LIMNOLOGY and OCEANOGRAPHY

ASLO

Limnol. Oceanogr. 65, 2020, S113–S133

doi: 10.1002/lno.11371

Microbial community composition and functional potential in Bothnian Sea sediments is linked to Fe and S dynamics and the quality of organic matter

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Abstract

The Bothnian Sea is an oligotrophic brackish basin characterized by low salinity and high concentrations of reactive iron, methane, and ammonium in its sediments, enabling the activity and interactions of many microbial guilds. Here, we studied the microbial network in these sediments by analyzing geochemical and microbial community depth profiles at one offshore and two near coastal sites. Analysis of 16S rRNA gene amplicons revealed a distinct depth stratification of both archaeal and bacterial taxa. The microbial communities at the two near coastal sites were more similar to each other than the offshore site, which is likely due to differences in the quality and rate of organic matter degradation. The abundance of methanotrophic archaea of the ANME-2a clade was shown to be related to the presence of methane and varied with sediment iron content. Metagenomic sequencing of sediment-derived DNA from below the sulfate–methane transition zone revealed a broad potential for respiratory sulfur metabolism via partially reduced sulfur species. The potential for nitrogen cycling was dominated by reductive processes via a truncated denitrification pathway encoded exclusively by bacterial lineages. Gene-centric fermentative metabolism analysis indicated a potential importance for acetate, formate, alcohol, and hydrogen metabolism. Methanogenic/-trophic pathways were dominated by *Methanosaetaceae*, *Methanosarcinaceae*, *Methanomassiliicoccaceae*, *Methanoregulaceae*, and ANME-2 archaea. Our results indicated flexible metabolic capabilities of core microbial community taxa, which could adapt to changing redox conditions, and with a spatial and depth distribution that is likely governed by the quality and input of available organic substrates and, for ANME-2, of iron oxides.

Sediment microbial communities drive biogeochemical cycles through their specific metabolic activities. The supply of organic carbon from primary production or terrestrial input via rivers, and electron acceptors such as nitrate (NO_3^-) and sulfate (SO_4^{2-}) in marine systems, will select for particular microbial guilds. Together, they will determine the establishment of environment-specific metabolic networks and geochemical profiles. Despite the critical role of coastal sediments in global biogeochemical cycling, for example, as a source of methane (CH_4) (Bange et al. 1994) and sink for nutrients (Asmala et al. 2017), our understanding of their microbial community composition and how this is linked to the cycling of sulfur (S), carbon (C), and nitrogen (N) is still not yet well explored.

The Bothnian Sea, a brackish basin located in the northern part of the Baltic Sea, is an ideal location to study the linkage between microbes and biogeochemistry because of the distinct sharp redox zonation of its surface sediments (Egger et al. 2015*a*; Rasigraf et al. 2017; Lenstra et al. 2018). The Bothnian Sea is oligotrophic and most organic matter in the sediment is

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Special Issue: Linking Metagenomics to Aquatic Microbial Ecology and Biogeochemical Cycles. Edited by: Hans-Peter Grossart, Ramon Massana, Katherine McMahon and David A. Walsh.

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supplied through rivers and is thus of terrestrial origin (Algesten et al. 2006). SO_4^{2-} concentrations in the bottom water are low (3–5 mM), which has allowed the development of a relatively shallow SO_4^{2-} reduction zone in the sediment at sites with relatively high sedimentation rates. At such sites, CH_4 is abundant in the lower part of the SO_4^{2-} reduction zone, and a distinct sulfate–methane transition zone (SMTZ) has developed (Egger et al. 2015*a*; Lenstra et al. 2018). The exact position of the SMTZ varies with space and time depending on the sedimentation rate and the input of organic matter (Slomp et al. 2013; Egger et al. 2015*a*; Rooze et al. 2016; Lenstra et al. 2018). The input of reactive iron (oxyhydroxides, henceforth termed Fe oxides) is in general higher than sulfide (H_2S) formation in the sediment resulting in net burial of Fe oxides below the SMTZ. Both modeling and incubation studies suggest CH_4 oxidation with Fe oxides as the electron acceptor in the SO_4^{2-}-depleted methanic layers below the SMTZ (Slomp et al. 2013; Egger et al. 2015*b*; Rooze et al. 2016). So far, the underlying pathways and responsible organisms for this process are largely unknown.

Several studies have investigated the microbial community composition in sediments of the Bothnian Bay and North Sea with 16S rRNA gene pyrosequencing techniques and speculated on possible microbial guilds involved in CH_4 and Fe cycling (Oni et al. 2015*a*; Reyes et al. 2016). In surface sediments from the Skagerrak and Bothnian Bay, various potential Fe-reducers belonging to *Desulfobulbaceae*, *Desulfuromonadaceae*, and *Pelobacteraceae* families were identified (Reyes et al. 2016). In deeper methanic sediment layers of the Helgoland area in the North Sea, microbial populations predicted to be involved in Fe and CH_4 cycling included uncultured lineages of *Atribacteria* (former candidate division JS1) and methanogenic/-trophic archaea belonging to *Methanohalobium*, *Methanosaeta*, and anaerobic methane oxidizing archaea clade 3 (ANME-3) (Oni et al. 2015*a*). Moreover, recent findings indicate that temperature is another factor which can influence the pathway of crystalline Fe utilization in these sediments (Aromokeye et al. 2018). Investigations of microbial communities involved in Fe cycling are challenging due to the absence of suitable "universal" biomarkers. Different microbial groups have evolved different mechanisms and underlying genes encoding responsible enzymes may be unrelated. Novel mechanisms with unknown enzymatic steps in Fe reduction may exist but would remain undetected.

Several processes involved in the N cycle were also investigated recently in sediments of the Bothnian Sea and the Bothnian Bay (Bonaglia et al. 2017; Hellemann et al. 2017; Rasigraf et al. 2017; Reyes et al. 2017). For example, dissimilatory nitrate reduction to ammonium (DNRA) and denitrification were shown to be of nearly equal importance at a coastal site in the oligotrophic Bothnian Bay (Bonaglia et al. 2017). In contrast, in more offshore areas in the same region, both the genetic potential (Reyes et al. 2017) and rates (Bonaglia et al. 2017) were greater for denitrification than for DNRA. Differences in nitrification potential were also observed, with ammonia-oxidizing archaea (AOA) being the dominant nitrifiers at an offshore site in the Bothnian Sea, while AOA and ammonia oxidizing bacteria (AOB) were of equal importance in Bothnian Bay sediments (Rasigraf et al. 2017; Reyes et al. 2017). Thus, large differences in measured activities and genetic inventory can occur between sediments in the same region. The environmental factors driving those differences are not well understood.

Here, we assessed the microbial community composition in sediments at three sites along a water depth gradient in the Bothnian Sea by various approaches including 16S rRNA gene amplicon sequencing and metagenomic sequencing of the iron-rich zone. Two sites are located near the coast in the Öre Estuary (N10 and NB8, Lenstra et al. 2018) while the third site is located in the central-basin of the Bothnian Sea (US5B, Egger et al. 2015*a*). Pore-water profiles of key geochemical constituents such as SO_4^{2-}, dissolved Fe, and CH_4 were used to determine the redox zonation. For two sites (NB8 and US5B), we also assess the occurrence of several key respiratory processes related to C, Fe, and S cycling as inferred from reactive transport modeling of the geochemical profiles (Rooze et al. 2016; Lenstra et al. 2018). Community comparisons suggest that the abundance of ANME-2 is regulated by the presence of methane and sediment Fe input. The core microbial community and its metabolic potential in the ferruginous methanic zone at one of the coastal sites are assessed through metagenome sequencing. Metagenome-assembled genomes (MAGs), which were recovered for most abundant microbial community members, indicated a flexible metabolic network with strong potential for fermentation and S cycling.

