Geomicrobiology Journal

ISSN: 0149-0451 (Print) 1521-0529 (Online) Journal homepage: http://www.tandfonline.com/loi/ugmb20

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To cite this article: Carolina Reyes, Dominik Schneider, Andrea Thürmer, Ajinkya Kulkarni, Marko Lipka, Saar Y. Sztejrenszus, Michael E. Böttcher, Rolf Daniel & Michael W. Friedrich (2017) Potentially Active Iron, Sulfur, and Sulfate Reducing Bacteria in Skagerrak and Bothnian Bay Sediments, Geomicrobiology Journal, 34:10, 840-850, DOI: 10.1080/01490451.2017.1281360

To link to this article: https://doi.org/10.1080/01490451.2017.1281360

GEOMICROBIOLOGY JOURNAL
2017, VOL. 34, NO. 10, 840–850
https://doi.org/10.1080/01490451.2017.1281360

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Potentially Active Iron, Sulfur, and Sulfate Reducing Bacteria in Skagerrak and Bothnian Bay Sediments

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ABSTRACT

In many marine surface sediments, the reduction of manganese (Mn) and iron (Fe) oxides is obscured by sulfate reduction, which is regarded as the predominant anaerobic microbial respiration process. However, many dissimilatory sulfate and sulfur reducing microorganisms are known to utilize alternative electron acceptors such as metal oxides. In this study, we tested whether sulfate and sulfur reducing bacteria are linked to metal oxide reduction based on biogeochemical modeling of porewater concentration profiles of Mn^{2+} and Fe^{2+} in Bothnian Bay (BB) and Skagerrak (SK) sediments. Steady-state modeling of Fe^{2+} and Mn^{2+} porewater profiles revealed zones of net Fe (0–9 cm BB; ~10 and 20 cm SK) and Mn (0–5 cm BB; 2–8 cm SK) species transformations. 16S rRNA pyrosequencing analysis of the *in-situ* community showed that *Desulfobacteraceae, Desulfuromonadaceae* and *Desulfobulbaceae* were present in the zone of Fe-reduction of both sediments. *Rhodobacteraceae* were also detected at high relative abundance in both sediments. BB sediments appeared to harbor a greater diversity of potential Fe-reducers compared to SK. Additionally, when the upper 10 cm of sediment from the SK was incubated with lepidocrocite and acetate, *Desulfuromonas* was the dominant bacteria. Real-time quantitative polymerase chain reaction (qPCR) results showed decreasing *dsrA* gene copy numbers with depth coincided with decreased Fe-reduction activity. Our results support the idea that sulfur and sulfate reducing bacteria contribute to Fe-reduction in the upper centimeters of both sediments.

ARTICLE HISTORY
Received 25 September 2016
Accepted 9 January 2017

KEYWORDS
16S rRNA; iron reduction; manganese reduction; marine sediments; qPCR; pyrosequencing

Introduction

Identifying the *in-situ* microbial community members responsible for Fe-reduction in marine sediments using molecular techniques is a current problem in the field of environmental microbiology. Fe-reducing microorganisms are difficult to track *in-situ* because they belong to different phylogenetic lineages (Lonergan et al. 1996; Lovley 1993) and they lack a unique molecular marker specific for Fe-reduction. Early investigations identified some microorganisms capable of reducing Fe oxides in pure culture studies (de Castro and Ehrlich 1970; Brock and Gustafson 1976; Lovley and Phillips 1988; Myers and Nealson 1988) including sulfur and sulfate reducing bacteria (Lovley et al. 1993; Roden et al. 1993). Subsequent microbial diversity studies using primers targeting the 16S ribosomal RNA (rRNA) gene demonstrated that members of the gamma and delta-*Proteobacteria*, including known isolates, were often detected in a variety of Fe-rich environments including sediments (DiChristina and DeLong 1993; Coates et al. 1996). *Geobacter* species, in particular, were found to make up the majority of known Fe-reducers in many freshwater sediment environments (Coates et al. 1996).

Moreover, some enrichment-based studies have tried to identify microbial communities responsible for Fe-reduction in different sedimentary environments including: aquifer (Rooney-Varga et al. 1999), mining-impacted lake (Cummings et al. 2000), freshwater pond (Lentini et al. 2012), tidal flat (Kim et al. 2014) and marine sediments (Coleman et al. 1993; Vandieken and Thamdrup 2012; Hori et al. 2015). Microorganisms identified in these enrichment studies with the potential to reduce Fe included: *Geobacter, Pelobacter* related isolates (Cummings et al. 2000; Hori et al. 2015), species belonging to the taxa *Desulfuromonadales* (Vandieken and Thamdrup 2012), *Desulfuromonas* (Kim et al. 2014) and those that are closely linked to *Geobacter, Enterobacter* and *Desulfovibrio* species (Lentini et al. 2012). However, a caveat with enrichment studies is that the enriched microbial community does not necessarily represent the *in-situ* community.

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Besides a microbial fuel cell study using marine sediments (Coleman et al. 1993), lipid biomarker and metagenome studies have also identified sulfate reducing bacteria (SRB) such as *Desulfobulbus, Desulfobacter, Desulfovibrio*, sulfur reducing *Desulfuromusa* spp. and *Desulfuromonas* with the potential to reduce Fe oxides (Holmes et al. 2004a,b; Kim et al. 2014). In contrast to the studies above, the goal of our study was to pinpoint active *in-situ* microbial community members potentially responsible for Fe-reduction in marine suboxic sediments. As with DNA-based studies, limitations in RNA-based studies also exist. One limitation is that we cannot directly relate 16S rRNA abundances to activity levels of individual taxa. However, the presence of 16S rRNA belonging to different taxa can tell us the metabolic potential for current, past and future microbial activity (Blazewicz et al. 2013).

Sites from the Baltic Sea and North Sea known for high Fe-reduction activity (Rajendran et al. 1992; Canfield et al. 1993; Jensen et al. 2003) and shown to potentially harbor Fe-reducing microorganisms (Reyes et al. 2016) were selected (Figure 1). Porewater concentrations of sediments showed ongoing Fe and Mn-reduction and Fe^{2+} and Mn^{2+} release providing the opportunity to relate these pathways to biogeochemical zones in the surface sediments. Bacterial 16S rRNA genes were transcribed to complementary DNA (cDNA), pyrosequenced and also quantified by real-time quantitative PCR (qPCR) to determine potentially active bacterial communities in sediments. We hypothesized that SRB capable of reducing Fe would be among the active microbial populations in the Fe-reduction zones.

Methods

Sample collection and storage

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 (Oktopus GmbH, Kiel, Germany). The two sampling locations considered in this study were Bothnian Bay (BB) Site At4 (65°26,71′N/23°17,92′E) and Skagerrak (SK) Site Geo 2a (58°29,513′N/9°35,855′E) (Figure 1). Samples were taken from a water depth of 75 m at Site At4 and from a water depth of 554 m at Site Geo 2a. 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 land-based laboratory. Sediments to be used for incubations were collected in parallel as described above. These cores were stored at 4°C following collection and were processed following transfer to the laboratory.

