FEMS Microbiology Ecology, 92, 2016, fiw054

doi: 10.1093/femsec/fiw054
Advance Access Publication Date: 8 March 2016
Research Article

RESEARCH ARTICLE

Bacterial communities potentially involved in iron-cycling in Baltic Sea and North Sea sediments revealed by pyrosequencing

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One sentence summary: This study gives insight into the bacterial community composition related to iron-cycling in suboxic marine sediments based on 16S rRNA gene pyrosequencing.

Editor: Tillmann Lueders

ABSTRACT

To gain insight into the bacterial communities involved in iron-(Fe) cycling under marine conditions, we analysed sediments with Fe-contents (0.5–1.5 wt %) from the suboxic zone at a marine site in the Skagerrak (SK) and a brackish site in the Bothnian Bay (BB) using 16S rRNA gene pyrosequencing. Several bacterial families, including Desulfobulbaceae, Desulfuromonadaceae and Pelobacteraceae and genera, including *Desulfobacter* and *Geobacter*, known to reduce Fe were detected and showed highest abundance near the Fe(III)/Fe(II) redox boundary. Additional genera with microorganisms capable of coupling fermentation to Fe-reduction, including *Clostridium* and *Bacillus*, were observed. Also, the Fe-oxidizing families Mariprofundaceae and Gallionellaceae occurred at the SK and BB sites, respectively, supporting Fe-cycling. In contrast, the sulphate (SO_4^{2-}) reducing bacteria *Desulfococcus* and *Desulfobacterium* were more abundant at greater depths concurring with a decrease in Fe-reducing activity. The communities revealed by pyrosequencing, thus, match the redox stratification indicated by the geochemistry, with the known Fe-reducers coinciding with the zone of Fe-reduction. Not the intensely studied model organisms, such as *Geobacter* spp., but rather versatile microorganisms, including sulphate reducers and possibly unknown groups appear to be important for Fe-reduction in these marine suboxic sediments.

Keywords: marine sediments; microbial communities; next-generation 16S rRNA gene amplicon sequencing; iron reduction; iron oxidation; marine microbial ecology

Received: 15 November 2015; **Accepted:** 7 March 2016

INTRODUCTION

Iron (Fe) and manganese (Mn) are commonly the most abundant solid-phase electron acceptors in aquatic sediments (e.g. Thamdrup 2000). The microbial communities involved in Fe-reduction are relatively well investigated in fresh water sediments (such as lake sediments or rice field soils; e.g. Stein *et al.* 2001; Hori *et al.* 2010). Under marine conditions Fe may also be reduced abiotically via sulphide oxidation (Canfield *et al.* 1993; Thamdrup *et al.* 2006; Raiswell and Canfield 2012), such that the study of Fe-reducing microbial communities is hampered by the difficulty to pinpoint locations where dissimilatory Fe-reduction is the predominant pathway. Most previous diversity studies focused on sites where either SO_4^{2-} is low (or absent), such as brackish water estuaries (Lin *et al.* 2007) or methanogenic zones (Oni *et al.* 2015), or on particular sediments with relatively high Fe-contents (>1 wt % Fe), such as in the surrounding of hydrothermal vents (Edwards, Bach and McCollom 2005; Nercessian *et al.* 2005; Forget, Murdock and Juniper 2010; Handley *et al.* 2010). Other studies, e.g. in tidal sediments (Kim *et al.* 2014) or the deep biosphere (e.g. Biddle *et al.* 2008), have focused mainly on SO_4^{2-}-reducers.

Nevertheless, geochemical studies provide strong evidence that dissimilatory Fe-reduction is the dominant electron accepting process in the sediments just below the Fe-redox boundary at locations with an expanded suboxic zone, as it occurs at several sites in the Baltic Sea (Rajendran, Dileepkumar and Bakker 1992; Canfield, Thamdrup and Hansen 1993; Jensen *et al.* 2003). Currently, only a few studies have characterized the microbial communities in surface sediments of the Baltic and North Sea. Edlund *et al.* (2008) performed a clone library study using bromodeoxyuridine (BrdU)-labelled DNA in surface sediments from Askö Island in the open Baltic Sea. There, Myxococcales, an order known to include Fe-reducing bacteria from the Myxococcaceae family (Treude *et al.* 2003) was detected. Another diversity study by Sinkko *et al.* (2011) in the Archipelago of the Northern Baltic Sea, using a 16S rRNA cloning method, revealed the presence of members of the order Desulfuromonadales containing S^0-, Fe- and Mn-reducers. Indeed, the 16S rRNA gene profile of this group positively correlated with total Fe and Fe-bound phosphorous in surface sediments in the estuary of the Paimionlahti Bay. Also, incubation studies by Vandieken *et al.* (2012) and Vandieken and Thamdrup (2013) showed that sediments from the Skagerrak (SK) harbour *Arcobacter*, *Colwellia* and *Oceanospirillaceae* capable of reducing Mn-oxides and that they can use lactate, acetate and hydrogen as electron acceptors. A single strain of *Arcobacter* was recently shown to reduce Fe-citrate in pure culture (Roalkvam *et al.* 2015). Based on results from these previous studies, we hypothesized that distinct microbial communities associated with Fe-cycling would be found in these sediments and the goal was to characterize these communities and screen them for microbial groups involved in Fe-cycling.

We received multicores from Site Geo 2a (Fig. 1; Jørgensen 1989; Canfield, Thamdrup and Hansen 1993) located in the SK to perform such a microbial community characterization. Marine salinity is maintained by water exchange with the North Sea (OSPAR 2000). The deep waters of the SK are always oxygenated maintaining a permanent redox gradient in the uppermost centimetres below the sediment surface (Canfield, Thamdrup and Hansen 1993; Croot *et al.* 2002; Bendtsen *et al.* 2009). Sediments are mainly derived from algal blooms and terrigenous input with a high Fe-content (Slomp *et al.* 1997). The abundances of reactive Mn and Fe are high (Rajendran, Dileepkumar and Bakker 1992; Thamdrup 2000), allowing microbial metal reduction to dominate over SO_4^{2-}-reduction despite marine SO_4^{2-}-concentrations (Canfield, Thamdrup and Hansen 1993). In fact, the location of Site Geo 2a projects laterally into a transect described by Canfield, Thamdrup and Hansen (1993) showing throughout the predominance of dissimilatory Fe-reduction in the top centimetres below the Fe-oxidation front.

As an intermediate site between marine and freshwater conditions we also studied Site At4 with brackish conditions located in the Bothnian Bay (BB; Fig. 1; Ingri and Pontér 1986; Yli-Hemminki, Jørgensen and Lehtoranta *et al.* 2014) in the northernmost part of the Baltic Sea. There, water exchange with the open Baltic Sea is strongly limited by the shallow Åland Sea (Fonselius 1981). The sediments in the BB are enriched in Fe and Mn (oxyhydr) oxides and even ferromanganese oxide nodules have been recovered (Glasby *et al.* 1997). The high levels of Fe (oxyhydr) oxides in BB sediments are most likely due to input by Finnish and Swedish rivers from leached acidic pine forest soils and peat lands (Burman 1983; HELCOM 2004; Pohl and Fernández-Otero 2012). Due to the low salinity, the BB area is characterized by low SO_4^{2-}-concentrations (Boström, Wiborg and Ingri 1982; Ingri and Pontér 1986). The water column in the BB is barely stratified and well oxygenated (Fonselius 1981) and a redox gradient is established in the top centimetres of the sediment showing prevalent Fe- and Mn-cycling. Brackish conditions and less reactive organic matter in the BB appear to be responsible for the low levels of bioturbating fauna in the BB (Elmgren 1984). Although the prevalence of dissimilatory Fe-reduction has not been demonstrated at this site yet, it shows a similar zonation in the porewater chemistry as Site Geo 2a. Even without precise knowledge of the predominant microbial activity, considering the high content of Fe, a comparison with Site Geo 2a appeared useful.

In the present study, we analysed the associated bacterial communities in sediments from the uppermost 30 cm at SK and BB sites using pyrosequencing of 16S rRNA genes. This technique now provides a much more representative analysis of the whole bacterial communities with unprecedented representation on a family or even genus level. Abundances of different Fe-, Mn- and SO_4^{2-}-reducing/oxidizing taxa were plotted versus depth and compared with the geochemical profiles. Using this approach, we were able to link patterns in the bacterial community to the redox stratification and zones of Fe-cycling within the suboxic zone of the sediments.