Materials and methods

Sampling and geochemical analysis

The two near-coastal sites, N10 and NB8, are located in the Öre Estuary in the Bothnian Sea at water depths of 21 and 33 m, respectively (Lenstra et al. 2018). Sediments at these sites were collected during a field campaign with R/V *Lotty* in August 2015 using a Gemini gravity corer (8 cm inner diameter). The offshore site US5B is located in the central basin of the Bothnian Sea at a water depth of 214 m and was sampled in August 2012 (Egger et al. 2015*a*). Sediments at this site were collected during a field campaign with R/V *Aranda* using a GEMAX gravity corer (10 cm inner diameter). Locations of all sampled sites are shown in Fig. 1. Pore-water depth profiles of SO_4^{2-}, CH_4, NH_4^+, H_2S, and dissolved Fe were measured either onboard or later in the laboratory as described previously (Egger et al. 2015*a*; Lenstra et al. 2018; Fig. 2). Sediment characteristics of sampled sites are summarized in Table 1. Sediment cores were kept at 4°C in the dark covered with a layer of bottom water until slicing. The slicing of sediment cores was performed in an anaerobic chamber under argon atmosphere. Sediment subsamples dedicated for DNA isolation were stored at −20°C until further processing. Sediment total Fe for sites NB8 and US5B was determined by Inductive Coupled Plasma Optical

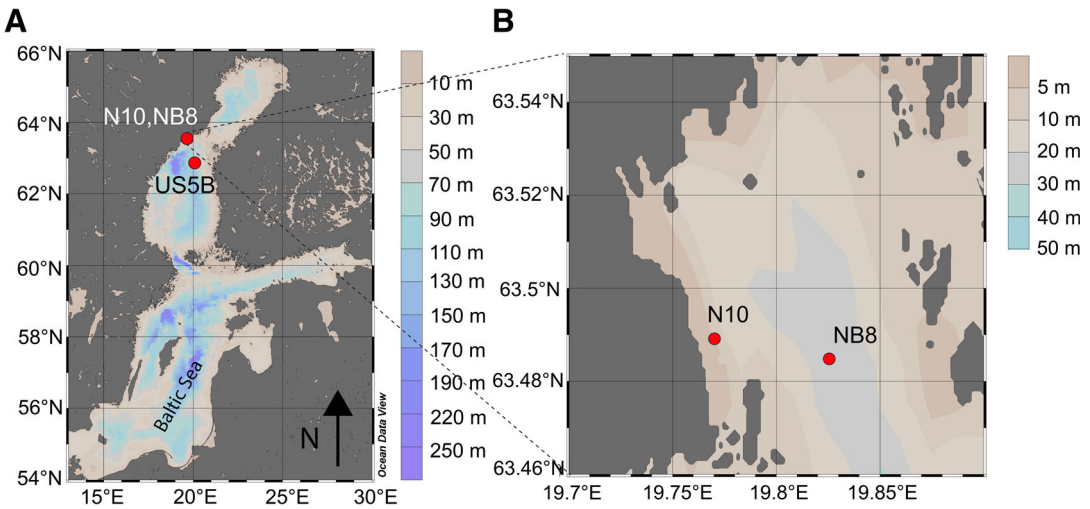

Fig. 1. (**A**) Locations of sampling sites N10, NB8, and US5B in the Bothnian Sea. (**B**) Locations of both coastal sites N10 and NB8 in the Öre Estuary in the Bothnian Sea. Figure drawn using Ocean Data View (Schlitzer 2015).

Emission Spectrometry after total digestion of ~ 125 mg of sediment in a mixture of strong acids (Egger et al. 2015*b*; Lenstra et al. 2018). For NB8, a small subsample from the core used for DNA isolation was used for this analysis, whereas for US5B, the sediment from the pore-water core was used.

The biogeochemistry of the analyzed sites at the time of sampling was discussed in detail previously (Egger et al. 2015*b*; Rooze et al. 2016; Lenstra et al. 2018).

DNA isolation

The frozen core sediment subsamples were defrosted on ice and vortexed to obtain homogenous slurry. Subsequently, 0.2–0.5 mL of original sediment slurry was filled into a bead beating tube from the PowerSoil DNA isolation kit (MoBio). Further isolation was performed according to manufacturer's instructions. The quantity of isolated DNA was assessed by NanoDrop 1000 (Thermo Scientific) and Qubit® 2.0 (Invitrogen, Life Technologies). After isolation, DNA was frozen at −20°C until further use.

16S rRNA and metagenome sequencing

The amplification of total archaeal and bacterial 16S rRNA genes was performed with the following primer pairs: Arch349F (5′-GYGCASCAGKCGMGAAW-3′) (Takai and Horikoshi 2000) and Arch806R (5′-GGACTACVSGGGTATCTAAT-3′) (Takai and Horikoshi 2000) for archaea, Bac341F (5′-CCTACGGGNGGC WGCAG-3′) (Herlemann et al. 2011) and Bac806R (5′-GGACTACHVGGGTWTCTAAT-3′) (Caporaso et al. 2012) for bacteria. 16S rRNA amplicon sequencing was performed on the Illumna MiSeq platform using the MiSeq Reagent Kit v3, yielding 2x300bp paired-end reads (Macrogen).

For metagenomic sequencing, DNA from separate depth samples was pooled in equimolar concentrations. Paired-end metagenomic sequencing with 2x300bp sequence chemistry was performed with Miseq reagent kit v3 on Illumina Miseq platform according to manufacturer's instructions at the Microbiology Department of Radboud University, Nijmegen.

16S rRNA gene amplicon analysis

Paired-end reads were processed with the Mothur v.1.36.1 software following the standard operation procedure (MySeq SOP) instructions (Kozich et al. 2013). The length of overlapped sequences was filtered for 400–500 base pairs (bp). Chimeric sequences were removed with the UCHIME algorithm (Edgar et al. 2011). Sequences were clustered into operational taxonomic units (OTU) with a 97% identity cutoff and classified using the SILVA 16S rRNA gene nonredundant reference database (version 123, SSURef123NR99) and the Bayesian classifier (wang) (Pruesse et al. 2007). After quality trimming, chimera removal and normalization ("subsampling" in Mothur) of data, each sample contained 5000 sequences for bacteria and 2000 sequences for archaea. Samples with fewer sequences were excluded from the analysis.

Statistical analysis was performed in R (https://www.r-project.org/) (R Development Core Team, 2013) with OTU tables obtained in Mothur using the package Vegan (Oksanen 2018). Data visualization was performed in Rstudio (RStudio Team 2015) using the package ggplot2 (Wickham 2016). The R package "OTUtable" was used to merge identical taxonomic groups classified as different OTUs in Mothur (Linz et al. 2017).

Metagenome analysis: Assembly, binning, and annotation

Sequencing data obtained from the sediment sample described in this study were analyzed together with data obtained from incubation samples which are part of another study (data not shown).

Quality-trimming, sequencing adapter removal, and contaminant filtering of Illumina paired-end sequencing reads

was performed using BBDuk (BBTools suite version 37.17) (Bushnell, BBMap, sourceforge.net/projects/bbmap/, unpubl), yielding 97,703,456 reads. Processed reads were co-assembled using MEGAHIT v1.1.1-2 (Li et al. 2015, 2016) using the "meta-sensitive" preset. MEGAHIT iteratively assembled the metagenome using k-mers of length 21, 29, 39, 59, 79, 99, 119, and 141. Reads were mapped back to the assembled metagenome for each sample separately using Burrows-Wheeler Aligner 0.7.15 (BWA) (Li and Durbin 2010), employing the "mem" algorithm. The sequence mapping files were processed using SAMtools 1.6 (Li et al. 2009). Metagenome binning was performed for contigs greater than 2000 bp. To optimize binning results, four different binning algorithms were used: COCACOLA (Lu et al. 2017),