Porewater analysis

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 et al. 2005). Porewater samples for Fe^{2+} and Mn^{2+} were acidified with 1% (v/v) nitric acid (65% suprapure, Merck, München, Germany). Porewater concentrations of Fe^{2+} and Mn^{2+} were measured by inductively coupled plasma-optical emission spectroscopy (ICP-OES) (Thermo, iCAP 6300 Duo, Thermo Fisher Scientific GmbH, Dreieich, Germany). Due to Site Geo2a samples having a higher salinity, these were diluted 6-fold prior to measuring them. Samples for Site At4 were not as saline and were left undiluted. Analytical accuracy and precision were determined using a 6-fold dilution of the international CASS-5 standard (Nearshore seawater reference material for trace metals; National Research Council of Canada, Ontario, Canada). They were found to be better than +/−6.7% and +/−8.2%, respectively. CASS-5 solutions were spiked with Fe^{2+} and Mn^{2+} because the porewater concentrations were much higher than that in the original reference material (Reyes et al. 2016). Additional porewater

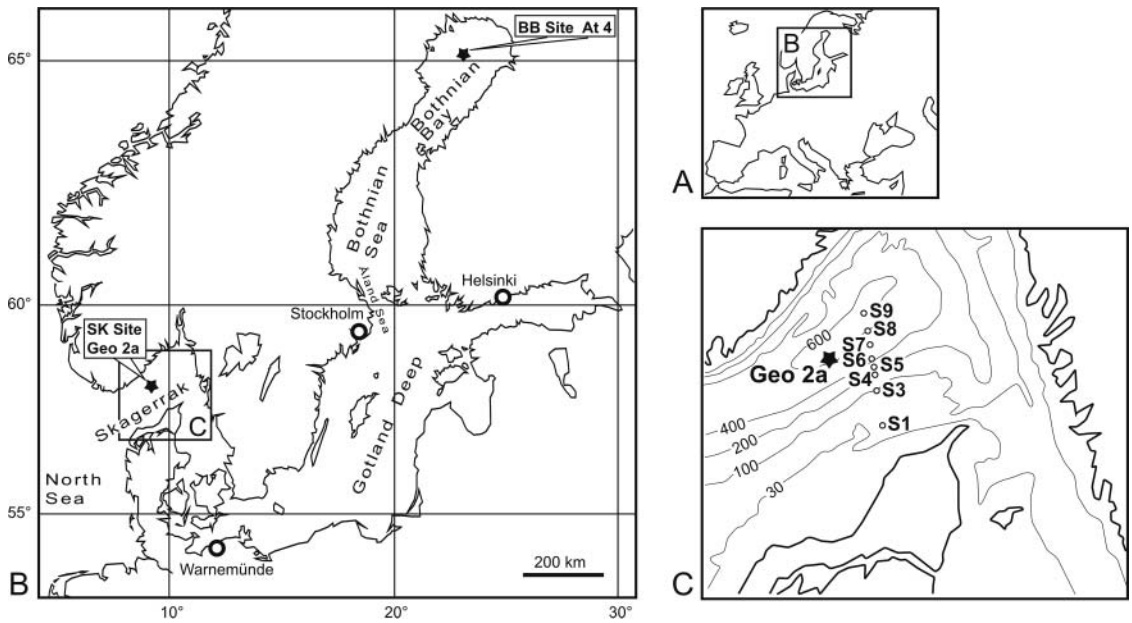

Figure 1. Map of the North Sea and Baltic Sea, modified after Reyes et al. (2016). Panel B shows the locations of the two sampling Sites Geo2a and At4. Panel C is from the Skagerrak showing the locations of Site Geo2a with respect to the locations of Sites S1 through S9 of the transect studied by Canfield et al. (1993).

measurements including nitrate, ammonium, sulfide and sulfate are reported in Reyes et al. (2016) and its supplementary material.

Porewater modeling

Interpretation of interstitial water profiles of dissolved Fe^{2+} and Mn^{2+} at Site Geo 2a and Site At4 were carried out by means of the modeling software packages PROFILE (Berg et al. 1998) and REC (Rate Estimation from Concentrations model; Lettmann et al. 2012) considering steady-state conditions. Local or non-local irrigation was neglected in the interpretation of porewater profiles. The diffusion coefficients in free solution at *in-situ* salinity and temperature were calculated according to Boudreau (1997) and Shulz and Zabel (2006). Molecular diffusivity in the sediment was corrected for tortuosity according to Iversen and Jørgensen (1993), considering sediment porosities. Both models calculate net instead of gross process rates.

RNA extraction and cDNA preparation

Frozen cores were sliced into 1 cm or 2 cm diameter discs with 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 disc were sectioned into aliquots for RNA extraction. Extractions were made from ~4–5 g of frozen BB sediments (2–3, 3–4, 6–7 cm) and ~9 g of frozen SK sediments (4–6, 6–8 cm) based on the method by Lueders et al. (2004) with the following modifications: pH 4–5 phenol:chloroform:isoamyl alcohol [PCI, 25:24:1 (vol:vol:vol)] (Carl Roth, Karlsruhe, Germany) and chloroform:isoamyl alcohol [CI, 24:1 (vol:vol)] (Carl Roth, Karlsruhe, Germany) were used to facilitate RNA recovery. Cells were lysed by bead-beating at 6.5 m/sec for 45 sec using a Fast Prep-24 instrument (MP Biomedicals, Irvine, USA). During the PCI and CI extraction steps, tubes were centrifuged at 17,949 × *g* for 1 min at 4°C. Between PCI and CI steps, before centrifugation, tubes were gently inverted. Finally, nucleic acids were precipitated from the aqueous phase by adding 2 volumes of 40% polyethylene glycol at 4°C for 1 h 45 min up to 2 h 30 min, followed by centrifugation at 12,000 × *g* at 4°C for 30 min. Pellets were washed with 2 ml of 4°C 70% ethanol, air dried for 5 min and resuspended in 200 μl of RNase-free water.

Total nucleic acids were checked for integrity using a 1% low melting agarose gel. Potential inhibitors such as humic acids were removed using the OneStep PCR Inhibitor Removal Kit (Zymo Research, Freiburg, Germany). RNA was obtained from the purified extracts by digesting samples with RQ1 RNase-free DNase (Promega, Mannheim, Germany) following the manufacturer's instructions. DNase was removed using the RNeasy Micro Kit (Qiagen, Hilden, Germany) following the RNA cleanup and concentration protocol. Total RNA was eluted with 20 μl of RNase-free water.

Complete removal of DNA from RNA was assessed by performing 16S rRNA PCR with 1 μl of isolated RNA as follows: 0.5 μM of primers 8F [AGAGTTTGATCCTGGCTCAG] and 1492r [CGGTTACCTTGTTACGACTT] (Eden et al. 1991), 200 μM dNTP mix, 1 mM $MgCl_2$, 0.025U Ampli-Taq DNA polymerase (Thermo Fisher Scientific, Darmstadt, Germany) and 1X Ampli-Taq buffer in a 50 μl reaction volume under the following conditions: 94°C for 3 min followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 2 min, and a final elongation step at 72°C for 7 min.