MATERIALS AND METHODS

Sample collection

Sediment cores of up to 38 cm in length (10 cm in diameter) were taken during RV Meteor cruise no. M86-1 in November 2011 using a multicorer device MC-8/100 (Oktopus GmbH, Kiel, Germany). The two sampling locations considered in this study are SK Site Geo 2a and BB Site At4 (Fig. 1; Table S1, Supporting Information). After collection, cores for microbiological analyses from each site were frozen at –20°C on board the ship and later transferred to –80°C in the shore based laboratory.

Parallel cores were recovered for geochemical analyses, and porewater was extracted directly after recovery by using Rhizon samplers (Rhizosphere Research Products B.V, Wageningen, the Netherlands) (Seeberg-Elverfeldt *et al.* 2005). Porewater samples for Fe^{2+}, Mn^{2+} and SO_4^{2-} analyses were acidified to 1% (v/v) with nitric acid (HNO_3) (65% suprapure, Merck, München, Germany). Samples for NO_3^- and NH_4^+ were kept frozen until analysis at the Leibniz Institute for Baltic Sea Research, Warnemünde

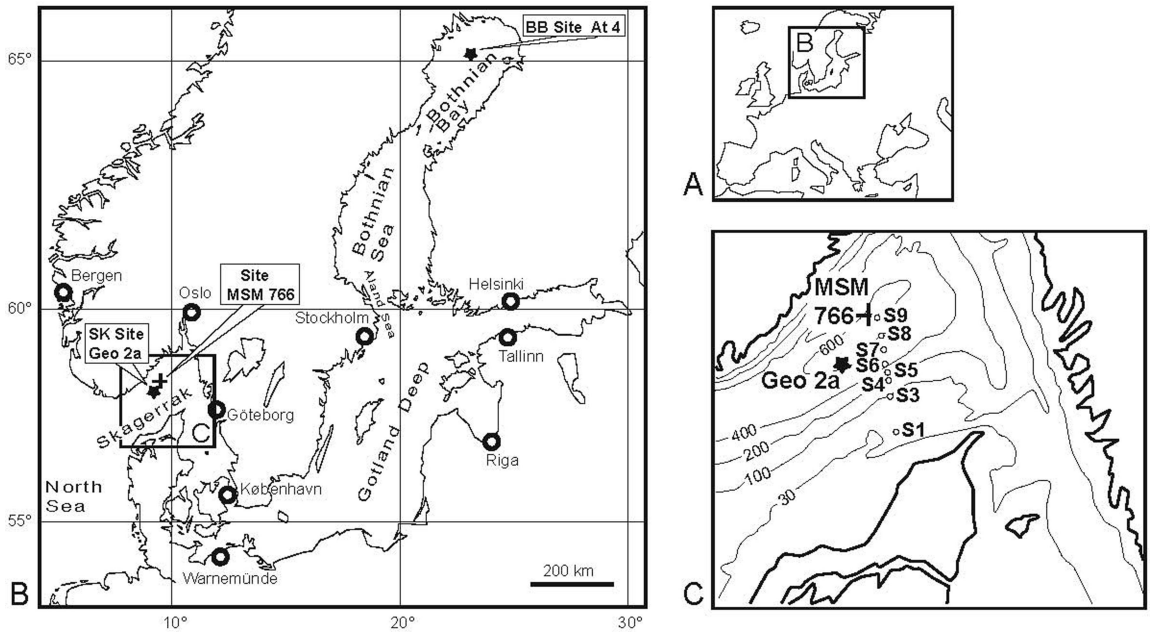

Figure 1. Map of the Baltic Sea (Panel B) showing locations of the three sampling Sites Geo 2a (SK), At4 (BB) and MSM 766. Panel C is from the SK showing the locations of Site Geo 2a with respect to the locations of Sites S1 through S9 of the transect studied by Canfield *et al.* (1993). Site MSM 766 corresponds to Site S9 in the transect.

(IOW). For the determination of H_2S, 2 ml of the porewater sample were mixed with 100 μl 5% zinc acetate.

A short core for S isotope and Fe/Mn studies was taken at SK Site MSM 766 during RV Merian cruise no. 12–4a in August 2009 (Fig. 1; Table S1, Supporting Information). This site is located 32 km northeast of Site Geo 2a. Following completion of the cruise, porewater from these samples was extracted after 38 days of storage at 4°C, by centrifuging 1 cm core slices at 4400 x *g* for 15 min. For Fe^{2+} and Mn^{2+} analysis, 1-ml porewater was filtered through a 0.45-μm polyvinylidene fluoride (PVDF) membrane filter into 25 μl of 6M HCl.

Geochemical analyses

Porewater concentrations of Fe^{2+}, $Mn^{2+}$and SO_4^{2-} (SO_4^{2-} was measured as total dissolved sulphur) for the 2011 samples were measured by inductively coupled plasma optical emission spectroscopy (ICP-OES; Thermo, iCAP 6300 Duo, Thermo Fisher Scientific GmbH, Dreieich, Germany). Depending on the salinity, 6-fold diluted aliquots were used for Site Geo 2a samples and non-diluted aliquots for Site At4 samples. Accuracy and precision were determined with the 6-fold diluted seawater standard CASS-5 (National Research Council of Canada, Ontario, Canada) and were better than 8.2% and 6.7%, respectively. CASS-5 solutions were spiked with Fe^{2+} and Mn^{2+} because the porewater concentrations were much higher than in the original reference material. The original CASS-5 concentrations were 1.4 μg/L Fe and 2.56 μg/L Mn, and these were spiked to a final concentration of 50 μg/L Fe and Mn when measuring samples from SK and 200 μg/L Fe and Mn for BB. The SO_4^{2-}-concentration of CASS-5 was calculated by rule of three by taking the SO_4^{2-}-concentration of the open ocean to be 35 ‰ (Bruland 1983) and the CASS-5 salinity to be 33.5 ‰. Since the dissolved H_2S concentrations of the porewater were extremely low, an interference with total dissolved S measured by ICP-OES can be excluded. Total H_2S in porewater was determined spectrophotometrically (Specord-40, Analytik, Jena, Germany) using the method of Cline (1969). NO_3^- and NH_4^+ were measured after the methods of Grasshoff, Kremling and Ehrhardt (1999a,b) by using a continuous flow nutrient analyser (QuAAtro, Seal Analytical GmbH, Norderstedt, Germany).

Freeze-dried and homogenized sediment samples for the analysis of Fe, Mn and total sulphur (TS) were digested with a mixture of concentrated HNO_3 (1 mL), perchloric acid ($HClO_4$, 1 mL) and hydrofluoric acid (HF, 2 mL) using a pressure digestion system PDS-6 (Loftfields Analytical Solutions, Neu Eichenburg, Germany; Heinrichs *et al.* 1986) at 180°C for 6 h. Two hundred fifty-fold diluted digestions were measured by ICP-OES (Thermo, iCAP 6300 Duo, Thermo Fisher Scientific GmbH, Dreieich, Germany). Accuracy and precision of the analyses were determined by measurements of the certified reference standard SGR-1 (Green River Shale, United States Geological Survey) and were better than ±5.3% and ±5.6%, respectively. Reactive Fe and Mn were extracted from the sediments by using 1M HCl applied for 24 h at room temperature (Leventhal and Taylor 1990) and were also measured by ICP-OES. Total carbon (TC) and total nitrogen (TN) were determined by using an elemental analyser EA 1110 (CE-instruments LTD, Wigan, UK) and total inorganic carbon (TIC) by an elemental multianalyser EA®2000 (Analytik, Jena, Germany) using half-concentrated phosphoric acid (H_2PO_4). TOC was calculated as the difference between TC and TIC. Precision and accuracy were better than ±2.3% and ±1.3%, respectively. TS measurements are reported in Fig. S1 (Supporting Information).

Porewater concentrations of Fe^{2+} and Mn^{2+} for the 2009 samples were measured by flame atomic absorption spectroscopy (AAS; AAnalyst 100, Perkin Elmer, Rodgau, Germany) and by the ferrozine assay as previously described (Thamdrup, Fossing and Jørgensen 1994). Samples below 5 cm were diluted 5—10-fold so that Mn concentrations would fall within the measuring range of the instrument.

