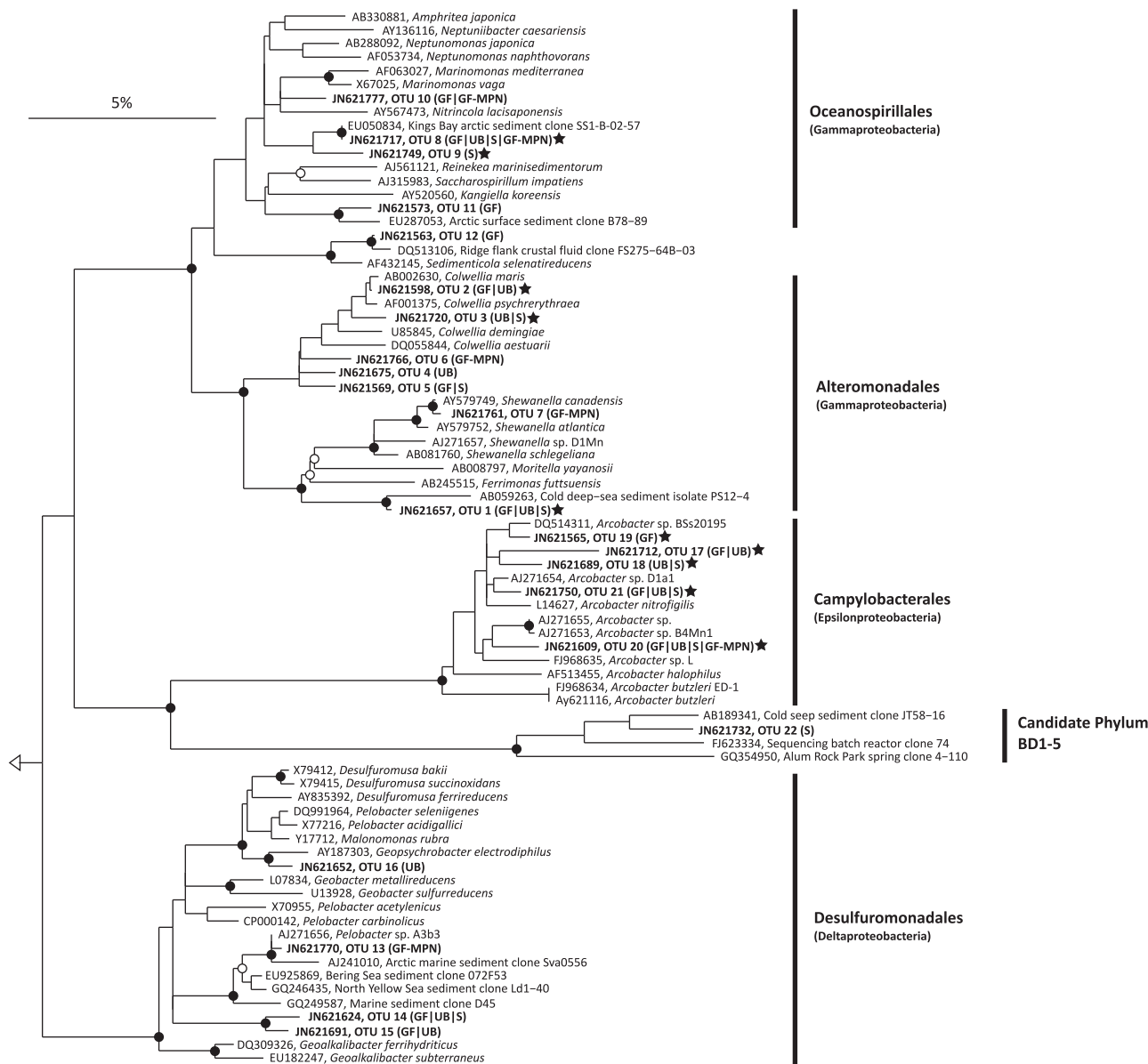


Figure 5 16S rRNA consensus tree showing the affiliation of OTUs detected in heavy SIP fractions of ^{13}C -actetate incubations of GF, UB and S sediments and in acetate-oxidizing manganese-reducing MPN cultures of GF sediment (GF-MPN). Sequences of *Pelobacter* sp. A3Mn2, *Arcobacter* sp. B4Mn1 and *Shewanella* strain D1Mn were clone sequences of manganese-reducing MPN counts of Black Sea sediment taken from Thamdrup *et al.* (2000). *Arcobacter*-L and *Arcobacter* ED1 sequences stem from acetate-fed microbial fuel cells of Fedorovich *et al.* (2009). Stars indicate OTUs for which ^{13}C -label incorporation was confirmed by comparative T-RFLP analysis of light and heavy SIP fractions (Figure 4, Supplementary Figure 4). OTUs that were represented by only one clone sequence are not shown. Relative abundance of OTUs is indicated in Table 2. Filled and open circles indicate lineages with >90% and 75–90% maximum likelihood bootstrap support, respectively. *Aquifex pyrophilus* (M83548) was used as outgroup. The scale bar indicates 5% estimated sequence divergence.