Purified RNA was checked for integrity using the Agilent 2100 Bioanalyzer, RNA 6000 Pico Chip and Prokaryotic Total RNA Pico Series II program. Total RNA concentration was measured using the NanoDrop Spectrophotometer ND-1000 (PeqLab VWR International GmbH, Erlangen, Germany) and Quant-iT Ribogreen RNA Assay Kit (Thermo Fisher Scientific) following the manufacturer's instructions. Fluorescence measurements were made using a Flourometer (Fluoroskan Ascent FC, Thermo Labsystems, Milford, USA). cDNA was synthesized using the High Capacity Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany) following the manufacturer's instructions.

16S rRNA pyrosequencing

For pyrosequencing by the sequencing center (Mr. DNA Molecular Research LP, Shallowater, TX, USA) 16S rRNA genes were PCR amplified from cDNA using primers 27fmod [AGRGTTTGATCMTGGCTCAG] (Eden et al. 1991) and 519modbio/Gray519R [GTNTTACNGCGGCKGCTG] (Frank et al. 2013) targeting the V1–V3 regions. Samples were prepared as described in Dowd et al. (2008). Briefly, cDNA samples were diluted to 100 ng/μl and 1 μl was used in a 50 μl PCR reaction. 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 sec, 53°C for 40 sec 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 using Roche 454 FLX titanium instruments and reagents following manufacturer's guidelines. Additionally, selected frozen samples from each site were sequenced using an alternative method targeting the V3–V5 region of 16S rRNAs to determine if additional taxa could be detected with this primer set (Table S1). We selected the above primer sets because previous studies have shown that using these primers can provide higher accuracy and classification of sequences compared to other primer sets (Kim et al. 2011; Vilo and Dong 2012).

Pyrosequencing data analysis

The QIIME software package, version 1.8.0 (Caporaso et al. 2010) was employed to analyze 16S rRNA sequences as described in Schneider et al. (2015) with the following exception: the Greengenes 13_5 database (McDonald et al. 2012) was used for operational taxonomic unit (OTU) determination and taxonomic classification. Briefly, sequences were filtered out if they were <250 bp, had an average quality score <25, contained unresolved nucleotides, had longer mismatches than 3 bp in the forward primer and possessed homopolymers

longer than 8 bp. Forward and reverse primer sequences were also removed. The program Acacia (Bragg et al. 2012) was used to remove pyrosequencing noise. Chimeric sequences were removed using UCHIME (Edgar et al. 2011). OTU determination was performed at 97% similarity with the script *pick_open_reference_otus.py* using the SILVA NR SSU 123 database as referenced in Quast et al. (2013). Alpha diversity indices including Chao1, Shannon and Simpson were calculated using the QIIME program and the script *alpha_diversity.py*. Alpha diversity rarefactions were analyzed using the sample with the lowest number of sequences. Rarefactions were generated with observations from randomized OTU draws (no replacement) using 10 iterations.

Comparison of 16S rRNA abundances

The 16S rRNA relative abundances, based on cDNA pyrosequencing, of select taxa from samples BB34, SK68 and SK810 were compared to previously published 16S rRNA relative abundances based on DNA pyrosequencing (Reyes et al. 2016). Both types of sequencing data were available only for the above samples. When comparing the two relative abundances we refer to those from this study as 16S rRNA and those from the previous study as 16S rDNA.

Incubation experiments

For the sediment incubations ~ 900 g of the top 10 cm of SK sediment was mixed with 300 ml of sterile anoxic artificial sea water (ASW) medium (devoid of sulfate) in a glass jar. ASW was buffered with bicarbonate (30 mM) and contained per liter of deionized water: 26.4 g NaCl, 11.2 g $MgCl_2{\cdot}6H_2O$, 1.5 g $CaCl_2{\cdot}2H_2O$, 0.7 g KCl and 0.023 g $MgSO_4{\cdot}7H_2O$. The medium was further supplemented with vitamins and minerals, which were prepared as described in Sztejrenszus et al. (2016). The slurry solution was continuously stirred and the headspace of the jar was sparged with N_2 for 15 min before transferring the jar into an Aldrich AtmosBag (Sigma–Aldrich, Munich, Germany) filled with N_2. Following transfer, while continuously stirring the slurry, 10 ml of slurry was transferred in triplicate to serum bottles. Slurries were amended with 500 μl of 200 mM lepidocrocite and 20 μl of 1M acetate. The serum bottles were sealed with rubber stoppers, crimped and incubated at 11°C in the dark. The sediment slurries were sub-cultured after 65 and 72 days of incubation by transferring 20% of the slurry to new serum bottles under N_2 gas. Samples that were sub-cultured at 65 days are referred to as SKEN65 and those sub-cultured at 72 days as SKEN72. Triplicate biological replicates were prepared for both time points. For sub-culturing 2 ml of slurry was mixed with 8 ml of modified ASW medium, acetate and lepidocrocite as described above. RNA extraction, cDNA preparation and sequencing were performed on sub-cultured samples as described above for frozen sediments. Soluble Fe was measured before and after sub-culturing by taking liquid samples anoxically using an N_2 flushed syringe, filtered through a 0.2 μm regenerated cellulose filter (Sartorius, Göttingen Germany) and transferred into 0.5 M HCl (trace metal grade, HN53.1, Carl Roth, Karlsruhe, Germany) (Sztejrenszus et al. 2016). Fe^{2+} was measured following the ferrozine assay (Stookey 1970; Hegler et al. 2008) with modifications (50% w:v ammonium acetate, 0.1% w:v ferrozine).

Synthesis of lepidocrocite was modified after Schwertmann and Cornell (2000). Lepidocrocite was prepared by adding 35.79 g of $FeCl_2{\cdot}4H_2O$ to a plastic beaker containing 900 ml of membraPure water. The pH of the solution was brought up to 6.7 while sparging vigorously with air and continuously stirring the solution with a magnetic stir bar at high speed. The pH was slowly brought up to 7.3–7.4 and was allowed to equilibrate. The pH was adjusted to 7.4 until constant. The minerology of the synthesized Fe oxide was analyzed by a Philips XPERT Pro X-ray diffractometer at the University of Bremen, Germany, using a CuKα radiation, and the samples were scanned from 3 to 100° (2θ). The identity of the Fe oxide was determined from integrated peak areas.

Quantitative PCR (qPCR)

Absolute quantification of bacterial 16S rRNA gene copy numbers (from cDNA) present in sediment samples was determined using the MESA BLUE qPCR kit for SYBR Assay (Eurogentec, Seraing, Belgium) and the Step One Plus (Applied Biosystems, Carlsbad, USA) qPCR thermocycler. Standard template was prepared by amplifying 16S rRNA genes from DNA extracts of *Escherichia coli* strain SB1 using bacterial primers 8F and 1492R (Turner et al. 1999). DNA standard templates were purified using the MiniElute PCR purification kit (Qiagen, Hilden, Germany) and their concentrations were determined using the Quanti-iT PicoGreen dsDNA Assay Kit (Invitrogen-Thermo Fisher Scientific, Steinheim, Germany). Standard curves were prepared using 10-fold serial dilutions of the standard templates (10 pg/μl to 0.1 fg/μl). Standard and sample templates were amplified using primers 338f and 518r (Muyzer et al. 1993). The qPCR efficiency was calculated using the slope of the standard curve with the following formula: Efficiency = $10^{(-1/\text{slope})} - 1$. Samples that were analyzed included: BB 2–3 cm, 3–4 cm and 6–7 cm depths (BB23, BB34 and BB67, respectively) and SK 4–6 cm and 6–8 cm depths (SK46 and SK68, respectively). Each 20 μl reaction mixture consisted of 10 μl of MESA Blue qPCR Master Mix, 2 μl of forward and reverse primers at a final concentration of 100 pM, 0.2 μl of 20 mg/ml bovine serum albumin (Roche, Mannheim, Germany), 3.8 μl nuclease free water and 2 μl of cDNA template. The following PCR protocol was used: 95°C for 5 min followed by 40 cycles of 95°C for 15 sec, 61°C for 20 sec and 72°C for 40 sec; melting curve stage: 95°C for 15 sec, 60°C for 60 sec followed by a ± 0.5°C increment up to 95°C. Gene copy number per gram of dry sediment was calculated the following way:

$$\frac{T \times C}{D} = FC$$

$$C = \frac{[qPCR\ quantity\ (g) \div 1.66054 \times 10^{-24}\ (g)(1\ Da)]}{914878\ Da}$$

$$\frac{FC}{DW} = copy\ number\ per\ gram\ sediment$$

T = total amount of initial RNA (ng)
C = number of copies

D = amount of cDNA used (ng)
FC = final gene copy number
DW = dry weight of sediment (g)

We also quantitated the abundance of the *dsrA* gene (from DNA) in samples to determine potential gene abundances of SRB. These methods are reported in the supporting material. Primers used for quantifying *dsrA* gene abundances are listed in Table S2.

Sequence accession numbers

Standard flowgram format (SFF) files were deposited in the DNA Data Bank of Japan (DDBJ) under the following sample names and accession numbers: BB23 (SAM00052072), BB34 (SAM00052071), BB67 (SAM00052070), SK46 (SAM00052073), SK68 (SAM00052074), SKEN65-1 (SAM00052075), SKEN65-2 (SAM00052076), SKEN65-3 (SAM00052077), SKEN72-1 (SAM00052078), SKEN72-2 (SAM00052079) and SKEN72-3 (SAM00052080).

Results

Geochemical model

The vertical profiles of Fe^{2+} and Mn^{2+} observed at BB (Figure 2A–D) and SK (Figure 2E and H) indicate different zones of net release or consumption. They followed the expected porewater redox zonation for sediments (e.g., Froelich et al. (1979)). At the BB site, Fe^{2+} occurred immediately below the surface to ~5 cm (Figure 2A). Below 5 cm, minor increases and decreases in Fe^{2+} were recorded (Figure 2A). Calculated rates of Fe^{2+} release (according to REC) show that the zone of maximum production is located at ~4 cm and minor peaks at ~15 cm and ~25 cm (Figure 2B). Slight consumption of Fe^{2+} occurred at ~10 and 19 cm depths (according to REC) (Figure 2B). Iron is liberated in the top ~9 cm according to both the models. Mn^{2+} also occurred directly below the surface and increased down to 5 cm (Figure 2C). Both models show the top 5 cm to be the zone of maximum release of Mn (Figure 2D).

At the SK site, Fe^{2+} occurred below 5 cm depths (Figure 2E). Modeled rates suggest that two zones of Fe^{2+} liberation were present at ~10 cm and ~20 cm (Figure 2F). Consumption of Fe^{2+} ensued closely below the surface down to ~8 cm and below ~12 cm down to 17 cm depths (Figure 2F). Dissolved Mn concentrations increased with depth below ~2 cm (Figure 2G) showing a concentration maximum at ~7 cm. Model results suggest that a zone of Mn liberation occurred between 5 and 8 cm (Figure 2H).

16S rRNA abundances of potential Fe-reducing groups present in in-situ

Several microbial groups that have members capable of Fe-reduction were detected in BB and SK samples by pyrosequencing analysis of 16S rRNA. Sample sequence information is reported in Tables 1 and S3. The groups reported here could not be resolved into known genera with our method. Bacteria belonging to the

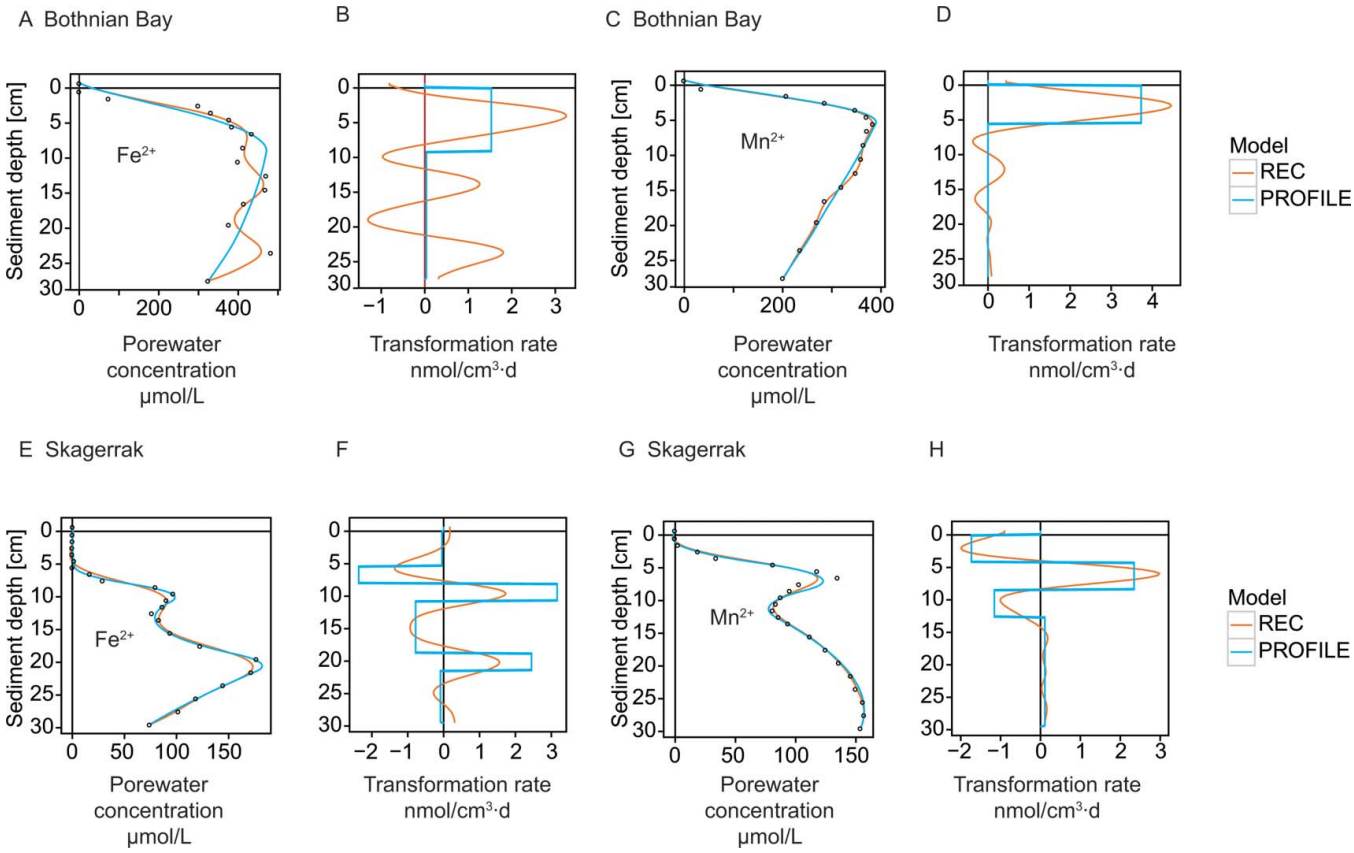

Figure 2. Model based on porewater concentrations showing predicted rates of Fe^{2+} and Mn^{2+} release and consumption in sediments. Porewater concentrations and predicted rates of Fe^{2+} and Mn^{2+} release and consumption in BB samples (A–D) and in SK samples (E–H).