Sulphur and oxygen isotopes

Dissolved SO_4^{2-} was precipitated as barium sulphate ($BaSO_4$) from filtered acidified solutions by the addition of barium chloride ($BaCl_2$) as described by Böttcher, Thamdrup and Vennemann (2001). S-isotope ratios were measured at IOW by means of combustion isotope-ratio monitoring mass spectrometry (c-irmMS) using a Thermo MAT 253 gas mass spectrometer (Thermo Fisher Scientific GmbH, Dreieich, Germany) coupled to a Thermo flash elemental analyser (Thermo Fisher Scientific GmbH, Dreieich, Germany) via a Thermo Conflo interface (Thermo Fisher Scientific GmbH, Dreieich, Germany) (Mann, Vocke and Kelly 2009; Saccon *et al.* 2013). Oxygen isotope measurements on precipitated $BaSO_4$ were carried out at the UFZ in Leipzig according to previously published methods (Gehre *et al.* 2004). Sulphur and oxygen isotope ratios are reported in the conventional δ-notation relative to the Vienna Cañon Diablo Troilite (VCDT) and Vienna Standard Mean Ocean Water (VSMOW) standards, respectively. Corresponding replicates agreed within ±0.3 ‰ and ±0.5 ‰.

Nucleic acid extraction

Frozen cores were sliced into 1 or 2 cm diameter discs using a band saw (K330S, Paul Kolbe GmbH, Elchingen, Germany) with a WIKUS blade (WIKUS DIAGRIT S Nr. 572 D254 VA, WIKUS-Sägenfabrik, Spagenburg, Germany). The blade was sterilized with ethanol after cutting each slice. The sediment that was in contact with the blade was removed and only the interior parts of the frozen discs were sectioned into aliquots for DNA extraction. Extractions were made from ~0.5–0.6 g of sediment based on the method of Lueders, Manefield and Friedrich (2004) with the following modification: pH-5 buffers and phenol–chloroform–isoamyl alcohol [PCI, 25:24:1 (vol:vol:vol)] and chloroform-isoamyl alcohol [CI, 24:1 (vol:vol)] mixtures were used as solvents to facilitate extraction of DNA/RNA. Following extraction, the nucleic acid pellet was dissolved in 50 μl of RNase/DNase-free water. Extracts were checked for nucleic acid integrity by agarose gel electrophoresis, and concentrations were estimated using a NanoDrop Spectrophotometer ND-1000 (PeqLab, VWR International GmbH, Erlangen, Germany). The DNA concentrations obtained after the extraction step above are reported in the (supplemental material).

Pyrosequencing

For pyrosequencing by the sequencing centre (Mr.DNA Molecular Research LP, Shallowater, TX, USA) 16S rRNA gene tags were PCR amplified using primers (V1–V3 regions) described in Table S2 (Supporting Information). Samples were prepared as described in Dowd *et al.* (2008). Following the dilution of each DNA sample to equal concentrations, PCR was carried out using HotStarTaq Plus Master Mix Kit (Qiagen, CA, USA) under the following conditions: 94°C for 3 min followed by 28 cycles of 94°C for 30 s, 53°C for 40 s and 72°C for 1 min and a final elongation step at 72°C for 5 min. Following PCR, all amplicon products from different samples were mixed in equal concentrations and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA). Samples were sequenced utilizing Roche 454 FLX titanium instruments and reagents following manufacturer's guidelines.

Pyrosequenced data analysis

The QIIME software package, version 1.6.0 (Caporaso *et al.* 2010) was employed to analyse 16S rRNA gene tag sequences using the default settings with the following exception: bacterial sequences were trimmed and denoised using the program AmpliconNoise version 1.27 (Quince *et al.* 2011) instead of the default program, and a truncation length of 500 bp was used to include as many long sequences as possible. The online program PrinSeq (Schmieder and Edwards 2011) was used to determine the average length of sequences for each sample following the denoising step. Denoised sequence files were deposited at the Metagenomics RAST (MG-RAST; Meyer *et al.* 2008) online database, and raw files were deposited at the DNA databank of Japan. Accession numbers are reported in Table S3 (Supporting Information). The Greengenes taxonomy database (McDonald *et al.* 2012) 12_10 release reference file (gg_12_10_otus/97_otu_taxonomy.rdp22_train.txt) was used to assign taxonomy to operational taxonomic units (OTU) up to family level for representative sequences. Representative sequences were clustered at a 97% similarity with uclust (Edgar 2010) as part of the pipeline. The alpha diversity (Chao 1) was calculated as described in the supplementary methods section. Briefly, Chao1 indices were based on the maximum rarefaction of 5420 sequences. OTU abundances were normalized to the total number of sequences in each sample and are reported as percent relative abundances.

RESULTS

Porewater chemistry

SK sediments show a NO_3^--concentration of more than 10 μM in the top 7 cm, while the Mn concentration increases downwards to reach a maximum of ca. 150 μM at 7 cm (Fig. 2A). A Fe-oxidation front is present at 7 cm, below which Fe^{2+}-concentration increases steeply to ca. 100 μM at 9.5 cm. Below, Fe and Mn only slightly increase and they decrease below 20 cm. NH_4^+-concentration steadily increases with depth below 6 cm to reach 300 μM at the bottom of the core (Fig. 2A). SO_4^{2-}-concentration is constant at ca. 30 mM and slightly decreases below 25 cm (Fig. 2B). H_2S-concentrations were below detection limit at all depths.

In BB sediments, maximum concentrations of NO_3^- of ca. 7 μM occurred at 1.5 cm (Fig. 2C). Concentrations of Mn^{2+} and Fe^{2+} both increase steeply from 0.5 and 1.5 cm, respectively, to reach a maxima around 400 μM near 6 cm. Accordingly, the Fe-oxidation front occurs within the top 1.5 cm. NH_4^+-concentration increases with depth to 400 μM within the top 10 cm and less steeply to 600 μM at the bottom of the core (Fig. 2C). SO_4^{2-}-concentrations decrease from 2 mM at the surface to nearly complete depletion at 27 cm (Fig. 2B and 2D). Dissolved H_2S-concentrations were at or below the detection limit.

At Site MSM 766, maximum concentrations of Mn^{2+} and Fe^{2+} occurred at 4–11 and 11–15 cm, respectively, and decreased linearly below 15 cm (Fig 3A). The Fe-oxidation front is located near 12 cm. In SK sediments from Site MSM 766, the $\delta^{34}S$ values of porewater sulphate were +20 ‰ at the surface and slightly increased to +23 ‰ at the bottom of the core (Fig. 3B). In the same samples, $\delta^{18}O$ (SO_4^{2-}) essentially showed constant values of +10 ‰.