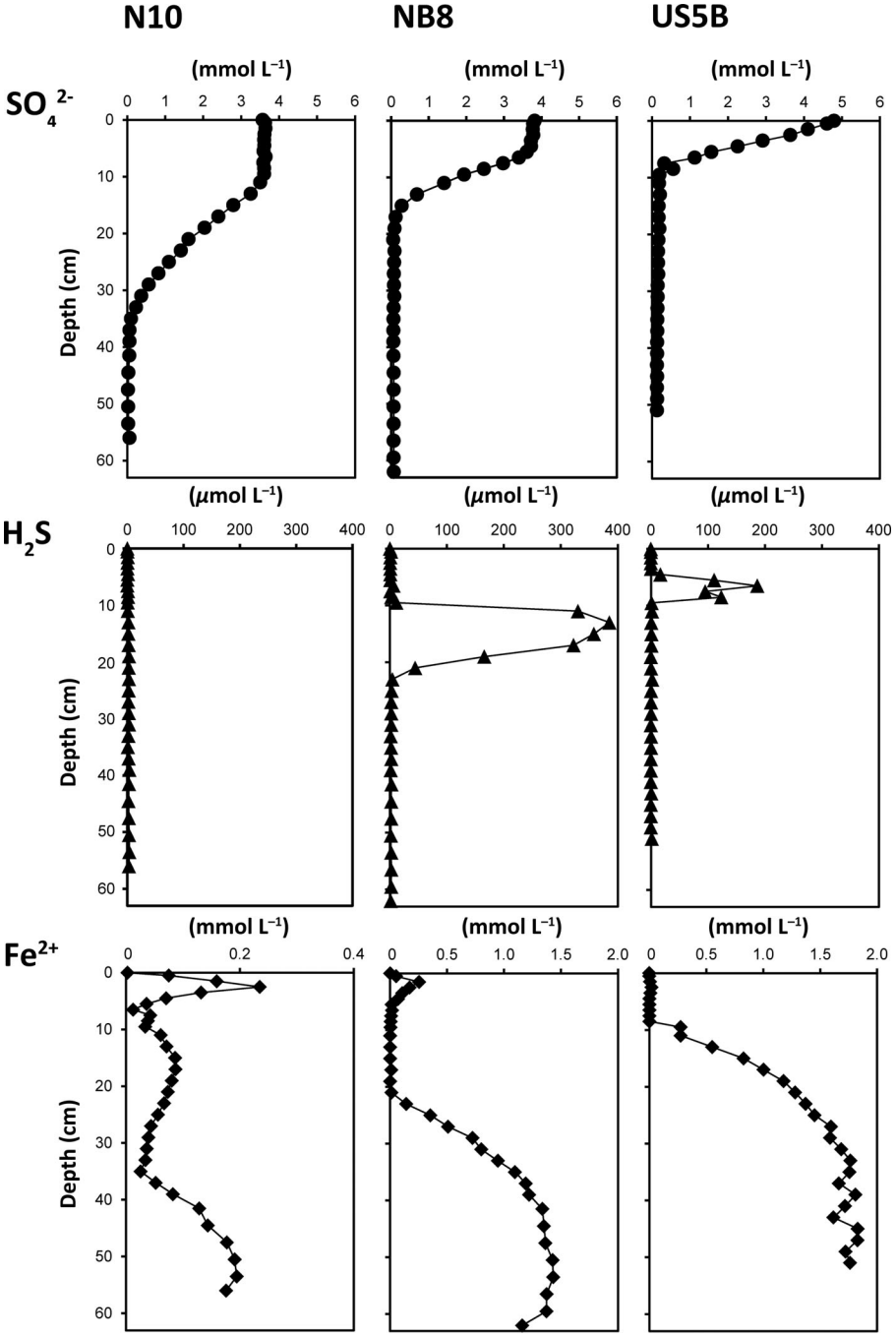

Fig. 2. Geochemical profiles for sites N10, NB8, and US5B. Pore-water profiles of SO_4^{2-}, sulfide ($\sum H_2S=H_2S + HS^- + S^{2-}$), Fe^{2+}, CH_4, and NH_4^+ are shown. Note the different scales for site N10.

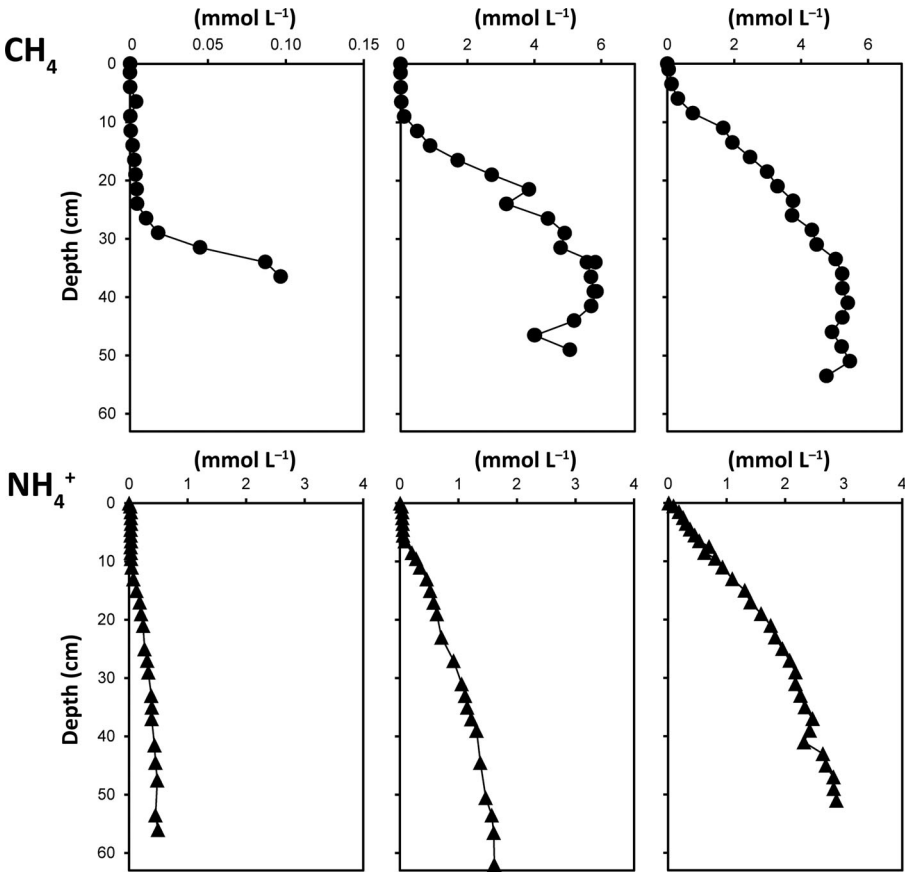

Figure 2 (Continued)

CONCOCT (Alneberg et al. 2014), MaxBin 2.0 2.2.3 (Wu et al. 2016), and MetaBAT 2 2.10.2 (Kang et al. 2015). The four bin sets were supplied to DAS Tool 1.0 (Sieber et al. 2018) for consensus binning to obtain the final bins. The quality of the genome bins was assessed through a single-copy marker gene analysis using CheckM 1.0.7 (Parks et al. 2015). A coarse taxonomic classification of the genome bins was performed using CheckM and further refined by placing bins in a phylogenetic tree using the UBCG pipeline for phylogenomic tree reconstruction (Na et al. 2018). Annotation and biomarker detection was performed with KEGG automatic annotation server with bit score threshold of 100 (Moriya et al. 2007) and the Microbial Annotation and Analysis Platform of MicroScope (MAGE) (Vallenet et al. 2006). All sequencing data obtained for this project were submitted to the GenBank under the BioProject PRJNA511814. The metagenome originating from the in situ sediment in the methanic Fe-rich zone at site NB8 described in this study is designated as sample BS5 (BioSample SAMN10644131).

Metagenome analysis: *mcrA* biomarker analysis

Functional biomarker analysis was performed as described previously (Lüke et al. 2016; Rasigraf et al. 2017). Following the procedures described in Lüke et al. (2016), metagenome data for the in situ sediment sample were quality trimmed with CLC Genomics Workbench 9.5.3 software using the following settings: quality score limit 0.01 (Q20), maximum number of ambiguous base pairs 0, min read length 100 [nt]. Metagenome size comprised 18,107,912 reads after quality trimming. Functional biomarkers were identified with blastx (release 2.4.0) using manually curated functional gene databases following the procedure described previously. Amino acid sequence data were aligned in ARB (Ludwig et al. 2004) and used for building an alternative classification taxonomy in MEGAN 5.11.3 based on manually curated *mcrA* gene database (Huson et al. 2007). Curated functional gene reads were re-blasted with a database file adapted for alternative taxonomic classification in MEGAN. Blast output was then imported into MEGAN and visualized for quantitative analysis. In total 288 *mcrA* gene reads were extracted from the metagenome. Quantified data were visualized with the R statistical package ggplot2.

For quantitative comparison, the analyzed gene reads were normalized to metagenome size and average gene length according to the following formula: normalized read count = (gene read count × 1,000,000,000)/(total metagenome read count × average gene length [nt]).

Table 1. Characteristics of the investigated sites N10, NB8, and US5B in the Bothnian Sea. The data for water depth, temperature, coordinates, organic carbon content, and sedimentation rates were compiled from Lenstra et al. (2018) and Egger et al. (2015*a*).

Site	Water depth (mbss)	Temperature °C	Latitude °N	Longitude °E	C_{org} (wt.%)	Sed. rate (cm year^{-1})
N10	20.8	7.8	63.293	19.462	3.40 (±0.70)	0.25
NB8	33.2	6.3	63.291	19.495	3.85 (±0.07)	1
US5B	214	5.0	62.351	19.581	2.58 (±0.21)	1.3

mbss, meters below sea surface.

For SSU rRNA quantification, raw reads were mapped to SILVA database (release 128) in CLC Genomics Workbench with the following settings: match score 1, mismatch cost 2, insertion cost 3, deletion cost 3, length fraction 0.5, similarity fraction 0.8. Mapped reads were extracted and submitted to SILVAngs online analysis pipeline (www.arb-silva.de/ngs/).

Results and discussion

Geochemistry of sites N10, NB8, and US5B

Pore-water profiles revealed the presence of a shallow SMTZ at all three sites (Fig. 2). At site N10, the SMTZ was located at a depth of about 25–35 cm. At this site, CH_4 and H_2S concentrations in the pore water were very low. At sites NB8 and US5B, in contrast, the SMTZ was located at depths of about 20–25 and 4–9 cm, respectively, and distinct maxima in H_2S were observed within the SMTZ. Maximum concentrations of NH_4^+ at depth in the sediment ranged from about 0.5 mM at site N10 to 1.5 and 3.0 mM at sites NB8 and US5B, respectively. All sediments were rich in dissolved Fe^{2+}, with concentrations increasing in the sequence N10, NB8, and US5B below the SMTZ (Fig. 2). This spatial trend was in accordance with the observed fivefold increase in sediment accumulation rates and corresponding increased input of organic matter with distance from the coast (Fig. 1 and Table 1). In other words, the further from the coast, the shallower the transitions between the different redox zones (such as the SMTZ) and the higher the concentrations of the various products of the respiration processes (e.g., Fe^{2+}, CH_4, and NH_4^+). In this region, riverine Fe inputs to the sediments are highly variable with time, as illustrated by the total sediment Fe profiles for sites NB8 and US5B (Fig. 3) and discussed in detail by Lenstra et al. (2018).