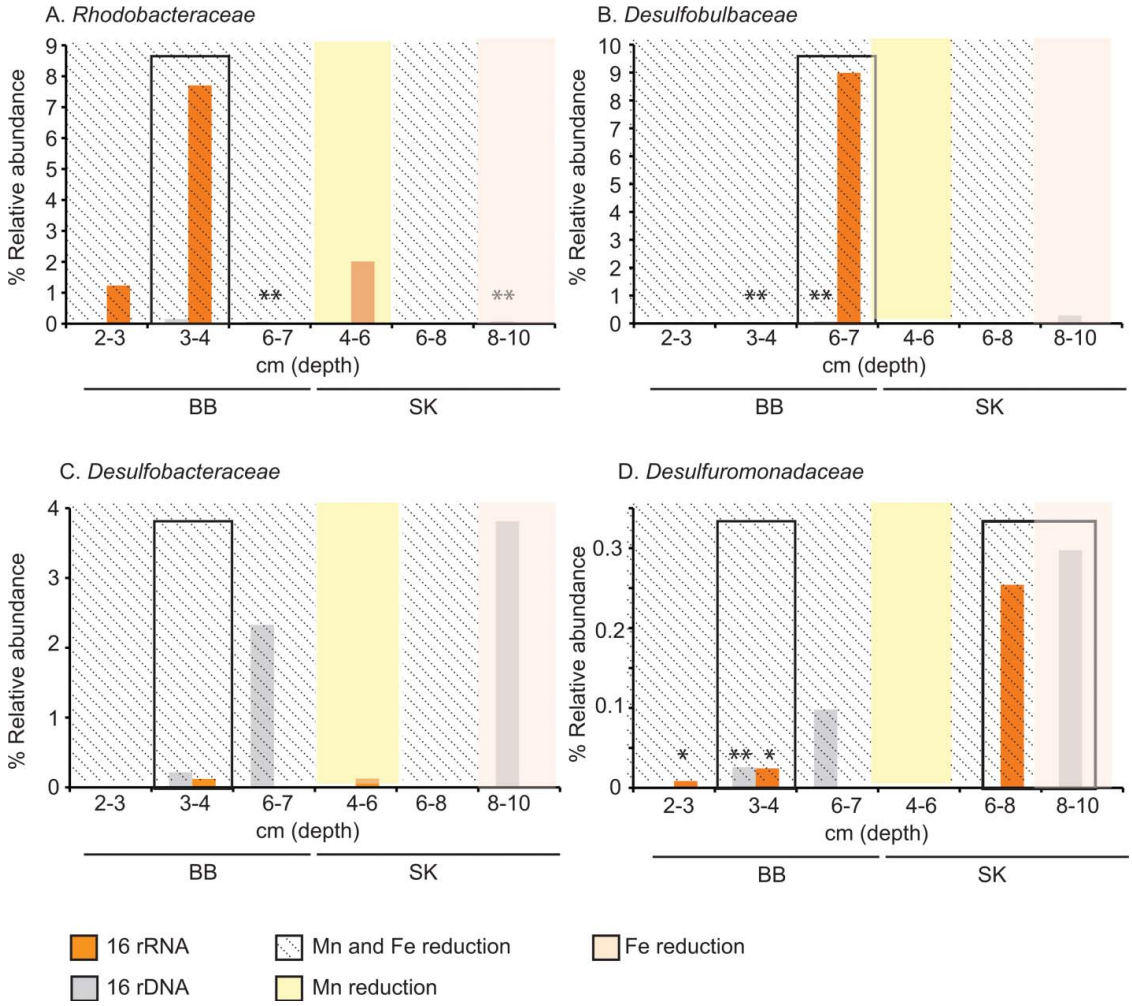

Figure 3. 16S rRNA and 16S rDNA gene relative abundances of bacterial families that were detected in BB and SK samples that could potentially be involved in Fe-reduction. (A) *Rhodobacteraceae*, (B) *Desulfobulbaceae*, (C) *Desulfobacteraceae* and (D) *Desulfuromonadaceae*. Outlined boxes refer to samples for which 16S rRNA and 16S rDNA gene data were available for comparison. Zones of Fe and Mn reduction based on modeling results are also specified. (*) Indicates 16S rRNA abundances were <0.1% and (**) indicates 16S rDNA gene abundances were <0.1%.

families *Rhodobacteraceae* (BB23: ~1%; BB34: ~8%; SK46: ~2%), *Desulfobulbaceae* (BB67: ~9%), *Desulfobacteraceae* (BB34: ~0.12%; SK46: ~0.13%) and *Desulfuromonadaceae* (BB23 and BB34: ≤0.02%; SK68: ~0.25%) (Figure 3A–D) were detected in samples. Percentages refer to relative abundances with respect to the total number of sequences per sample here and throughout the rest of the manuscript. Although *Desulfobulbaceae* were not detected in the 6–8 cm SK sample with the V1–V3 sequencing, they were detected with an alternative V3–V5 sequencing method (Figure S1). The V1–V3 sequencing produced 11,000 more sequences for the BB and 25,000 more sequences for the SK compared to the V3–V5 sequencing (Tables 1 and S1).

Only a few groups known to harbor members capable of Fe-reduction were resolved to the level of genera. Members of the families *Clostridiaceae* and *Bacillaceae* could partially be resolved into the genera *Clostridium* and *Bacillus*. *Bacillus* was detected in the SK68 (0.1%) sample (Figure 4A) and *Clostridium* (<0.03%) was detected in BB34 and SK46 samples (Figure 4B). Sequences belonging to the family *Geobacteraceae* were resolved into the genus *Geobacter* which was present in BB34 and SK68 sediments at abundances ≤0.06% (Figure 4C). Part of the family *Myxococcaceae* was resolved into the genus *Anaeromyxobacter* which was present only in BB23 (0.01%). Also, *Rhodoferax* was detected in low abundances (<0.03) in BB23 and BB34 samples. Other genera that were detected in BB samples with the V3–V5 sequencing included *Pelobacter, Rhodobacter, Desulfuromonas* and *Desulfovibrio* (Figure S1).

16S rRNA comparisons to 16S rDNA genes

The 16S rRNA abundances of *Rhodobacteraceae, Desulfobulbaceae, Bacillus* and *Clostridium* were higher compared to their 16S rDNA gene abundances (Figures 3A, B and 4A, B). Similar 16S rRNA and 16S rDNA gene abundances were observed for *Desulfobacteraceae* (Figure 3C), *Desulfuromonadaceae* and *Geobacter* (Figure 3D and 4C).