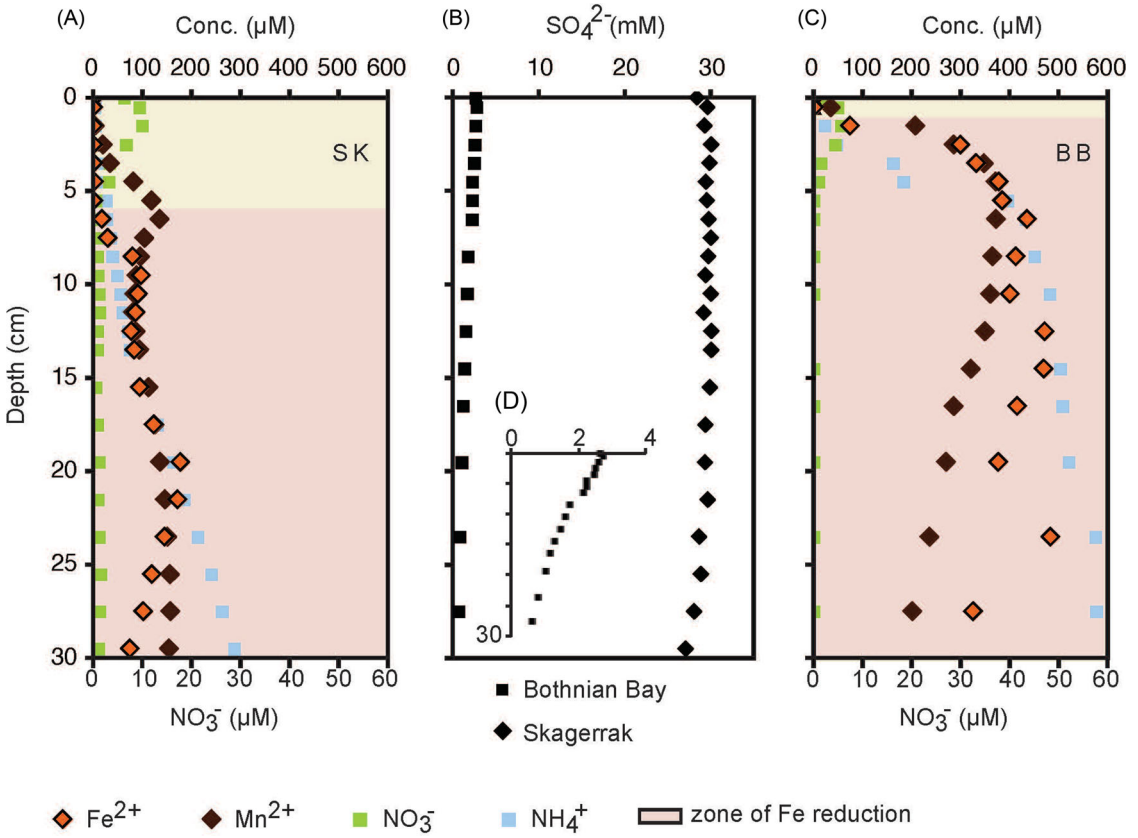

Figure 2. Porewater profiles of SK and BB sediments. (**A**) Fe^{2+}, Mn^{2+}, NO_3^- and NH_4^+ at SK. (**B**) SO_4^{2-}-concentration profiles at both sites. (**C**) Fe^{2+}, Mn^{2+}, NO_3^- and NH_4^+ at BB (**D**) Inset: close-up of SO_4^{2-}-concentration profile at BB. Shaded areas showing zones where free Fe^{2+} is present in the porewater. These cores are from the November 2011 Meteor cruise no. M86-1.

Solid-phase chemistry

In SK sediments, the extracted solid-phase Fe-content is above 1 wt % in the top 6.5 cm. The Fe-content decreases steeply below a maximum at the Fe-oxidation front and shows some scatter between 0.5 and 1 wt % below ca. 8 cm (Fig. 4A). Mn-contents were highest around 0.3 wt % in the top 4.5 cm and stay around 0.1 wt % throughout the remainder of the core (Fig. 4B). TOC contents decrease linearly from 2.5 wt % at the top to 2 wt % at the bottom of the core (Fig. 4C). TN contents also decrease linearly from 0.3 to 0.25 wt % over the same interval, indicating a constant C/N ratio throughout the core (Fig. 4C).

In BB sediments, the extracted Fe-contents decreased from above 1 wt % to below 0.5 wt % at 7 cm and remain constant to ca. 20 cm (although with some scatter in the lower part; Fig. 4D). Extracted Mn decreased downwards asymptotically from 0.15 wt % below 2 cm (Fig. 4E). One measurement at 10 cm showed a much higher Fe content relative to the average content. This peak in Fe was reflected in a less pronounced peak in Mn^{2+} (Fig. 4D and E). The content of TOC was 3.2 wt % at the top and decreased downwards to asymptotically reach a value of 0.7 wt % (Fig. 4F). Total N was 0.3 wt % at the top and decreased to 0.03 wt % at 27.5 cm (Fig. 4F). Due to the fact that TN is almost entirely depleted, the C/N ratio increases strongly with depth (not shown in the plot).

Bacterial community composition

Following analyses of bacterial 16S rRNA genes, a total of 68 470 sequences were assigned to between 1415 and 1698 OTU for the SK site, and 134 158 sequences were assigned to between 428 and 1184 OTU for the BB site (Table S3, Supporting Information). The α-, β-, δ- and γ-Proteobacteria were the predominant bacterial phyla at both sites (Fig. 5). Additional phyla present and abundant in the samples included Planctomycetes, Chloroflexi, Actinobacteria, Acidobacteria, Bacteroidetes and Nitrospirae (Fig. 5).

At the family level, Desulfobacteraceae (SK: 2%–12%, BB: 0.1%–6%), Rhodospirillaceae (SK: 0.6%–4%, BB: 1%–6%), Hyphomicrobiaceae (SK: 0.7%–3%, BB: 2%–5%) and Pirellulaceae (SK: 1%–5%, BB: 0.9%–3%) had the highest relative abundances at both sites (percentages with respect to taxonomic groups refer to relative abundances of OTU's in each sample).

Potential Fe-cycling bacteria

Several bacterial families of which members are capable of Fe-reduction were detected: Desulfobacteraceae (SK: 2%–12%, BB: 0.1%–6%), Desulfobulbaceae (SK: ≤0.8%, BB: ≤0.2%), Desulfuromonadaceae (SK: <0.1%–0.3%, BB: <0.1), Geobacteraceae (SK and BB: ≤0.1%) and Pelobacteraceae (BB: ≤0.2%) (Fig. 6A and B). They all belong to the δ-Proteobacteria and contain a majority of genera capable of Fe-reduction. Taxa that are able to reduce Fe were selected from published data (e.g. Lovley 2013; Röhling 2015 etc.; Table S4, Supporting Information). Among those, Desulfobacteraceae were the most abundant group detected. They could be resolved into the genera *Desulfobacter* (SK: 0.3%–0.6%, BB: ≤2%), *Desulfococcus* (SK: 2%–5%, BB: ≤1%) and *Desulfobacterium* (SK: 0.4%–2%, BB: ≤0.6%). The family

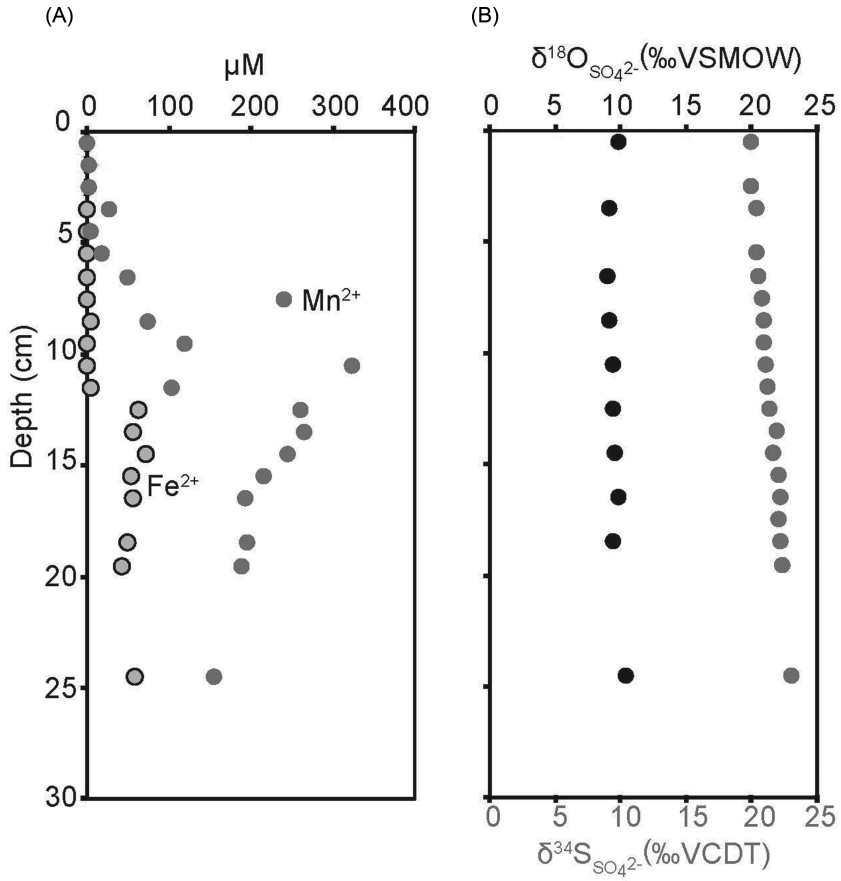

Figure 3. (**A**) Fe^{2+}- and Mn^{2+}-concentrations and (**B**) $\delta^{34}S$ and $\delta^{18}O$ of SO_4^{2-} in porewater samples collected from the SK Site MM766. This core is from the August 2009 RV-Merian cruise no. 12–4a.