Chemical redox zonations provide only partial insight in the location of the corresponding respiratory processes because products can rapidly react and be removed (e.g., Canfield and Thamdrup 2009). Thus, while a decrease in SO_4^{2-} with depth is indicative of sulfate reduction, the accumulation of H_2S is additionally determined by the availability of Fe oxides in the sediment (Canfield et al. 1992; Lenstra et al. 2018). This explains the variability in H_2S concentrations at our three study sites, and its lack of correlation with the gradient in the SO_4^{2-} profile (Fig. 2).

To obtain further insight in the zonation of respiratory processes, key rates relevant to N, C, S, and Fe cycling inferred from reactive transport modeling of both pore-water and solid phase profiles for sites NB8 and US5B were assessed (Fig. 4; Rooze et al. 2016; Lenstra et al. 2018). Since this is a dynamic system, subject to variations in input of organic matter and metal oxides, the rates provide only a "snapshot" in time. These results suggest that, at both sites, at the time of sampling, nitrification and denitrification were limited to the upper 3 to 5 cm of the sediment. Two zones of Fe oxide reduction can be distinguished, above and at the lower end of the SMTZ. Since, in the model, all Fe oxide reduction at depth is assumed to be coupled to methane oxidation, rates of Fe oxide reduction coupled to organic matter degradation may be underestimated (Rooze et al. 2016; Lenstra et al. 2018). At both sites, the pathways of anaerobic degradation of organic matter show significant overlap in their depth zonation in the model. For example, the model suggests that methane is produced close to the sediment–water interface (Fig. 4) in zones where it is not detected (Fig. 2). The model results further illustrate that, while the pore-water profiles at sites NB8 and US5B are broadly similar, the higher rates of organic matter

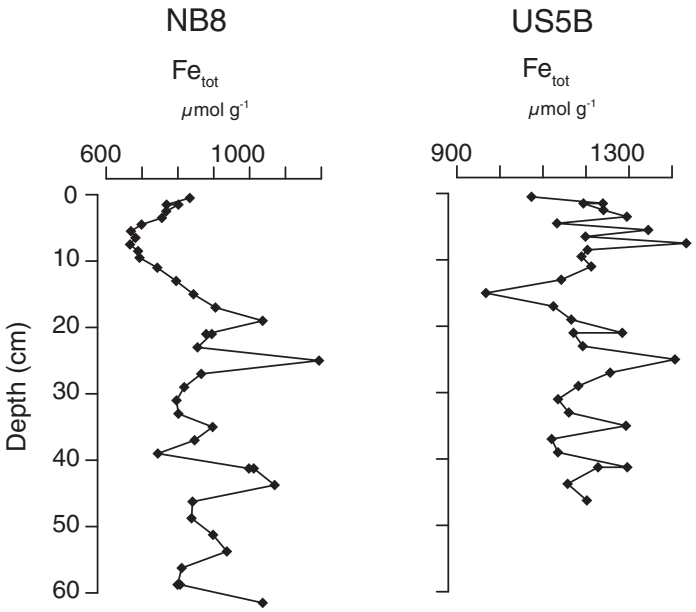

Fig. 3. Sedimentary solid phase Fe profiles at sites NB8 and US5B in the Bothnian Sea.

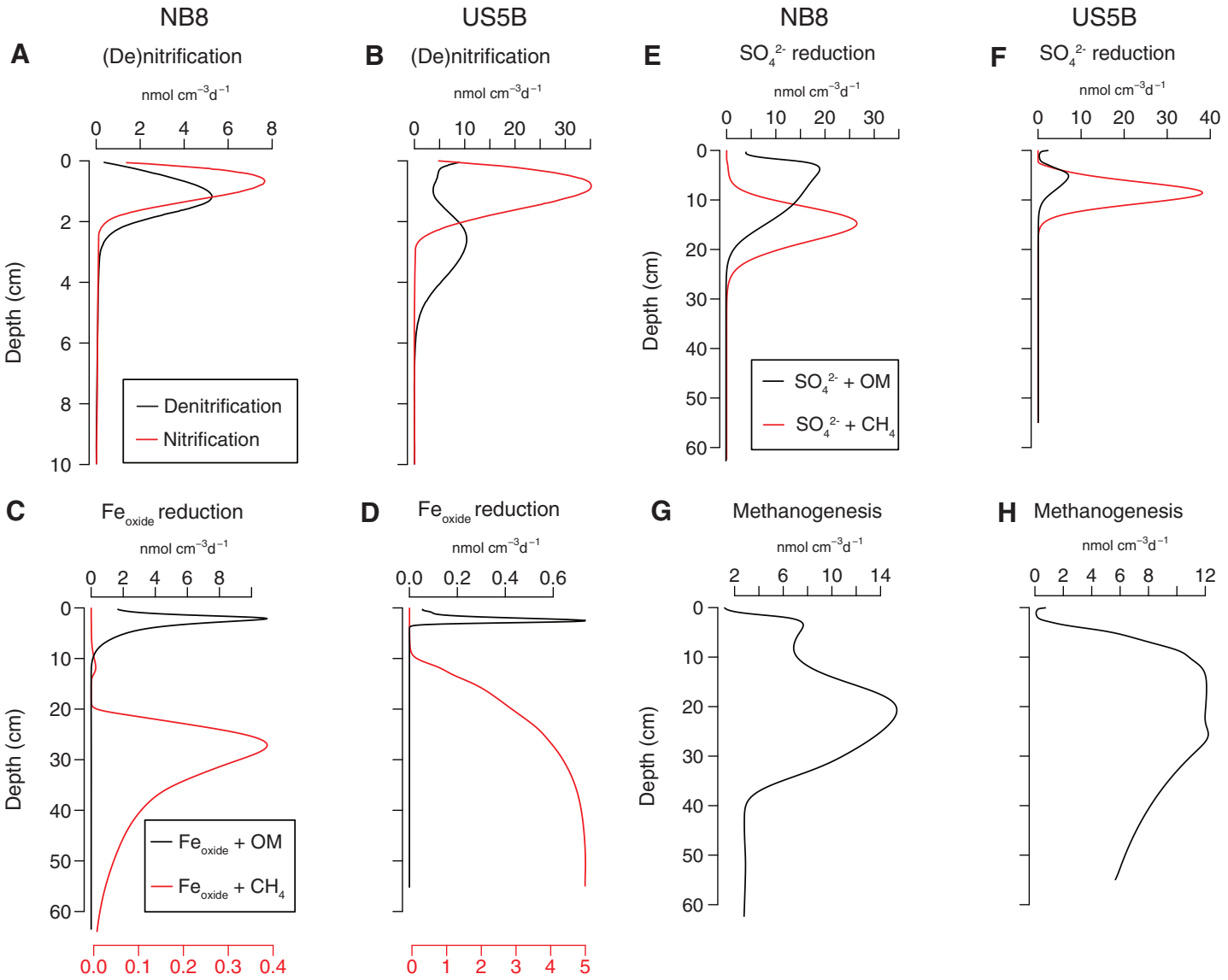

Fig. 4. Rates of key biogeochemical processes in the sediments at sites NB8 and US5B in the Bothnian Sea from reactive transport modeling (Rooze et al. 2016; Lenstra et al. 2018). A-B: (De)nitrification, C-D: Fe_{oxide} reduction, E-F: SO_4^{2-} reduction, G-H: Methanogenesis.

degradation at the latter site are associated with higher rates of, for example, denitrification and SO_4^{2-}-AOM, when compared to NB8.

Bacterial and archaeal community composition follows geochemical gradients

The depth distribution of most dominant bacterial and archaeal taxa followed the geochemical gradients at all three sites, indicating a close connection to the different redox zones (Figs. 2, 5, 6). The microbial communities at the two coastal sites were more similar in composition than those at the offshore site (Supplementary Fig. S1).

At all sites, the archaeal communities were dominated by *Thaumarchaeota* Marine Group-I (MG-I) in the upper non-methanic sediment zone. Although their relative abundance decreased with depth, they were still detected throughout the whole sediment profile at each site. MG-I are assumed to be involved in aerobic ammonia oxidation (Pester et al. 2011), although they are also found in anaerobic sediments (Rasigraf et al. 2017, and references therein). Some members of MG-I can also use organic N compounds for growth, without possessing the aerobic ammonia-oxidizing enzyme complex and the ability for ammonia oxidation (Weber et al. 2015). Given that aerobic ammonium oxidation at these sites is limited to the upper few centimeters of the sediment (Fig. 4),

Bothnian Sea MG-I are likely to possess the capacity for an anaerobic lifestyle.