16S rRNA abundances of bacteria present in incubations

In the incubation experiment with lepidocrocite and acetate, *Desulfuromonas* was the most abundant genus (Figure 5A and B) coinciding with increased Fe^{2+} concentrations in the enrichments (Figure 5C). The highest Fe^{2+} concentration was observed 54 days after the initial onset of the incubation

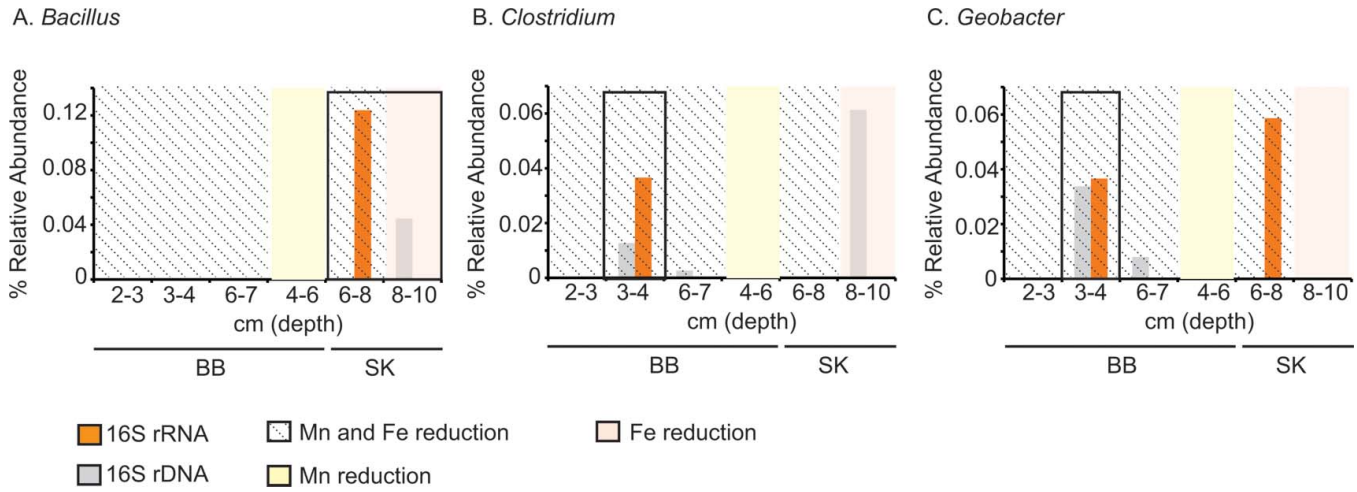

Figure 4. 16S rRNA and 16S rDNA relative abundances of bacterial genera that were detected in BB and SK samples that could potentially be involved in Fe-reduction. (A) *Bacillus*, (B) *Clostridium and* (C) *Geobacter*. Outlined boxes refer to samples for which 16S rRNA and 16S rDNA data were available for comparison. Zones of Fe and Mn reduction based on modeling results are also specified.

experiments (Figure 5C). Fe^{2+} concentrations decreased after each subculturing step and increased after 5 days (Figure 5C). Fe^{2+} concentrations were the lowest 72 days after the initial onset of the incubations, coinciding with a decrease in the abundance of *Desulfuromonas* (Figure 5A and B).

Alpha diversity of samples

The *in-situ* sequenced BB34 sample contained the most diverse, most abundant and most evenly distributed 16S rRNA sequences based on Shannon and Simpson diversity indices (Table 1).

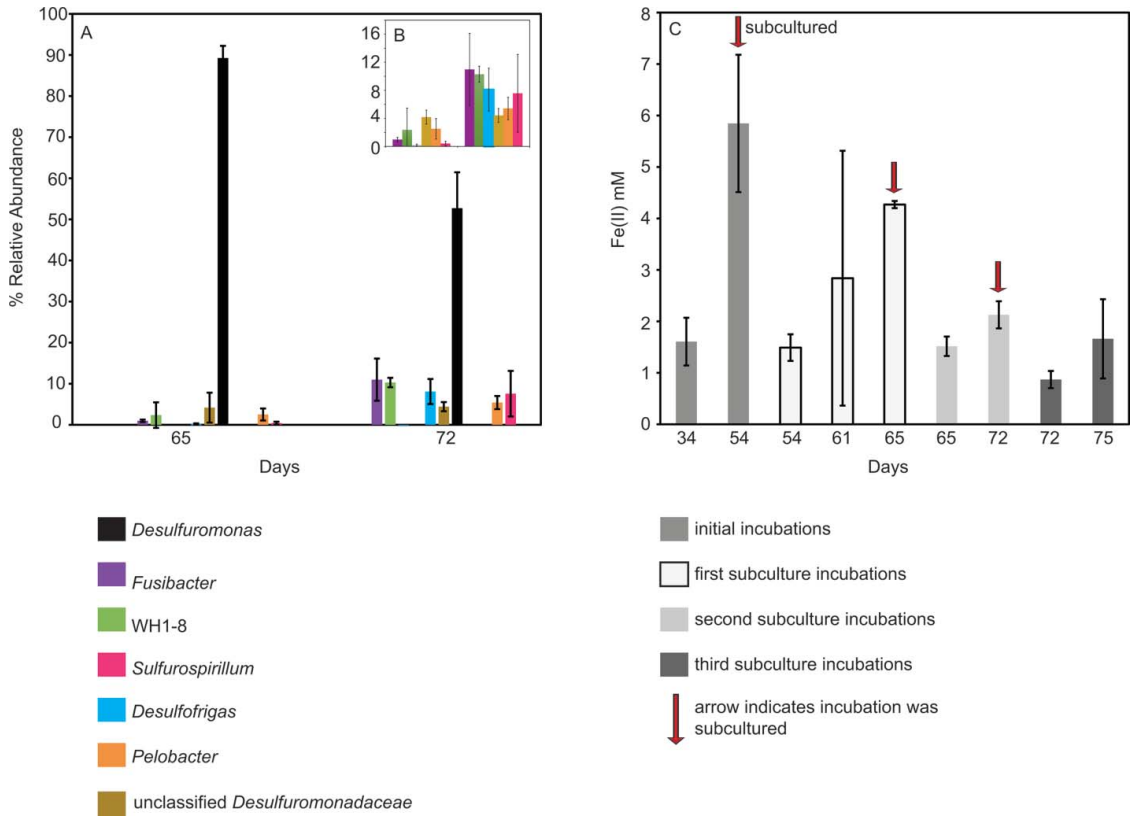

Figure 5. (A) Major bacterial groups that were detected in incubations with sediments from the Skagerrak and lepidocrocite. (B) Bacterial groups excluding *Desulfuromonas* to show the presence of the low abundant groups in samples (C) Fe^{2+} concentrations measured at the time of sampling. Arrow indicates that samples were taken for RNA extraction, Fe^{2+} measurements, and then sub-cultured at the designated day. Days indicate time elapsed since the onset of experiment. Initial incubations refer to the first set of incubations since the onset of the experiment. First, second and third incubations refer to subsequent incubations following the subculturing step. The data represents averages and standard deviations based on triplicate measurements.