Geobacteraceae could be further resolved into the *Geobacter* genus (SK: 0.006%). The Desulfobulbaceae, Desulfuromonadaceae and Pelobacteraceae could not be resolved beyond family level.

Besides these dissimilatory Fe-reducers, some bacteria known to transfer extra electrons generated during fermentation to Fe(III) (Lovley, Phillips and Lonergan 1991; Dobbin *et al.* 1996, 1999) were observed. These families were comprised of the Clostridiaceae (SK: ≤0.2%, BB: ≤0.5%), Bacillaceae (SK: ≤0.2%, BB: ≤0.1%–1%) and Rhodobacteraceae (SK and BB: ≤0.2%) and could be further resolved into the *Clostridium* (SK: ≤0.06%, BB: ≤0.18%), *Bacillus* (SK: ≤0.09%, BB: ≤0.4%) and *Rhodobacter* genera. *Rhodobacter* was only observed at extremely low abundance (<0.0005%) at 3–4 cm in BB. In particular, many species of *Bacillus* and *Clostridium* are capable of Fe- and Mn-reduction in pure culture (Table S4, Supporting Information and references therein).

Bacterial distribution with depth

To compare microbial distributions with geochemical profiles, the relative sequence abundances of the families and genera with members capable of Fe-reduction in pure culture were plotted versus depth for the SK (Fig. 7A–C; Fig. S2A, Supporting Information) and the BB sites (Fig. 7D–F; Fig. S2B, Supporting Information). At both sites, by far the most abundant bacterial family was *Desulfobacteraceae*. It showed a higher abundance at greater depths at both sites. *Desulfococcus*, *Desulfobacterium* and unclassified genera belonging to this family also showed a higher abundance with depth (Fig. 7A–F). In contrast, the genera *Desulfobacter* and *Geobacter* as well as the other families Desulfobulbaceae, Desulfuromonadaceae, and Pelobacteraceae showed highest abundance at the shallow depths and were less abundant at greater depths (Fig. 7A–F). Pelobacteraceae was not present at SK and *Geobacter* only at extremely low abundance (0.006%) at 16–23 cm. *Bacillus* and *Clostridium* remained constant with depth at SK (Fig. S2A, Supporting Information) and showed lower abundance at greater depth at BB (Fig. S2B, Supporting Information)

Potential Fe-oxidizing bacteria

Mariprofundaceae (≤0.1%) occurred in the SK sediments in zones of Fe-reduction at 8–12 cm and 16–23 cm depths. Gallionellaceae (≤0.3%) were only found in BB sediments above the zone of Fe-reduction at 3–4 cm.

DISCUSSION

Geochemical evidence for Fe-reduction at Site Geo 2a and At4

High Fe- and Mn-contents in the porewater (Fig. 2) and in the solid phase (Fig. 4) generally indicate Fe and Mn-reduction prevailing throughout the sampled intervals at both Site Geo 2a and At4. While it is not clear based on Fe^{2+}- and SO_4^{2-}-concentration profiles, how much Fe is abiotically reduced via sulphide-oxidation, several pieces of evidence support that dissimilatory Fe-reduction is indeed the predominant process, at

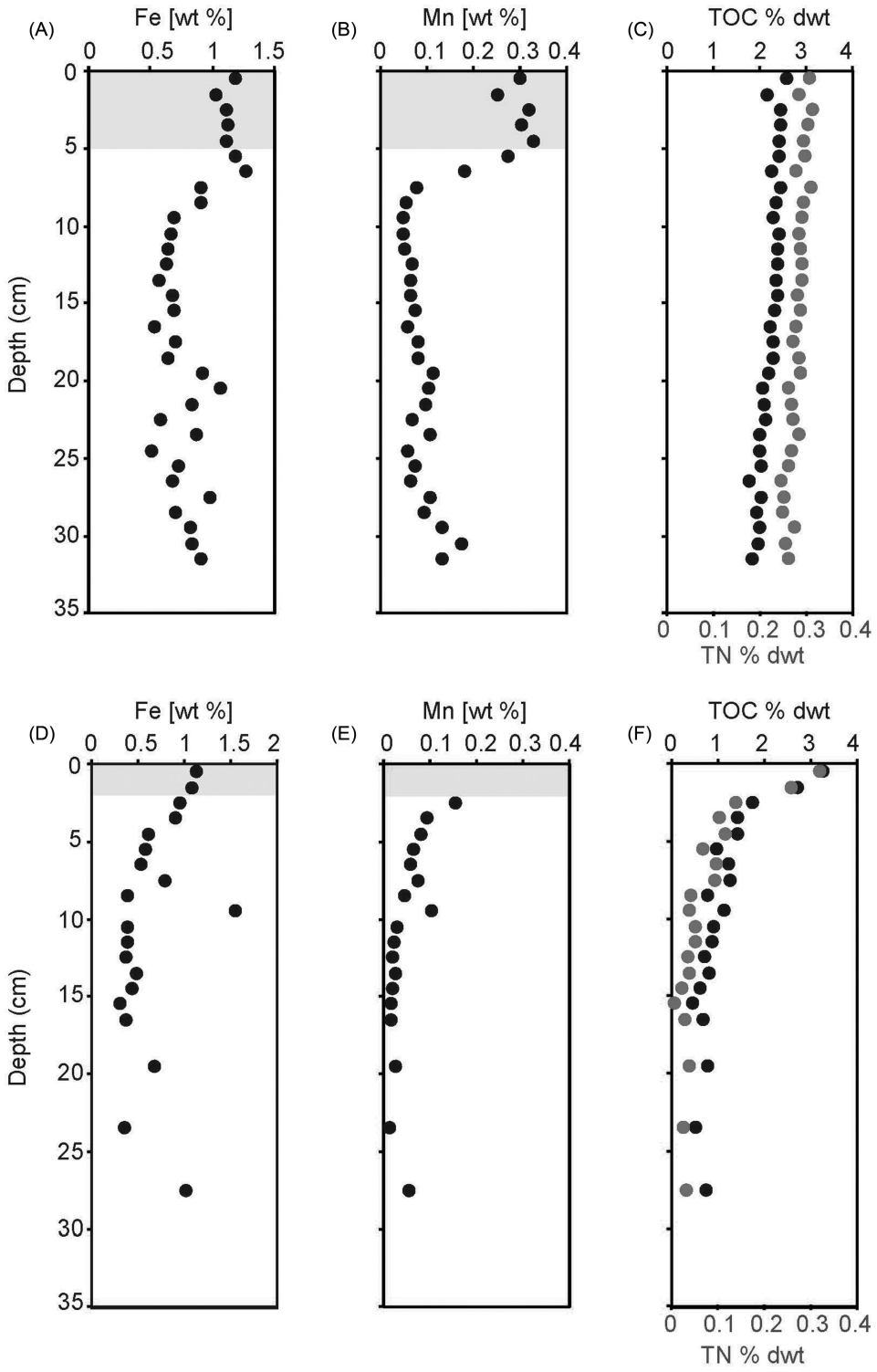

Figure 4. Depth profiles of solid-phase element concentrations (Fe, Mn, TOC and TN) from SK (panels A–C) and BB sites (panels D–F) reported in wt % of total dry sediment. Reactive Fe- and Mn-concentrations were determined from acid extractions. Shaded and unshaded areas indicate the zones of free NO_3^- and Fe^{2+} in the porewater, respectively. These cores are from the November 2011 Meteor cruise no. M86-1.

least in the shallower interval of the SK site. The porewater profile of dissolved Fe concentration indicates the strongest net production of Fe^{2+} in the depth range of the shallow sample interval (5–10 cm; Fig. 2A). As can be observed in the map (Fig. 1) the sampling Site Geo 2a can be laterally projected onto a transect analysed by Canfield, Thamdrup and Hansen (1993), showing decreasing carbon turnover rates from south to north. The geochemical zonation of Site Geo 2a falls between those of Site S6 and Site S9 of the Canfield, Thamdrup and Hansen (1993) study. Canfield showed that SO_4^{2-}-reduction rates were a magnitude lower than carbon turnover rates (derived from rates of NH_4^+ liberation), leaving Fe and Mn-reduction as the dominating