Interestingly, both coastal sites revealed relatively high abundances of *Bathyarchaeota* and *Thaumarchaeota* Group C3 (G-C3), while at site US5B these groups were in the minority. Available genomes of *Bathyarchaeota* suggest their involvement in methylotrophic methanogenesis, anaerobic methanotrophy (Evans et al. 2015; Harris et al. 2018), detrital protein degradation, and/or fermentative acetate production (Lloyd et al. 2013; He et al. 2016; Lazar et al. 2016). The metabolism of *Thaumarchaeota* G-C3 remains somewhat enigmatic since no enrichments or genomic sequences are available yet. Some members are, however, involved in acetate consumption in SO_4^{2-}-reducing marine and estuarine sediments (Webster et al. 2010; Na et al. 2015). Hence, their distribution may reflect the quality and quantity of fermentable organic matter in the sediments.

Euryarchaeota were dominated by the methanotrophic clade ANME-2a/b at all sites while no other ANME clades were detected. The ANME-2a/b clade has been detected in many marine and brackish sediments including the Baltic Sea (Treude et al. 2005). Some studies suggest a preference for sediments with low CH_4 and H_2S concentrations (Roalkvam et al. 2011, 2012) as also observed in our study. At our sites, the relative abundance of ANME-2 peaked in the SMTZ (Fig. 5). Below the SMTZ, ANME-2 16S rRNA biomarkers showed variations with depth. Strikingly, the variations in relative abundance of ANME-2 coincide with variations in sediment Fe (Figs. 3, 5). ANME-2a have been linked to the oxidation of CH_4 in both the presence and absence of SO_4^{2-}, in the latter case through direct electron transfer onto artificial shuttles (McGlynn et al. 2015; Scheller et al. 2016). Previous research using sediment incubations has indicated that Fe oxides stimulate CH_4 oxidation in methanic sediments at site US5B (Egger et al. 2015*b*). Whether the Bothnian Sea ANME-2a organisms are able to use Fe oxides as an electron acceptor is not known. However, our results for this site and NB8 also point toward a link between anaerobic methanotrophy and Fe availability.

Other members of *Euryarchaeota* prevalent in the Bothnian Sea sediments included the families *Methanosaetaceae*, *Methanosarcinaceae*, *Methanoregulaceae*, and *Methanobacteriaceae*. Members of these groups possess methanogenesis pathways which differ in their substrate utilization. *Methanobacteriaceae* and *Methanoregulaceae* are mainly hydrogenotrophic (Oren 2014; Imachi and Sakai 2015), *Methanosaetaceae* are strict acetotrophs which are adapted to low acetate concentrations (Jetten et al. 1992) and *Methanosarcinaceae* are metabolically flexible and are able to utilize a variety of substrates for CH_4 production, but appear to possess lower affinities for acetate (Jetten et al. 1992; Liu and Whitman 2008). Acetate and hydrogen are major fermentation products in marine sediments, thus their availability will control the diversity and activity of resident methanogens. Previous research for US5B using CH_4 isotopes has shown that hydrogenotrophic methanogenesis is the most likely methanogenic pathway at station US5B (Egger et al. 2015*b*), in line with work for other areas in the Baltic Sea (Parkes et al. 2007; Pimenov et al. 2010, 2012; Beulig et al. 2018).

There was a substantial difference in bacterial populations in the upper sediment layers between the near coastal and

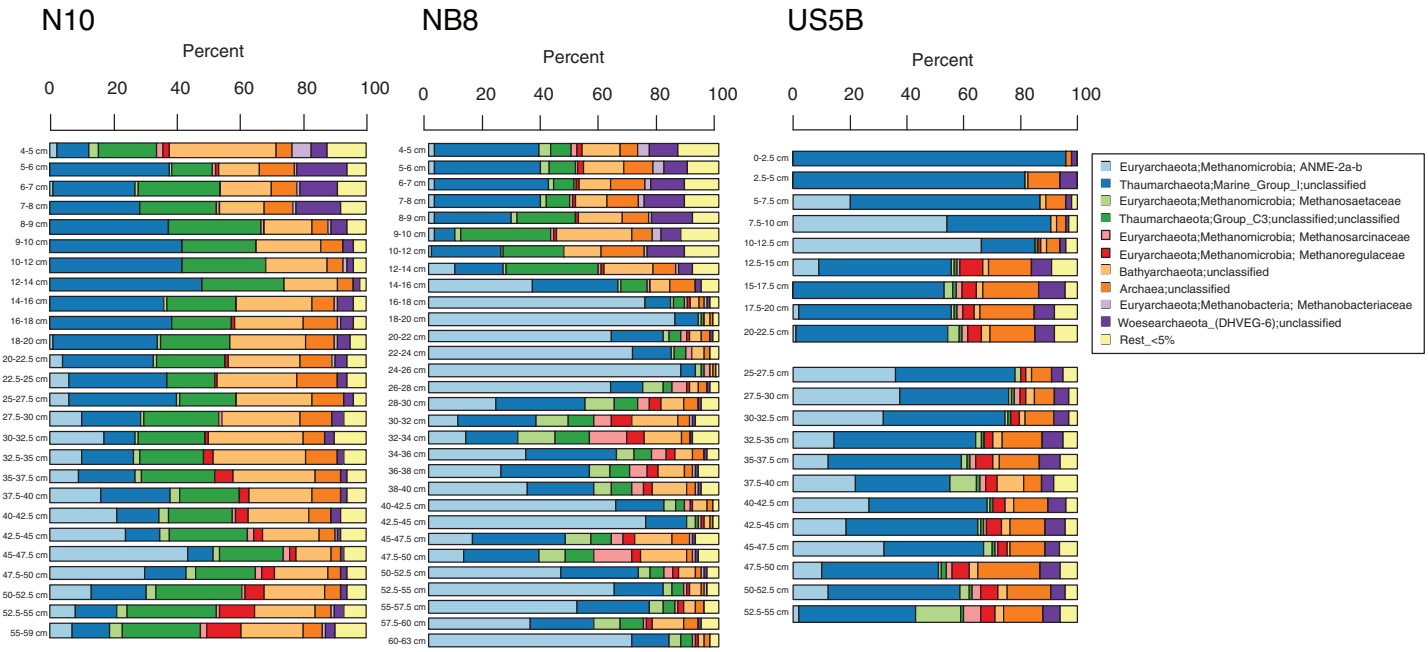

Fig. 5. Distribution of archaeal taxons based on 16S rRNA amplicon analysis for sediment transects from N10, NB8, and US5B sites in the Bothnian Sea.

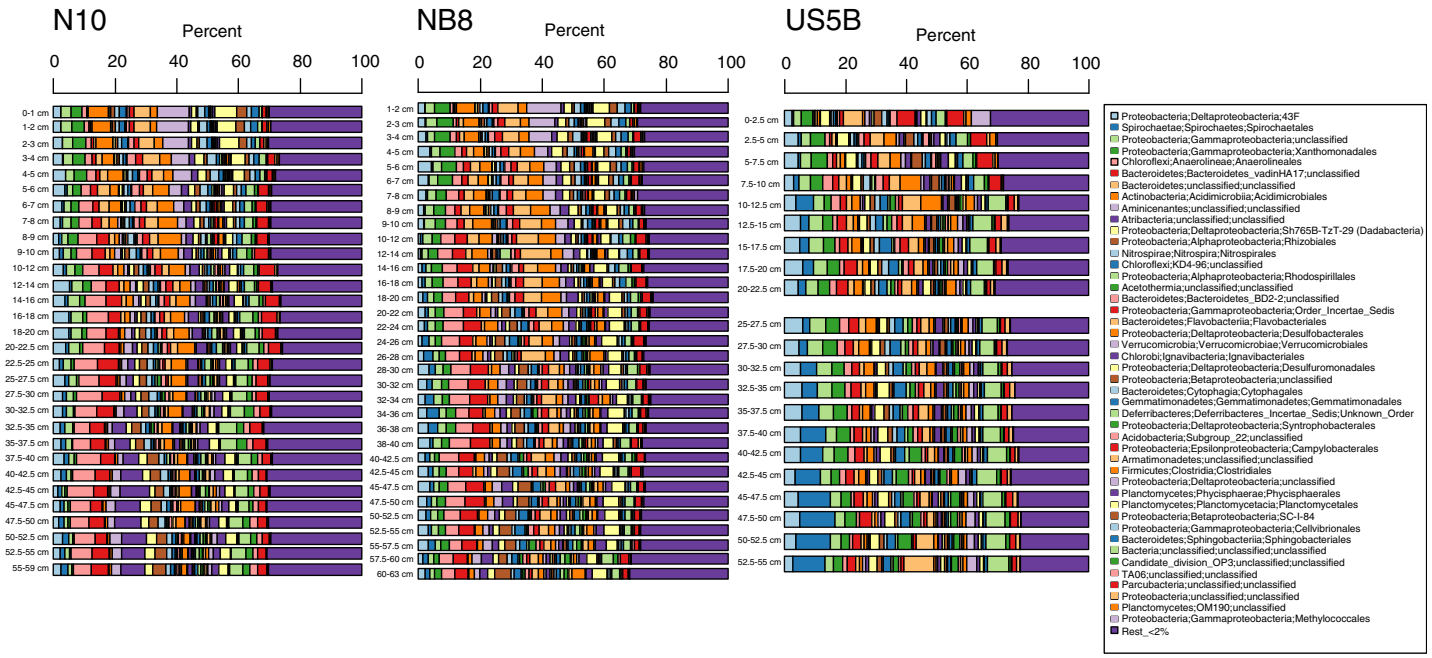

Fig. 6. Distribution of bacterial taxons based on 16S rRNA amplicon analysis for sediment transects from N10, NB8, and US5B sites in the Bothnian Sea.