Table 1. Sequencing and diversity information for samples sequenced with V1–V3 primers.

Site	Depth (cm)	Samples	Number of sequences	Number of OTUs	Average sequence length (bp)	Chao1	Shannon	Simpson
Bothnian Bay (At4)	2–3	BB23	25,835	10,212	440 ± 46.3	1356 ± 76*	6.8 ± 0.05*	0.96 ± 0*
	3–4	BB34	11,852	7054	455 ± 48.7	1754 ± 25*	9.0 ± 0.02*	0.99 ± 0*
	6–7	BB67	5434	3086	447 ± 49.4	217 ± 0.7*	5.2 ± 0.00*	0.94 ± 0*
Skagerrak (Geo 2a)	4–6	SK46	13,212	5885	444 ± 46.6	1587 ± 63*	8.4 ± 0.04*	0.98 ± 0*
	6–8	SK68	18,148	5561	439 ± 46.4	1628 ± 107*	7.5 ± 0.04*	0.96 ± 0*
Incubations	1–10	SKEN65-1	8672	1837	473 ± 26.9	2384 ± 29‡	4.1 ± 0.04‡	0.84 ± 0‡
	1–10	SKEN65-2	4902	545	473 ± 27.5	197 ± 25‡	4.4 ± 0.02‡	0.89 ± 0‡
	1–10	SKEN65-3	5777	225	475 ± 25.1	171 ± 5‡	4.1 ± 0.0‡	0.88 ± 0‡
	1–10	SKEN72-1	13,155	493	466 ± 32.7	100 ± 20‡	1.4 ± 0.06‡	0.31 ± 0‡
	1–10	SKEN72-2	3688	494	465 ± 32.4	153 ± 23‡	2.1 ± 0.04‡	0.48 ± 0‡
	1–10	SKEN72-3	2045	370	468 ± 30.9	105 ± 16‡	1.3 ± 0.04‡	0.29 ± 0‡

*Diversity indexes based on 4500 sequences.
‡Diversity indexes based on 1950 sequences.

Diversity indices showed the following pattern of diversity, abundance and distribution between samples: BB34 > BB23 > BB67, SK46 > SK68. In contrast, the Chao1 index showed SK68 having greater richness than SK46. Among the SK incubations, diversity indices showed that sample SKEN65 contained the most diverse, most abundant and most evenly distributed 16S rRNA sequences compared to the other subcultures (Table S1). Diversity indices showed the following pattern of diversity, abundance and distribution between incubation samples: SKEN65-1 > SKEN65-2, SKEN65-3 > SKEN72-2 > SKEN72-1, SKEN72-3.

Quantitative PCR

The amplification efficiency of the 16S rRNA qPCR was 82% ($R^2 > 0.99$). BB sediments showed a higher estimate of bacterial (10^8 vs. 10^6) 16S rRNA gene copy numbers per gram of sediment compared to SK sediments. In BB samples, bacteria had the highest 16S rRNA gene copy numbers per gram of sediment (1.8×10^9) at 3–4 cm depth. In contrast, the 16S rRNA gene copy numbers in the SK sediments were similar between depths for bacteria ($\sim 4–5 \times 10^6$ gene copy numbers per gram of sediment). The amplification efficiency of the *dsrA* qPCR was 89.9% (see supporting material). *dsrA* gene copy numbers appeared to decrease with depth in samples from both sites (Figure S2).

Discussion

In a previous DNA-based pyrosequencing study in which the biogeochemistry of the same samples was extensively analyzed, results suggested that dissimilatory SRB contribute to Fe-reduction in sediments from both sites (Reyes et al. 2016). In this study, geochemical modeling results support ongoing Fe and Mn reduction in sediment samples analyzed for pyrosequencing of 16S rRNA, a marker thought to be more relevant for estimating the activity of microorganisms. In BB and SK samples, Fe and Mn reduction overlapped in all samples that were pyrosequenced (Figure 2A–D). Based on the modeling results, Fe and Mn reduction appear to dominate within the top 5 cm of BB and 9 cm depths of SK samples.

Previous geochemical results (discussed in detail in reference Reyes et al. 2016) support ongoing dissimilatory Fe-reduction at both sites. Briefly, both Fe and sulfate reduction have been shown to contribute to Fe^{2+} in SK sediments from neighboring Sites S4 and S6 (Rajendran et al. 1992; Canfield et al. 1993; Figure 1C). The Canfield et al. (1993) study showed that (with the exception of Site S9 which showed very low Fe-reduction and no sulfate reduction) in Site S4, there was significant Fe^{2+} liberated in the porewaters that could not be due to sulfate reduction and concluded that it was produced via dissimilatory Fe-reduction. Furthermore, Rysgaard et al. (2001) and Jensen et al. (2003) measured *in-situ* rates of Fe, Mn and sulfate reduction in sediments from the Baltic Sea–North Sea transition and found that the rates of Fe-reduction were highest in sediments with high concentrations of poorly crystalline Fe(III) (e.g. 20–60 μmol/cm^3). Geochemical data from SK (Rajendran et al. 1992; Canfield et al. 1993; Reyes et al. 2016) sediments show that they have a high Fe(III)-oxide content and that the Fe oxides are reactive. The surface of the sediments from the BB also has high Fe(III) content ($\geq$1%) (Ingri and Pontér 1986; Reyes et al. 2016) and it is for the most part composed of amorphous Fe(III) (Ingri, personal communication). The oxygen availability in the upper surface layers, bioturbation and high concentrations of bioavailable Fe oxides should favor Fe-reduction despite the presence of sulfate. Collectively, these previous geochemical evidences support that dissimilatory Fe-reduction is an important process in these sediments. Moreover, based on past geochemical results, a transition from Fe-reduction to sulfate reduction could occur at depths below 10 cm (Reyes et al. 2016).

In a previous study, we hypothesized that certain SRB were likely contributing to Fe-reduction in the upper centimeters of both sediments but were not necessarily the dominant Fe-reducers in these layers. In this previous study, we observed an increase in the DNA abundance of SRB concomitant with a decrease in Fe-reducing activity (Reyes et al. 2016). Here, the occurrence of 16S rRNA from *Desulfobulbaceae, Desulfobacteraceae* and *Desulfuromonadaceae*, in the zone of Fe-reduction of both sediments (Figures 3B–D and S1) shows that they are potentially active and contributing to Fe-reduction *in-situ*. From pure culture studies it is known that the above families contain members capable of reducing Fe (Table S4).

In our incubation experiments, the diversity and abundance of bacteria in the sediment decreased until 16S rRNA abundances of *Desulfuromonas* became the dominant taxa (Figure 5A and B). An

increase in the abundance of *Desulfuromonas* correlated with an increase in Fe^{2+} release suggesting that sulfate reducers could play an important role in Fe-reduction. Future incubation experiments will allow us to determine if the above microorganisms remain highly abundant and active in these sediments in the absence of Fe oxides.