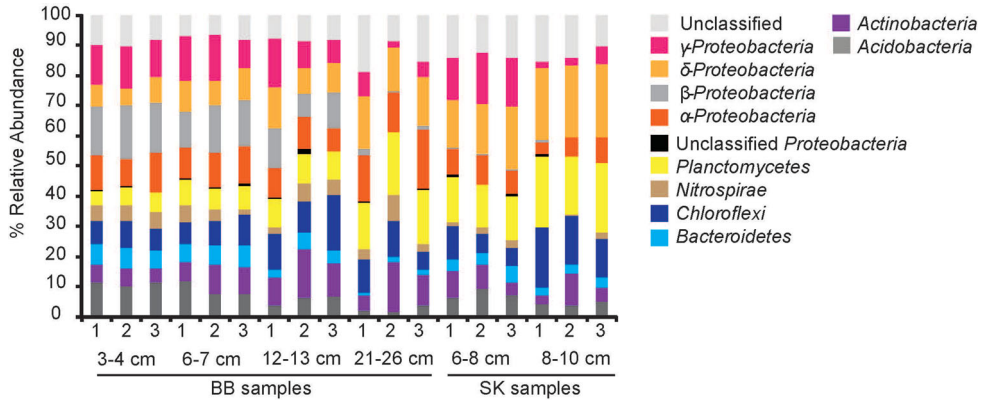

Figure 5. Bacterial diversity in SK and BB samples. Stacked bar graphs represent the relative abundance of major phyla in the different samples.

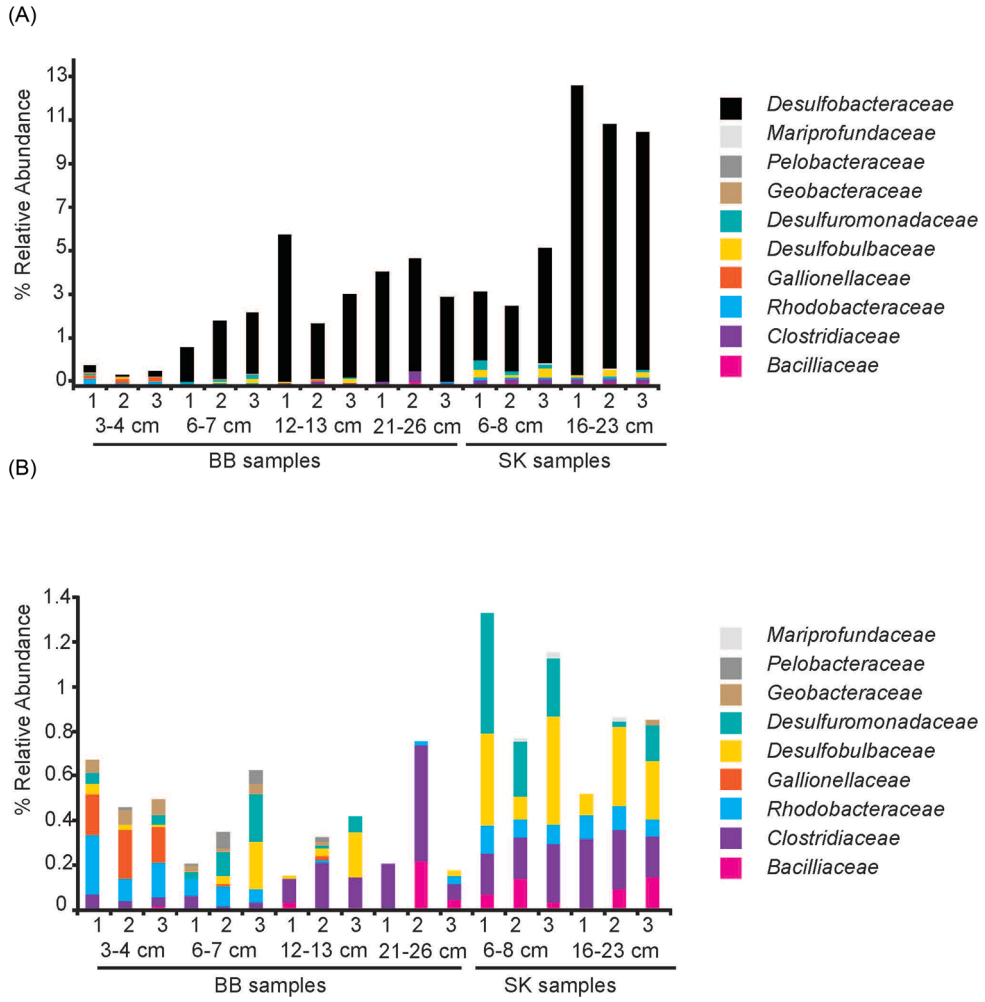

Figure 6. Bacterial diversity of families potentially linked to Fe-cycling in SK and BB samples. Stacked bar graphs represent the relative abundance of families in the different samples. (**A**) SK and BB samples, including Desulfobacteraceae and (**B**) excluding Desulfobacteraceae so that less abundant taxa are easily discerned.

terminal electron accepting pathways in the top centimetres of sediment from Site S6. This interpretation by the Canfield, Thamdrup and Hansen (1993) study is supported by a number of studies at diverse sites all over the SK and Kattegat (Rysgaard, Henrik and Jensen 2001; and Jensen *et al.* 2003) showing that rates of Fe-reduction were highest in sediments with high Fe(III) content. It is further supported by unaltered S and oxygen isotope values in SO_4^{2-} (Fig. 3B) measured in a core we analysed from an additional Site MSM 766. This is the same location as Site S9 of the Canfield, Thamdrup and Hansen (1993) transect (Fig. 1) where SO_4^{2-}-reduction was absent. Oxygen isotopes in SO_4^{2-} have been shown to be sensitive to S-cycling even if no changes in SO_4^{2-}-concentration or S-isotopes are observed (Van Stempvoort and Krouse 1994; Böttcher *et al.* 1998; Böttcher and