(*Gammaproteobacteria*, OTU 8–11) and *Alteromonadales* (*Gammaproteobacteria*, OTU 1), accounting for 26%, 23% 31% and 10% of the clones, respectively, as an average for all stations (Table 2, Figure 5). Out of these four groups, the acetate-oxidizing manganese reducers identified by the combination of all three approaches (clone library and T-RFLP analyses with both restriction enzymes) were the bacteria affiliated to *Arcobacter* (OTU 18–22)

and *Oceanospirillaceae* (OTU 8–11) for all three sediments whereas bacteria affiliated to *Colwellia* (OTU 2–3) were identified only in the Gullmar Fjord and Ulleung Basin sediments and bacteria affiliated to *Alteromonadales* (OTU 1) in the Skagerrak sediment (Figures 4 and 5, Supplementary Figure 4). In support of our biogeochemical analyses, we did not find sequences related to known sulfate-reducing bacteria in clone libraries of heavy

fractions (Table 2). This result corroborates our conclusion that sulfate reduction was of no importance for acetate oxidation during the incubations.

Interestingly, sequences similar to the four groups of active manganese reducers (same affiliation and same *MspI/XapI* restriction site) in clone libraries of the original sediments were only detected within the *Oceanospirillaceae* for Gullmar Fjord and Skagerrak, and for *Alteromonadales* for Ulleung Basin and Skagerrak (Supplementary Table 1). Although the coverage of the original sediment clone libraries were only between 39% and 62% (on 90% sequence identity level) (Supplementary Table 1) and, thus, sequences of the identified acetate-reducing manganese reducers might have been missed in the original samples, it is also possible that other electron donors, possibly of greater importance than acetate, were used by different groups of manganese reducers *in situ*. Thus, only a subset of the manganese-reducing communities in the three sediments would have been identified by SIP.

Manganese reducers in MPN counts of Gullmar Fjord sediment

Growth of manganese-reducing microorganisms was obtained with all tested electron donors (acetate, lactate, propionate, ethanol, butyrate and formate) in MPN counts of Gullmar Fjord sediment with the highest cell numbers on lactate (43×10^5 cells cm^{-3}) (Table 3). MPN counts of acetate oxidizers (9.3×10^5 cells cm^{-3}) were similar to cell counts (up to 5 and 1.1×10^5 cells cm^{-3} , respectively) of two studies by Nealson *et al.* (1991) and Thamdrup *et al.* (2000) with manganese oxide-rich sediments of the Black Sea.

Interestingly, a bacterial 16S rRNA gene clone library constructed from the highest active MPN dilutions with acetate as substrate revealed that the majority of sequences were affiliated to the genera *Colwellia*, *Arcobacter* and *Shewanella* as well as new genera within the order *Desulfuromonadales* and the family *Oceanospirillaceae* (Table 2). In a similar study with Black Sea sediment clone libraries from the highest dilution of the acetate-oxidizing manganese reducers revealed 16S rRNA gene sequences related to *Arcobacter* and *Pelobacter* within the *Desulfuromonadales* and additional, after subcultivation on lactate, to *Shewanella*

(Thamdrup *et al.*, 2000), which were related to the sequences of this study (90 to >97% sequences identity to the respective sequences) (Figure 5). *Shewanella* and members of the *Desulfuromonadales* are two groups with the ability to reduce iron and manganese, which are recovered frequently through cultivation for example, with iron oxides as electron acceptor or acetate as electron donor (Caccavo *et al.*, 1992; Venkateswaran *et al.*, 1998; Snoeyenbos-West *et al.*, 2000; Holmes *et al.*, 2002; Vandieken *et al.*, 2006a). *S. putrefaciens* is one of the few strains isolated directly by manganese oxide reduction (Myers and Nealson, 1988; Nealson *et al.*, 1991). However, sequences have been rarely detected in clone libraries from marine sediments, just as in our study, and the *in situ*-importance of this group remains unclear (Lovley *et al.*, 2004). This might indicate that the high substrate concentrations of cultivation-dependent methods favor organisms that are able to grow fast but might not be abundant in high numbers and/or active in the environment. Similar problems may arise for SIP experiment as fast growing organisms (like it is indicated for *Arcobacter* species in this and other studies (for example, Webster *et al.*, 2010)) that would dominate an enrichment culture also become first ^{13}C -labeled, although added acetate concentrations for the SIP incubations were lower than in the MPN counts. However, our combined results of cultivation-independent and cultivation-dependent methods together with the results of Thamdrup *et al.* (2000) give strong evidence that the taxa *Arcobacter*, *Colwellia* and *Oceanospirillaceae* include organisms that are capable of growing on acetate and manganese oxide and are present in distinct manganese-oxide rich sediments.