offshore sites. At the offshore site US5B, an apparent population of aerobic CH_4 oxidizing bacteria (MOB) represented by *Methylococcaceae* was detected (6% of total bacterial reads). In contrast, this group was significantly lower in abundance at coastal sites with only few detected sequences. An enrichment of MOB close to the sediment surface points to a less efficient CH_4 removal in deeper anoxic layers at US5B. CH_4 that is not consumed in the SMTZ diffuses toward the sediment surface and fuels aerobic methanotrophic communities. The absence of surface sediment MOB communities at the coastal sites could be a result of either more efficient removal within the SMTZ (NB8) or a lower production by methanogens in deeper layers as seen at site N10. At site US5B, also an abundance of *Campylobacterales* was observed in the uppermost sediment layer, as already detected in our previous study (Rasigraf et al. 2017). Members of this group use sulfide and other reduced S species as electron donors and are indicative of sulfide availability near the sediment surface. Thus, the detection of a high abundance of sulfide and methane oxidizing bacterial taxa points to key geochemical differences between the locations in the Bothnian Sea. Geochemical profiles of sulfide and methane show that, in contrast to the coastal sites, the SMTZ is located quite close to the sediment surface at the offshore site US5B. The composition of the surface bacterial community is thus indicative of the diffusion of sulfide and methane up to oxic layers where they can be scavenged by aerobic/facultative aerobic taxa.

The putatively fermentative bacterial taxa were represented by *Spirochaetales*, *Anaerolineales*, *Bacteriodetes* VadinHA17, and *Atribacteria* (former candidate divisions "OP9" and "JS1"). Members of these groups make up a bulk of sedimentary anaerobic communities in mesophilic sediments and were shown previously to employ chemo-organotrophic lifestyles (Yamada et al. 2006; Breznak and Warnecke 2008; Hug et al. 2013; Bolhuis et al. 2014; Miyazaki et al. 2014; Carr et al. 2015; Shivani et al. 2015; Harrison et al. 2016; Nobu et al. 2016). Their distribution differed between the coastal and offshore sites corroborating differences observed in putatively fermentative archaeal taxa. *Spirochaetales* sequences were relatively more abundant in US5B sediments, while *Anaerolineales* and *Bacteroidetes* prevailed at both coastal sites. In contrast, *Atribacteria* were more prevalent in the sediments of N10, while showing lower abundance at NB8 and US5B. Sequences of *Atribacteria* have been detected previously in anaerobic environments where their abundance strongly correlated with concentrations of dissolved methane and iron suggesting an involvement in Fe-dependent AOM (Oni et al. 2015*a*) and sediments dominated by SO_4^{2-}-dependent AOM (Harrison et al. 2009). This correlation was also evident at site N10 where *Atribacteria* showed the highest abundance in depths below 30 cm coinciding with measurable CH_4 and the abundance of ANME. Carr et al. 2015 also identified a strong correlation of *Atribacteria* abundance and dissolved CH_4 profiles in Arctic marine sediments. This correlation was suggested to be based on metabolic cooperation with methanogens which would scavenge fermentation products of *Atribacteria*, primarily acetate (Carr et al. 2015).

Other abundant bacterial groups putatively involved in respiration of oxidized S and N compounds included members of *Xanthomonadales* and *Desulfobacterales*. These organisms appear to be ubiquitous in sediments of the Bothnian Sea and other brackish sediments (Leloup et al. 2007; Ruff et al. 2015;

Dyksma et al. 2016; Mußmann et al. 2017; Rasigraf et al. 2017). *Xanthomonadales* were shown to be the major players in sedimentary dark CO_2 fixation while being involved in sulfide oxidation (Dyksma et al. 2016). In the Bothnian Sea, sequences of *Xanthomonadales* were detected at all sites and throughout the whole sediment profiles pointing to their important role in the S cycle. Also, *Desulfobacterales* which employ sulfate reduction as the main metabolic lifestyle (Pfennig et al. 1981) were abundant at all sites and prevailed in sediment layers where SO_4^{2-} was still available. The peak of their abundance also coincided with the SMTZ and the abundance of ANME archaea at all sites. Some members of the *Desulfobacterales* are frequently observed partners in ANME/SRB consortia, where they perform SO_4^{2-} reduction and scavenge the reducing equivalents from ANME (Schreiber et al. 2010). Different ANME clades prefer certain SRB groups as partners, and it has been shown previously that ANME-2a are often detected together with SEEP-SRB1a—a clade belonging to *Desulfobacterales* (Schreiber et al. 2010). Our results are in line with previously published studies as the dominant ANME clade observed so far at all sites in the Bothnian Sea sediment belonged to ANME-2a. However, despite SO_4^{2-} being under the detection limit (75 μM) below the SMTZ at NB8 and US5B, a zone where Fe-dependent CH_4 oxidation was postulated to occur (Egger et al. 2015*b*), the presence of SRB was indicative of either a presence of a high flux of oxidized S-species or SRB performing other types of metabolisms (e.g., fermentation). Previous research has shown that SRB can switch their respiratory metabolism to fermentation when suitable electron acceptors are not available (Plugge et al. 2011). In such a situation, a cooperation with H_2-scavenging methanogens is feasible (Plugge et al. 2011). Thus, a sudden introduction of SO_4^{2-} could potentially activate their SO_4^{2-} metabolism.

At all sites, the distribution of *Desulfobacterales* was tightly followed by sequences assigned to *Flavobacteriales*, with one exception at site US5B where their abundance increased again below the 50-cm sediment depth. Members of this group were detected previously in an anaerobic methanotrophic enrichment originating from marine sediments (Jagersma et al. 2009). In active AOM cultures dominated by an ANME-2a archaeon, *Flavobacteriales* and *Desulfobacterales* together made up the bulk of the total bacterial sequences (Jagersma et al. 2009). Their metabolic role remained unclear, and possible involvement in S-compound transformations was discussed (Jagersma et al. 2009).

At both coastal sites, the upper sediment column with detectable SO_4^{2-} was dominated by the members of *Verrucomicrobiales*, while this group was hardly detectable at the offshore site US5B. Their distribution gradient within the sediment suggests an adaptation to a higher redox potential and possibly an aerobic/denitrifying lifestyle. *Verrucomicrobia* were previously shown to be abundant in marine water columns and sediments and to be mostly involved in polysaccharide degradation (Martinez-Garcia et al. 2012; Cardman et al. 2014). A similar distribution of *Verrucomicrobia* sequences was observed previously in surface sediments of the North Sea and was linked to degradation of fresh algal biomass (Oni et al. 2015*b*). Thus, based on previous studies, the difference in the distribution of *Verrucomicrobiales* further corroborates differences in organic matter quality with water depth in the Bothnian Sea. The differences in prevalence of core community archaeal and bacterial groups with depth in the sediment and between sites as described above is likely linked to the offshore increase in rates of sedimentation and degradation of organic matter (Figs. 2, 4). This affects the redox zonation and processing of organic matter (Figs. 2, 4) including the production of intermediary metabolites. Increased inputs of organic matter lead to compression of redox zones and a more limited vertical distribution of associated microbial communities. This is particularly evident when comparing the geochemical and microbial profiles: for example, at N10, the zonation over the full depth of 55 cm is broadly similar to that over the upper 20–25 cm of sediment at site NB8. However, this does not imply that the redox zones are identical: this is most evident when comparing the geochemical and microbial profiles for NB8 and US5B. Here, offshore changes in the quality and quantity of the organic matter may play a role, with possibly a lower contribution of relatively refractory terrestrial material and higher overall rates of organic matter degradation at the offshore site US5B.