Previous studies that involved the enrichment and isolation of sulfur or sulfate reducing bacteria from marine sediments with ongoing Fe-reduction (Vandieken et al. 2006; Vandieken and Thamdrup 2012; Kim et al. 2014; Hori et al. 2015) showed strong evidence that they contribute to Fe-reduction in those sediments. Lovley and Phillips (1987) observed that in marine sediment incubations ammended with H_2 and acetate, Fe-reducers metabolized H_2 and acetate at concentrations lower than sulfate reducers and methanogens, therby outcompeting them for substrates. In our study, the majority of potential Fe-reducers were detected mostly in BB samples with both sequencing methods (Figures 3 and S1). Perhaps a more diverse community of Fe-reducers could potentially contribute to Fe-reduction in BB vs. SK sediments.

Besides sulfur and sulfate reducing bacteria, unknown members of the *Rhodobacteraceae* were detected at high abundance in BB and SK samples (Figure 3A). In a previous pyrosequencing study, members of the *Rhodobacteraceae* were also detected in both sediments at depths where nitrate and Fe-reduction overlapped (Reyes et al. 2016). *Rhodobacteraceae* relative abundance was shown to decrease with depth, coinciding with a decrease in Fe-reduction activity (Reyes et al. 2016). Although some *Rhodobacteraceae* could be capable of denitrification, these are all known to be phototrophic (Shapleigh 2013). Recently, a non-phototrophic *Rhodobacter* marine sediment species was shown to reduce various Fe oxides coupled to organic carbon oxidation in pure culture experiments (Yang et al. 2013). Thus, it is possible that members of this genus could contribute to Fe-reduction at these sites.

In a previous study, we hypothesized that dissimilatory Fe-reducers were likely to contribute the most to Fe-reduction in the upper centimeters of both sediments (Reyes et al. 2016). The occurence and decrease in abundance of *Geobacter*, to which many of the dissimilatory Fe-reducers belong to (Table S2) in both sediments, coincided with a decrease in Fe-reducing activity (Reyes et al. 2016). Results from this study like those for the SRB show that they are potentially active in both sediments in the upper layers.

Moreover, potential Fe-reducers such as unknown members of the *Desulfuromonas, Geobacter, Anaeromyxobacter, Clostridium* and *Bacillus* could also be contributing to Mn-reduction in zones where Fe^{2+} and Mn^{2+} overlap. Pure cultures of certain members of the above groups have been shown to be capable of Mn(IV)-reduction (Lovley 2013; Yang et al. 2013).

We attempted to amplify a different 16S rRNA region to determine whether groups that were not detected with the V1–V3 primers would be detected with V3–V5 primers. Because the V1–V3 primers provided more sequences, our main interpretations in terms of abundances and diversity are based on this dataset. Despite being a smaller dataset, additional taxa potentially involved in Fe-reduction including *Pelobacter* and *Desulfovibrio*, which were not detected with the V1–V3 primers, were detected with the V3–V5 primers (Figure S1).

Compared to 16S rDNA relative abundances, which were low for *Rhodobacteraceae* and *Desulfobulbaceae* in both sediments (Figure 3A and B), 16S rRNA abundances were higher (e.g. $\geq$1%). This observation shows that while previously reported 16S rDNA abundances were low for these taxa, they appear to be active. Whether specific taxa are more active than others *in-situ* cannot be elucidated from these pyrosequencing results. Different taxa can vary in their growth rate and rRNA abundance. Therefore, differences in rRNA abundances between taxa may not necessarily indicate that certain taxa are more active than others (Blazewicz et al. 2013).

Our qPCR data show that the *dsrA* gene copy numbers also decrease with depth at both sites (Figure S2). The presence of high *dsrA* gene copy numbers (6×10^8 and 4×10^7) at depths with increased Fe-reduction rates, points toward a potential involvement of SRB in Fe-reduction. However, these results should be interpreted with caution since various bacteria that oxidize internal storages of sulfur can also harbor the *dsrA* gene (Müller et al. 2015). Also, a high margin of error was observed for the 8–10 cm SK sample. The high margin of error in the sample could be attributed to the large differences in the total amount of DNA extracted from one of the three biological replicates (2.26 μg $\pm$ 2.32 μg/g wet sediment) thereby showing higher differences in gene copy numbers when the data was normalized.

A higher 16S rRNA gene copy number in the upper centimeters of BB sediments compared to the deeper layers implies that more active microbial communities inhabit the top centimeters of the BB. This high activity in the upper layers of the BB suggests that some substrates could become limiting with depth. As to the nature of the available substrates, organic matter could provide a source of nutrients to microorganisms. Organic matter in the BB is mostly terrestrial in origin making it more refractory than the organic matter in the SK (which is mostly marine in origin) (Anton et al. 1993; Piiparinen et al. 2010). Alpha diversity results from this study based on 16S rRNA (Table 1) and a previous study based on 16S rDNA (see supplementary material in reference Reyes et al. 2016) also show decreasing trends in the BB supporting the idea that the quality of the organic matter could decrease with depth and limit microbial activity. Decreasing microbial activity with depth due to less labile organic matter availability has also been observed in other marine sediment environments (Arndt et al. 2013). The pronounced peak in bacterial activity at 3–4 cm depth could occur in this zone due to availability of electron acceptors such as Fe(III), Mn(IV) and NO_3^- in this layer (Reyes et al. 2016).

Although less samples were available for analysis from the SK site, estimates of bacterial gene copy numbers (based on 16S rRNA) in the SK ($\sim 5.1 \times 10^6$ gene copies/g sediment) agree with acridine orange bacterial cell counts observed in the upper centimeters of other sites in the SK (Iversen and Jørgensen 1993). They are lower than the total bacterial cell counts ($\sim 8.6 \times 10^8$ cells/g sediment) in the Norwegian trench of SK sediments (see supporting material in reference Trimmer et al. 2013). However, the estimates extrapolated in Trimmer et al. (2013) could be less accurate than acridine orange and qPCR methods as they are based on NanoDrop measurements. Compared to qPCR gene copy estimates for specific bacterial groups

such as anammox bacteria in surface sediments, total bacterial gene copy estimates based on cDNA measurements have not been previously reported for the Baltic Sea or North Sea.

Conclusions

This study shows that in the zone of Fe-reduction, sulfate and sulfur reducers are active and have the potential to contribute to Fe-reduction in both sites. Results also suggest that the BB may harbor a greater diversity of Fe-reducers compared to SK. Increased bacterial activity in BB sediments could indicate that bacteria capable of using more refractory material are active in the BB compared to SK.

Acknowledgments

The authors thank Gerhard Kuhn (Alfred-Wegener-Institut für Polar- und Meeresforschung, Bremerhaven) who helped with subsampling of the cores, Olaf Dellwig (Leibniz Institute for Baltic Sea Research, Warnemünde) for contributing to porewater measurements, Patrick Meister (University of Vienna) for helping to transport cores and for contributing the map of the study sites. They also thank Johannes Birkenstock (University of Bremen) for XRD measurements.

Funding

National Science Foundation (NSF), International Research Fellowship Program (project no. 1064521 to C.R.), the Fulbright fellowship programme (fellowship to C.R.), the Hanse–Wissenschaftskolleg, Delmenhorst (HWK; fellowship to C.R.). Modeling work was supported by BMBF during the SECOS project. Financial support was also provided by the Leibniz IOW and the University of Bremen. The funders had no role in study design, data collection and interpretation or the decision to submit the work for publication.

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