Relevance of manganese reduction for the identified acetate oxidizers

Among the taxa identified with SIP and MPN enrichments, only members of the *Alteromonadales* and *Desulfuromonadales* have been linked previously to the reduction of manganese and iron oxides in pure culture (Lovley *et al.*, 2004). Sequences of OTU 1 within the *Alteromonadales* were related to cultured species of the genera *Ferrimonas* and *Shewanella* (Figure 5), in which *F. balearica*, *F. kyonanensis* and *F. futtsuensis*, as well as many *Shewanella* species are able to reduce iron and manganese oxides in pure culture (Rosselló-Mora *et al.*, 1995; Lovley *et al.*, 2004; Nakagawa *et al.*, 2006). All members of the *Desulfuromonadales* are able to reduce iron and manganese oxides (Lovley *et al.*, 2004) and are also frequently found in clone libraries of marine sediments (for example, Ravensschlag *et al.*, 1999; Bowman and McCuaig, 2003; Mußmann *et al.*, 2005). *Desulfuromonadales*-related organisms can be easily enriched in sediments or microbial fuel cells by acetate addition (Snoeyenbos-West *et al.*, 2000; Bond *et al.*, 2002;

Table 3 Most probable number (MPN) counts (10^5 cells cm^{-3}) of manganese-reducing microorganisms in surface sediment of Gullmar Fjord

Electron donor	MPN counts	95% confidence interval
Lactate	43	9–180
Acetate	9.3	1.8–42
Propionate	7.5	1.7–20
Ethanol	1.5	0.37–4.2
Butyrate	0.93	0.18–4.2
Formate	0.75	0.17–2

Holmes *et al.*, 2002; Holmes *et al.*, 2004; Vandieken *et al.*, 2006a) but have also been identified as iron-reducing bacteria by cultivation-independent SIP studies with ^{13}C -acetate in rice field soil and uranium-contaminated aquifer (Chang *et al.*, 2005; Hori *et al.*, 2010). Our finding of *Desulfuromonadales* in MPN enrichments coincided with their detection in clone libraries of heavy SIP fractions. However, their low abundance in clone libraries of the SIP experiments (2–11%, Table 2) and absence of prominent T-RFs representing these microorganisms suggests that they contributed less to acetate turnover than other bacteria.

Bacteria affiliated to *Colwellia*, *Oceanospirillaceae* and *Arcobacter* were identified as important manganese reducers in acetate-fed SIP incubations of all three sediments. This is astonishing because metal reduction has not yet been shown in pure culture studies of members of any of these taxa. Sequences of *Oceanospirillaceae* and *Colwellia* are frequently recovered in molecular studies of marine waters and sea ice (DeLong *et al.*, 1993; Gosink and Staley, 1995; Bowman *et al.*, 1997; Brown and Bowman, 2001; Koskinen *et al.*, 2011). One additional report is from a sediment (Borin *et al.*, 2009), but judging from the eutrophic coastal setting, manganese reduction is not likely to be important there either. Isolates of these two gammaproteobacterial taxa are mainly of marine origin (Deming *et al.*, 1988; Gosink and Staley, 1995; Bowman *et al.*, 1998; Yumoto *et al.*, 1998; Hedlund *et al.*, 1999; Nogi *et al.*, 2004; Jung *et al.*, 2006). Most isolates are facultative anaerobes, some are able to use acetate as sole carbon and energy source and to reduce nitrate. However, in most of these studies manganese and iron reduction was not tested so that manganese reduction might be a potential metabolism for these organisms. Only four *Colwellia* species have been tested negative for iron reduction with acetate (Bowman *et al.*, 1998).