Metagenomic analysis of the Fe-rich methanic sediment at site NB8 in the Bothnian Sea

Metagenome assembly and binning of sediment DNA from coastal site NB8 resulted in a retrieval of 53 bacterial and 11 archaeal genomic bins with variable degree of completeness (Supplementary Table S1, only bins with > 20% completeness, contamination level < 10% and > 0.1% proportion of total sequenced community were analyzed). In line with the abundance frequency in the 16S rRNA gene amplicon sequencing data, genome bins could be obtained for most abundant bacterial lineages including *Spirochaeta*, *Aminicenantes*, *Atribacteria*, *Chloroflexi*, *Actinobacteria*, *Bacteroidetes*, *Gemmatimonadales*, *Nitrospira*, *Planctomycetes*, *Parcubacteria*, *α*-, *β*-, *γ*-, *δ*-Proteobacteria, and archaeal lineages including *Thaumarchaeota*, *Bathyarchaeota*, *Thorarchaeota*, *Methanomassiliicoccales*, *Methanosaeta*, *Methanosarcina*, and ANME-2a. We analyzed all bins and draft genomes for the presence of marker genes involved in fermentation, autotrophy/acetogenesis, methanogenesis/-trophy and respiratory N and S cycles (Figs. 7, 8 and Supplementary Table S2).

Respiratory sulfur (S) cycle metabolism

In anaerobic sediments, Fe and manganese (Mn) oxides can undergo abiotic reactions with H_2S leading to its oxidation to either partially reduced sulfur species (PRSS, comprising thiosulfate, polysulfide, tetrathionate, sulfite, and elemental sulfur) or completely to SO_4^{2-} (Zopfi et al. 2004). Anoxic Bothnian Sea sediments below the SMTZ have been shown to contain high concentrations of Fe oxides which consist for

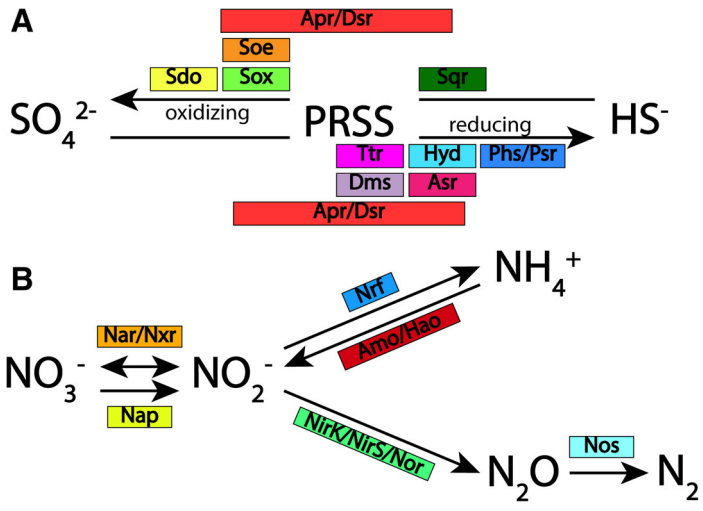

Fig. 7. Metabolic potential for respiratory sulfur (**A**) and nitrogen (**B**) cycle reactions identified in metagenomic bins obtained from the iron-rich methanic sediment at site NB8 in the Bothnian Sea. Key enzymes catalyzing each process are shown. PRSS, partially reduced sulfur species (include tetrathionate, thiosulfate, sulfite, polysulfide, elemental sulfur); Sdo, sulfur dioxygenase; Sox, sulfur-oxidizing multi-enzyme complex; Apr, adenylylsulfate (APS) reductase; Dsr, dissimilatory (bi)sulfite reductase; Sqr, sulfide:quinone oxidoreductase; Ttr, tetrathionate reductase; Asr, sulfite reductase; Hyd, sulfhydrogenase; Phs, thiosulfate reductase; Nar, nitrate reductase; Nxr, nitrate:nitrite oxidoreductase; Nap, periplasmic nitrate reductase; Nrf, nitrite reductase (NH_4^+ forming); Amo, ammonia monooxygenase; Hao, hydroxylamine oxidoreductase; Nos, nitrous oxide reductase; NirK, Cu-containing nitrite reductase (NO forming); NirS, Fe-containing nitrite reductase (NO forming); Nor, nitric oxide reductase.

> 50% of ferric (oxy)hydroxide (Slomp et al. 2013; Egger et al. 2015*a*; Lenstra et al. 2018). Thus, any free H_2S is likely to react with the Fe oxides or precipitate as FeS with Fe^{2+}. Partial oxidation of free H_2S with Fe oxides may lead to formation of SO_4^{2-} and thiosulfate (or other PRSS) which could act as an electron acceptor for organisms which would reduce the PRSS and SO_4^{2-} with donors such as acetate or H_2 back to H_2S (Zopfi et al. 2004). This is termed a cryptic S cycle (Holmkvist et al. 2011; Brunner et al. 2016).

In general, genes potentially involved in PRSS transformations were detected in most of the retrieved bacterial MAGs indicating a potential for an active S cycle in the methanic zone below the SMTZ (*see* Supplementary Information for analyzed genes). Among archaeal MAGs, the most widespread PRSS metabolism biomarkers encoded sulfhydrogenase (Hyd)-like proteins. However, genes encoding all four subunits (HydABDG) were only detected in bins assigned to *Thorarchaeota*, corroborating recent findings (Seitz et al. 2016). Other bacterial and archaeal MAGs mostly only encoded one or two of the four Hyd-like comprising subunits.

Biomarkers for dissimilatory SO_4^{2-} reduction to H_2S (Apr/Dsr) were detected in bacterial lineages *Bacteroidales*, *Xanthomonadales/Chromatiales*, *Aminicenantes*, *Syntrophobacterales*, and *Gemmatimonadales*. Many members of the *Syntrophobacterales* are SO_4^{2-} reducers that are frequently detected in anaerobic SO_4^{2-}-containing sediments (Plugge et al. 2011). The detection of Apr and Dsr in a *Gemmatimonadales* MAG was more surprising. Recently, similar observations were reported for *Gemmatimonadales* MAGs obtained from estuarine sediments (Baker et al. 2015), however no SO_4^{2-} reducers have been described so far from this group.

The detected Apr in one *Xanthomonadales/Chromatiales* MAG indicated its potential involvement in sulfite oxidation (Ghosh and Dam 2009; Müller et al. 2015). Members of *Chromatiales* and particularly *Ectothiorhodospiraceae* family are common in marine anoxic sediments and can employ a chemolithoautrophic lifestyle of either Fe- or reduced S compound oxidation (Hallberg et al. 2011; Dyksma et al. 2016).

Interestingly, homologues of a desulfoviridin-type dissimilatory sulfite reductase were detected in MAGs classified as ANME-2a and *Lokiarchaeota*. This type of sulfite reductase is involved in an energy-yielding reduction of sulfite to H_2S. This finding is particularly relevant in view of the anaerobic CH_4 oxidation potential in these sediments. ANME are typically associated with SRB in order to perform AOM, in which the bacterial partner would perform the reduction of SO_4^{2-} or PRSS to H_2S (Knittel and Boetius 2009). However, this finding indicates the potential of Bothnian Sea ANME to perform the reduction of sulfite intrinsically. The ability of some ANME to reduce sulfur species has been observed earlier (Milucka et al. 2012).

Several core community members of putative fermenters including *Anaerolineales*, *Bacteroidales*, *Atribacteria*, and *Aminicenantes* also encoded gene homologues for enzymes involved in PRSS transformations. This indicated flexible metabolic strategies providing the ability to adapt to fluctuating environmental conditions. Thus, the potential for reductive respiratory S cycle metabolisms seems widespread in core community bacterial taxa.

In the presence of energetically more favorable electron acceptors like NO_3^- or Mn^{4+}, reduced sulfur compounds can be completely oxidized to SO_4^{2-} (Zopfi et al. 2004). This process would represent a source of SO_4^{2-} and thus electron acceptor for SRB. In general, the potential for oxidative processes in the S cycle were less widespread than those involved in PRSS reduction. This redundancy in the potential for oxidative processes could be further explained with the lack or shortage of electron acceptors in this sediment layer. Thus, the residing microbial community would over time lose the ability for PRSS oxidation.

The results of respiratory S cycle analysis indicated the potential for PRSS reductive processes in many analyzed MAGs while that for oxidative ones was scarcer. These findings point to a possibility of a shorter operational S cycle where the abiotic oxidation of H_2S by the reactive Fe would create a pool of PRSS which would be reduced back to H_2S.