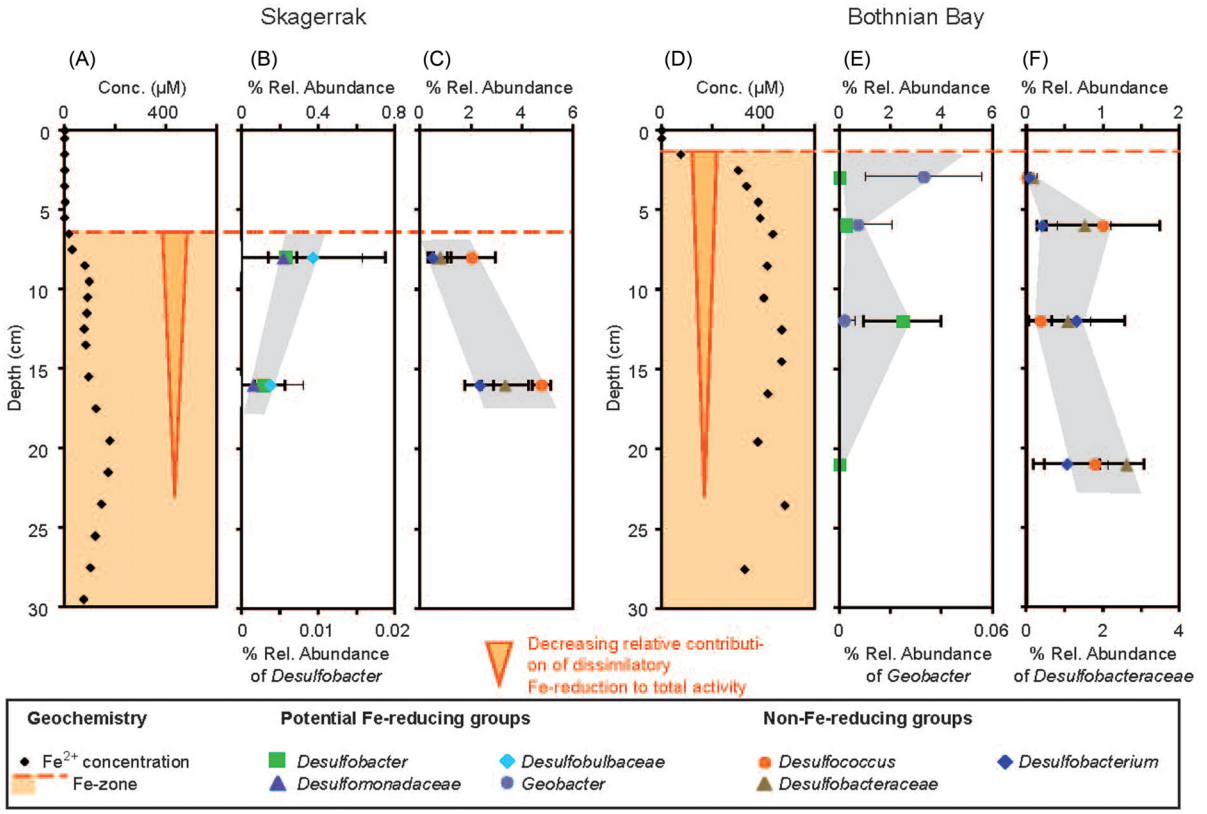

Figure 7. Relative sequence abundances of several bacterial genera and family plotted versus sediment depth in sediment cores taken from the SK and BB. (**A**) Fe^{2+}-concentrations in the porewater at SK site. Arrow indicates the downward decreasing relative contribution of dissimilatory Fe-reduction to total activity. (**B**) Relative sequence abundances of bacterial groups showing a decreasing trend with depth at Site SK. (**C**) Relative sequence abundances of bacterial groups showing an increasing trend with depth at Site SK. (**D**) Fe^{2+}-concentrations in the porewater at BB site. Arrow indicates the downward decreasing relative contribution of dissimilatory Fe-reduction to total activity. (**E**) Relative sequence abundances of bacterial groups showing a decreasing trend with depth at Site BB. (**F**) Relative sequence abundances of bacterial groups showing an increasing trend with depth at Site BB. Points indicate averages of triplicates analysed from three different aliquots of the same sediment core sample. Error bars indicate standard deviations. Genus taxonomy assignment was performed at a 94% similarity level. For the BB, the following samples were analysed: BB (3-4, 6–7, 12–13 and 21–26 cm) and for SK (8–10 and 16–23 cm). A missing value for a particular depth in a sample indicates that no sequences for this group were detected.

Thamdrup 2001; Böttcher, Thamdrup and Vennemann 2001; Brunner *et al.* 2005; Wortmann *et al.* 2007). The predominant Fe-reducing activity in the top 10 cm of Site Geo 2a is likely to be due to freshly buried Fe-oxides as the sediment becomes buried below the Fe-redox boundary (Fig. 4A). Hence, even if some microorganisms may perform SO_4^{2-}-reduction as well, some of the potential Fe-reducers observed in this zone must be responsible for the Fe-reduction.

The Fe-concentration profile at Site A4 suggests strong net Fe-reduction just below the Fe-oxidation front, with Fe^{2+} reaching up to 0.5 mM (Fig. 2C). Furthermore, several studies have reported a high abundance of reactive Fe-oxyhydroxides in BB sediments (HELCOM 2004; Pohl and Fernández-Otero 2012; J. Ingri, pers. comm.). Moreover, organic matter in the BB is mostly refractory and known to originate from peatland and forests (Vonk, van Dongen and Gustafsson 2008; 2010). Indeed, a high TOC (around 2 wt %; Fig. 3C) (showing high C/N ratios) suggests a more refractory organic matter pool in the Bothnian Bay. Together, these geochemical conditions would favour a microbial pathway for Fe-reduction at Site A4.

Bacteria known for Fe-reduction

Geobacteraceae are the only well-known model organisms commonly used for Fe-reduction laboratory studies that we could find in Baltic Sea sediments, but they occur at low abundance. Most of the observed Fe-reducing bacterial groups are versatiles. In particular, Desulfobacteraceae, Desulfobulbaceae and Desulfuromonadaceae are well-known SO_4^{2-}-reducers. Also two species belonging to the family Pelobacteraceae are known to reduce Fe and SO_4^{2-}. Thus, it is possible that these SO_4^{2-}-reducers also perform Fe-reduction in the SK and BB sediments. Fermentative bacteria and SRB, which have been shown to contribute to Fe-reduction (Lentini, Wankel and Hansel 2012), were also low in abundance. Considering that dissimilatory Fe-reduction is a major process in SK sediments and likely also at BB, we can only hypothesize at this point that some of the versatiles or perhaps a consortium of several different bacteria, including bacteria that have not yet been shown to reduce Fe (defined here as unknowns), could be contributing to Fe-reduction. A further explanation could be that known Fe-reducers are low in abundance but performing at high rates, perhaps by synergistically using multiple electron donating pathways.

Comparison with other sites of Fe-reduction

The Fe-reducing groups we observed in the upper layers of both settings, the normal marine SK and the brackish BB, were similarly found in other suboxic marine sediments (see compilation in Table S5, Supporting Information). For example,

Lin *et al.* (2007) found two genera of Geobacteraceae (0.01%), *Geothrix* and *Anaeromyxcobacter* (0.001%), and *Shewanella* (1%), in samples from two sites in the Scheldt Estuary (located in Belgium and the Netherlands) with on-going Fe-reduction in the top 15 cm depth. Sinkko *et al.* (2011) observed SRB capable of Fe-reduction (e.g. *Desulfobacter*, Desulfuromonadales and Desulfobulbaceae) in sediments from the top cm in the Finnish Archipelago and the open Baltic Sea. Fe-reduction has been shown to occur in the top centimetres at those sites in previous studies by Lukkari, Leivuori and Kotilainen (2009); Lukkari et al. (2009). Powell *et al.* (2003) found SRB (0.01%) (including some capable of Fe-reduction) and *Shewanella* (0.01%) in the top 10 cm in a heavy-metal enriched marine site located in a bay in the Antarctic. Edlund *et al.* (2008) observed that SRB (Desulfobulbaceae, Desulfuromonadales and Desulfobacteraceae), Myxococcales and Rhodobacterales within the top 2 cm at a coastal site near the Askö marine research station in the Baltic Sea. Myxococcales, were not present in our samples. Also *Arcobacter* (a member of the Campylobacteraceae family, part of the ε-Proteobacteria) present at Site S9 (cf. Table 2; Vandieken *et al.* 2012) was not recorded at Site Geo 2a. However, Site S9 differs from our site in that it shows mainly Mn-reduction, while no Fe- or SO_4^{2-}-reduction has been recorded by Canfield, Thamdrup and Hansen (1993). In general, all these sites show similar organisms as in our study.

We also compared our community to SO_4^{2-}-reduction zones and SMTZ, where Fe-reduction is clearly not microbially driven (Table S5, Supporting Information). While our detected communities show overlap with other sites of Fe-reduction, they show lower relative abundances and diversity of SRB compared to zones of SO_4^{2-}-reduction and lower abundances of *Clostridium* compared to the SMTZ of one site.

Comparison with various marine sediments in which Fe-reduction occurs confirms our finding of low abundances of known Fe-reducing bacteria. In order to find further indication as to whether these bacteria low in abundance are responsible for Fe-cycling, we compare their downcore distribution with the geochemical depth zonation.

Association of known Fe-reducing bacteria with zones of Fe-reduction

In the samples taken from the shallower intervals (above 10 cm) where dissimilatory Fe-reduction occurs, potential Fe-reducers *Desulfobacter*, *Geobacter*, Desulfobulbaceae, Desulfuromonadaceae and Pelobacteraceae were detected. They showed highest abundances at the shallow depths and were less abundant at greater depths (Fig. 7A–F; Fig. S2A and B, Supporting Information). In contrast, for the samples taken from the deeper interval (16–23 cm), a greater importance of SO_4^{2-}-reduction cannot be excluded. It is striking that *Desulfococcus, Desulfobacterium* and other SRB show a higher abundance in the deeper intervals. Members of the genus *Desulfococcus* are not capable of Fe-reduction. The rather constant Fe-concentrations indicate that the rate of Fe-reduction is much lower, while the net release of NH_4^+ to the porewater (as indicated by the porewater profile) suggests on-going organic carbon turnover (Fig. 2A). Under these conditions, SO_4^{2-}-reduction may become the dominating process, while Fe might be reduced via cryptic S-cycling if not hampered by the rapid precipitation of insoluble Fe-sulphides. A transition to increased SO_4^{2-}-reduction is plausible since the availability of reactive Fe becomes exhausted over time, while reactive organic matter, which stimulates heterotrophic activity, is still available (cf. total organic carbon, TOC, data; Fig. 3C). In this respect, it also makes sense that bacteria that couple Fe-reduction to fermentation, *Bacillus* and *Clostridium*, remained constant with depth at the SK site. Also α-diversities of bacteria are high at both depth intervals at the SK site (Fig. S2A, Supporting Information).