In contrast to *Colwellia* species and members of the *Oceanospirillaceae*, indication for the involvement of *Arcobacter* species in manganese reduction stems from MPN enrichments of manganese oxide-rich Black Sea sediments, as discussed above (Thamdrup *et al.*, 2000). *Arcobacter* species have also been found in other sediments, with a depth distribution that could match that of manganese reduction. Manganese oxides are typically restricted to the upper one to few cm in coastal sediments (Aller, 1980; Sundby *et al.*, 1981; Thamdrup *et al.*, 1994), and fluorescence *in situ* hybridization counts showed high abundance of *Arcobacter* in surface sediment of the Wadden Sea whereas they were virtually absent below 3 cm depth (Llobet-Brossa *et al.*, 1998). Similarly, 16S rRNA gene sequences related to *Arcobacter* were only retrieved from Antarctic shelf surface sediment (0–0.4 cm) but not from deeper layers (Bowman and McCuaig, 2003). However, isolates of *Arcobacter* were obtained from both surface and deeper sediment layers of the

Wadden Sea (Freese *et al.*, 2008). Recently, *Arcobacter* strains were isolated from the graphite electrode of a microbial fuel cell inoculated with marine sediment and fed with acetate (Fedorovich *et al.*, 2009) (Figure 5), indicating that *Arcobacter* species are able to use acetate and transfer electrons onto solid-phase electron acceptors. In a recent cultivation-independent SIP study with estuarine sediment, *Arcobacter*-related bacteria incorporated ^{13}C into their DNA from acetate and glucose under various terminal electron acceptor conditions (aerobic, nitrate-, sulfate-reducing, methanogenic) (Osaka *et al.*, 2006; Webster *et al.*, 2010). Under sulfate-reducing conditions with ^{13}C -labeled acetate, *Arcobacter* became enriched fast and although the authors suggested that *Arcobacter* was involved in sulfur cycling, iron and manganese reduction during the sediment incubations were indicated by increasing concentrations of dissolved manganese and iron (Webster *et al.*, 2010). *Arcobacter*-related sequences have been found in clone libraries of many marine and terrestrial habitats (Campbell *et al.*, 2006), for example, Arctic and Antarctic surface sediments and the anoxic water column of the Cariaco Basin, the Black Sea, the African shelf and an anoxic fjord (Nitinat Lake) (Madrid *et al.*, 2001; Bowman and McCuaig, 2003; Vetriani *et al.*, 2003; Lavik *et al.*, 2009; Schmidtova *et al.*, 2009). The metabolism of these organisms in the anoxic water columns is somewhat uncertain but sulfide oxidation has been repeatedly suggested (Lin *et al.*, 2006). Thus, the genus *Arcobacter* appears highly versatile and is found under many different environmental conditions though the physiology of most of its members remains unclear.

Conclusions

This study shows that microbial manganese reduction completely dominates anaerobic carbon oxidation in the surface of the three studied sediments similar to other sites with high manganese oxide concentrations (Aller, 1990; Thamdrup *et al.*, 2000; Vandieken *et al.*, 2006b; Nickel *et al.*, 2008). However, manganese-reducing microorganisms have been rarely studied and directly isolated on manganese oxides, and most properties of iron reducers and iron-reducing communities have been assumed also to apply to manganese reducers. Our results of cultivation-independent SIP experiments and cultivation-based MPN counts identified *Arcobacter*-, *Colwellia*- and *Oceanospirillaceae*-affiliated bacteria as the main acetate-oxidizing manganese reducers. Thus, this study gives strong evidence that dissimilatory manganese reduction in the environment is conveyed by a different assemblage of bacteria than the extensively explored iron reducers, and suggests a new function for bacteria affiliated to *Arcobacter*, *Colwellia* and *Oceanospirillaceae*, namely the reduction of manganese oxides.

The finding of highly similar populations of manganese reducers in all three manganese oxide-rich sediments is intriguing as the locations, a Swedish fjord, the deep Skagerrak and a continental basin in the northwest Pacific, are distinct in terms of geographical distance and environmental conditions. This further leads to the conclusion that species of *Arcobacter*, *Colwellia* and new genera within the *Oceanospirillaceae* and *Alteromonadales* represent globally distributed marine bacteria, which contribute to dissimilatory manganese reduction in manganese oxide-rich sediments. As most of the groups have not been associated with iron reduction but rather metabolisms of higher energy gain (aerobic respiration and nitrate reduction), this raises fundamental questions, such as whether the mechanisms involved in extracellular electron transfer are similar to those used by iron reducers and whether the substrate spectrum of manganese reducers is as restricted as that of iron reducers, or more diverse as for aerobes.

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