At Site At4 in the BB, the depth distribution of potential Fe-reducers shows a very similar pattern as at Site Geo 2a in the SK. A similar redox zonation has been measured at this site (Fig. 2C), although it is somewhat more condensed than at Site Geo 2a. Similar to Site Geo 2a, SO_4^{2-} remained constant throughout the measured interval at Site At4, although at a lower concentration (Fig. 2B and D). At present it has not been determined how much SO_4^{2-}-reduction contributes to carbon turnover, but it is striking that the same depth zonation of the Fe-reducing community composition occurs as at Site Geo 2a. Moreover, the occurrence of the genus *Geobacter* (to which many of the well-known model microorganisms for Fe-reduction belong to) in the upper interval is consistent with a predominantly Fe-reducing community. Bacteria that couple Fe-reduction to fermentation, *Bacillus* and *Clostridium*, show lower abundance at greater depth at Site At4 (Fig. S2B, Supporting Information). Lower activity in the lower part of the core is also reflected in decreasing α-diversity with depth (Fig. S2B, Supporting Information). An inability to efficiently use the refractory organic matter that prevails in the BB could also explain the lower abundance of certain microbial taxa and α-diversity at greater depth.

In general, the depth distribution of microbial communities needs to be interpreted with caution. It needs to be taken into account that living or dead microorganisms or extracellular DNA (cf. Corinaldesi, Beolchini and Dell'Anno 2008; Corinaldesi *et al.* 2011) can be preserved in the sediment and persist during burial, or can be homogenized by bioturbation. Also, it is well-known that Baltic Sea bottom water conditions vary over time, which may cause upwards and downwards migration of redox zones within the sediment column. Despite these effects that blur the stratification of microbial communities, the depth distributions we found at the two sites appear consistent with a redox stratification in which different microbial groups are responsible for Fe-reduction and SO_4^{2-}-reduction. The classical model organisms used for laboratory experiments were absent or only occurred at low relative abundance. Whether taxa with low abundances are also the least active remain to be determined. Perhaps, several of the versatiles, including the SO_4^{2-}-reducers with members capable of Fe-reduction or unknowns, could mainly be executing Fe-reduction, while other SRB could be responsible for SO_4^{2-}-reduction, each playing its role in a complex ecological network.

A possible explanation as to why the commonly cultured Fe-reducers do not thrive in these sediments could be that they have a metabolic disadvantage for organic substrate usage. Microbially degraded organic matter in marine sediments consists to a great part of saturated hydrocarbons (Tissot and Welte 1984). The high abundance of Desulfobacteraceae, Rhodospirillaceae and Hyphomicrobiaceae in these sediments, described as hydrocarbon-degrading bacteria (Beazley *et al.* 2012), may indicate that hydrocarbons could indeed impose a selective constraint. Baltic Sea and SK sediments also receive high inputs of polycyclic aromatic hydrocarbons (Magnusson *et al.* 1996; Biselli *et al.* 2004) that were shown to serve as substrates, e.g. for *Pseudomonas* (Edlund and Jansson 2006). Perhaps, a less efficient use of these or other types of organic substrates could limit the growth of commonly cultured Fe-reducers.

Fe-oxidizing bacteria

In addition to Fe-reducers, the proteobacterial families Mariprofundaceae and Gallionellaceae that have been linked to Fe-oxidation were observed at Site Geo 2a and At4, respectively. A study by Scott *et al.* (2015) reports that Zetaproteobacteria, including the Mariprofundaceae, to be generally restricted to marine aquatic habitats where Fe is readily available for oxidation. Gallionellaceae are known to oxidize Fe in freshwater or brackish environments (Hallbeck and Pedersen 1991; Emerson and Moyer 1997; McBeth, Fleming and Emerson 2013). Hence, its occurrence at Site At4 matches its environmental preference and contrasts with Mariprofundaceae in the open marine setting. Both Gallionellaceae and Mariprofundaceae are microaerophilic and perform aerobic Fe-oxidation at the oxic–anoxic interface. Both families co-occurred with an enrichment in solid-phase Fe and Mn at ~5 cm (Fig. 4A, SK) and at ~9.5 cm (Fig. 4B, BB), which is indicative of Fe- and Mn-oxidation. However, the presence of dissolved Mn^{2+} shows that conditions are free of oxygen at the NO_3^-/Fe^{2+} front at the time of sampling (Fig. 2A and C). That is to say aerobic Fe-oxidation is not occurring at present, while Fe-oxidation more likely occurs via an anaerobic pathway. Nitrate reduction coupled to Fe-oxidation may occur abiotically through catalytic reactions at Fe-oxide surfaces (Sørensen and Thorling 1991; Ottley, Davison and Edmunds 1997; Picardal 2012) or may be mediated by bacteria (Straub and Buchholz-Cleven 1998; Benz, Brune and Schink 1998; Weber, Achenbach and Coates 2006), but these bacteria were not identified at the studied sites. A likely explanation for the solid-phase Fe/Mn peaks would be that oxygen was introduced into the sediment in the past, possibly by episodic phases of bioturbation (Canfield, Thamdrup and Hansen 1993). Thus, microaerophilic conditions could also have led to biotic Fe/Mn-oxidation at some time in the past. Taking this temporal aspect into account, Mariprofundaceae and Gallionellaceae very well match their geochemical environments with episodically microaerobic, ferrous and marine or brackish conditions, respectively, implying that they do contribute to Fe-oxidation at these sites.

CONCLUSIONS

Pyrosequencing of marine suboxic sediments from the SK and the BB revealed several bacterial groups of which members are capable of reducing Fe. The overall microbial community compositions at both sites were comparable to other marine sites, in particular, sites showing Fe-reduction. The detected families Desulfobulbaceae, Desulfuromonadaceae, Geobacteraceae (*Geobacter*), Pelobacteraceae and the genus *Desulfobacter* showed the highest abundance near the surface, where fresh Fe-oxides are buried into the zone of Fe-reduction. Apparently, not the well-known model organisms, commonly used for laboratory experiments, are dominating in this zone. Instead, versatiles capable of using both Fe and SO_4^{2-} as electron acceptors were abundant. At greater depth (below 15 cm), where SO_4^{2-}-reduction is most likely a dominating process, different organisms, mainly the genus *Desulfococcus* belonging to the family Desulfobacteraceae, were more abundant. Furthermore, bacterial groups capable of coupling fermentation to Fe-reduction showed variable abundances at greater depth. The Fe-oxidizing bacteria Mariprofundaceae and Gallionellaceae were present, consistent with their marine vs. brackish preference, in the upper parts of the sampled intervals. They most likely resided near the oxic–anoxic boundary but became buried over time.

A clear downward change in community structure in ferruginous, suboxic sediment at a normal marine and a brackish site may be partially due to the distribution of reactive Fe but also organic substrate availability and a complex ecological network. Our study demonstrates that a far greater effort has to be made to understand phylogenetic and functional microbial community composition in marine sediments, one of the largest microbial habitats on Earth.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

ACKNOWLEDGEMENTS

We thank Helge W. Arz (IOW) and cruise members of Meteor Cruise M86-1A for providing the sediment cores for this study. Gerhard Kuhn (Alfred-Wegener-Institut für Polar- und Meeresforschung, Bremerhaven), Tetsuro Miyatake, Ajinkya Kulkarni and Saar Sztejrenszus helped with subsampling of the cores. Special thanks also go to F. Pollehne for organizing sediment sampling on board Merian (12-4a) in 2009 and to P. Escher for mass spectrometry support.

FUNDING

This work was supported by the National Science Foundation (NSF), International Research Fellowship Program (IRFP, project no. 1064521 to CR), the Fulbright fellowship programme (fellowship to CR.), the Hanse-Wissenschaftskolleg, Delmenhorst (HWK; fellowship to CR), the Danish National Research Foundation (grant DNRF53), the Deutsche Forschungsgeminschaft (project DA1207/1-1 to KD), the Leibniz Institute for Baltic Sea Research (IOW) and the University of Bremen.

Conflict of interest. None declared.

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