Respiratory nitrogen (N) cycle

In a recent study of Öre estuary sediment, nitrification–denitrification was shown to be the dominant sink for reactive

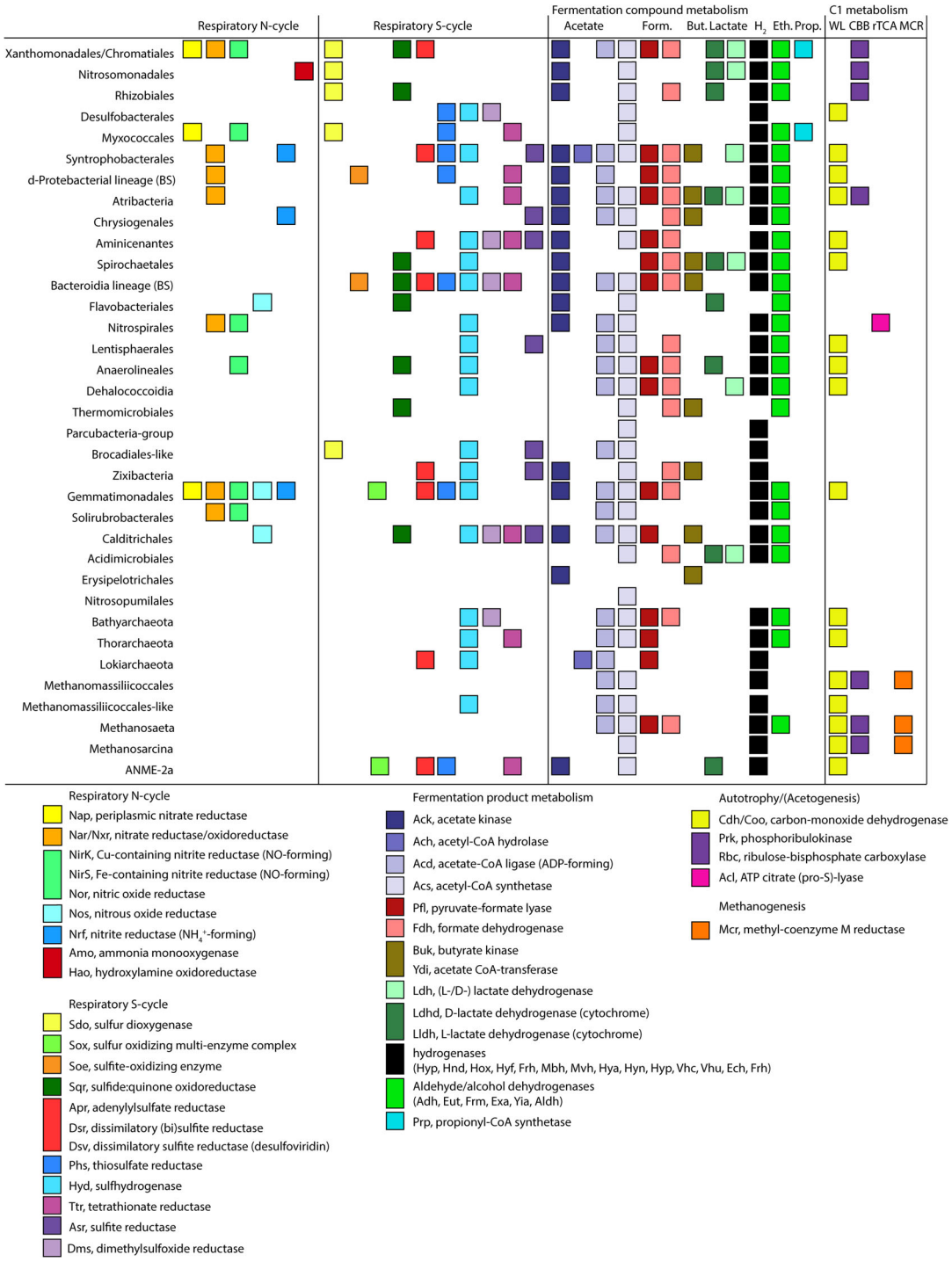

Fig. 8. Overview for presence of functional biomarkers in genome bins obtained from the Fe-rich methanic sediment layer at coastal site NB8 in the Bothnian Sea. Analysis was performed for key genes encoding enzymes involved in processes of respiratory nitrogen (N) and sulfur (S) cycles, fermentation product metabolism, autotrophy/acetogenesis, and methanogenesis. Form., formate; But., butyrate; Eth., ethanol; Prop., propionate.

N in the ecosystem while anaerobic ammonium oxidation (anammox) was not detectable (Hellemann et al. 2017). These activities are corroborated by the genetic potential of dominant taxa identified in this study.

Respiratory NO_3^- reduction can proceed via two types of reduction systems: a periplasmic (Nap) and membrane-bound nitrate reductase (Nar). The abundance of both Nap and Nar was similar among analyzed bacterial taxa. Only two groups,

Xanthomonadales/*Chromatiales* and *Gemmatimonadales*, contained both Nap and Nar. In addition, some *Gemmatimonadales* MAGs also contained genes encoding for enzymes catalyzing reduction of NO_2^- to nitric oxide (NO) (NirK) and reduction of nitrous oxide (N_2O) to N_2 (Nos). Thus, next to a wide spectrum of genes involved in fermentation product metabolism, SO_4^{2-} and PRSS reduction, the order *Gemmatimonadales* appears to possess high metabolic diversity.

The putative SO_4^{2-} reducing *Syntrophobacterales* encoded a Nar and cytochrome c nitrite reductase (Nrf). Previous studies with characterized SRB have shown the preferred use of NO_3^- as an electron acceptor when available (Krekeler and Cypionka 1995). The capability for DNRA seems to be a common trait among SRB and indicates a flexible metabolism depending on the availability of electron acceptors and a coupling of dissimilatory N and S cycles.

Functionally, dissimilatory NO_3^- reduction can be decoupled from further steps of NO_2^- reduction and an organism can excrete NO_2^- which can be further used as an electron acceptor by other community members. The fate of NO_2^- then differs depending on genomic potential. It can be either reduced to NO and then to N_2O or reduced in one step to NH_4^+ by Nrf. NO is a toxic and very reactive metabolite which is usually processed by the cell immediately. Thus, we analyzed the presence of NO-forming nitrite reductases (NirK/NirS) in combination with NO reductases (Nor) as one module for denitrification to N_2O. The product of this process is N_2O which again can be either excreted into the environment or further reduced to N_2. N_2O reduction can be performed by a different functional group of denitrifiers. Both denitrification modules, to N_2O and to N_2, were spread among the retrieved MAGs indicating functional redundancy and truncation in metabolic denitrification potential. However, activity measurements performed by Hellemann et al. (2017) revealed that the contribution of N_2O production to total denitrification in Öre Estuary sediments was below 1% and was thus negligible.

The potential for DNRA was only present in *Syntrophobacterales*, *Gemmatimonadales*, and *Chrysiogenales*. The activity of either DNRA or denitrification for NO_2^- reduction would depend on the quality and availability of electron donors in the estuary sediment system.

Nitrification potential was assessed by the presence of Amo/Hao and Nxr encoding genes. Amo/Hao catalyzes the oxidation of ammonia to NO_2^-, which can then be used by NO_2^- oxidizers such as *Nitrospira* for further oxidation to NO_3^- by an Nxr. Both processes require a potent electron acceptor such as oxygen (O_2). Two MAGs assigned to putative ammonia oxidizers could be retrieved from the analyzed sediment. One was assigned to *Nitrosomonadales*, a bacterial order which includes many characterized ammonia oxidizers, and another to archaeal *Thaumarchaeota*. However, only the *Nitrosomonadales* MAG contained both Hao and Amo, the thaumarchaeal MAG lacked the genes encoding for Hao/Amo. However, since the latter was only 46% complete, it is likely that ammonia oxidation pathway encoding genes were not binned into the MAG. Both AOB and AOA might be participating in the oxidation of NH_4^+ to NO_2^- in the coastal sediment. Nxr was only detected in one MAG classified as *Nitrospirales*, an order which includes many characterized NO_2^- oxidizers widespread in natural ecosystems (Lücker et al. 2010). However, no O_2 is available for nitrification metabolism at the analyzed depth in the methanic zone below the SMTZ. It has been shown previously that O_2 penetration depths are restricted to the upper centimeter in the Öre Estuary sediments (Hellemann et al. 2017; Lenstra et al. 2018). The presence of aerobic nitrifiers in anoxic environments has been frequently observed in the past and an alternative anaerobic metabolism was discussed as a possible lifestyle strategy (Abeliovich and Vonshak 1992; Weber et al. 2001; Schmidt et al. 2002). The possibility of nitrification coupled to metal oxide reduction involving, for example, Fe and Mn oxides, has also been suggested (Luther et al. 1997; Hulth et al. 1999; Thamdrup and Dalsgaard 2000; Mogollón et al. 2016). An alternative explanation would be a dormant nitrifier community which was preserved at depth due to fast sedimentation and slow degradation rates. Importantly, we find that even in the complete absence of O_2 and despite the activity of alternative anaerobic processes, the analyzed sediment still contains a genetic potential for O_2-dependent nitrification. Anammox biomarkers were not detected in the analyzed sediment which is consistent with the results of Hellemann et al. (2017).

Fermentative metabolism

Fermentative processes are of central importance in sediment ecosystems since anaerobic degradation of deposited organic matter yields a variety of short chain fatty and carboxylic acids and H_2 which can be further used in respiratory processes for the reduction of oxidized N, S, and Fe species, methanogenesis, and homoacetogenesis (Finke et al. 2007). The production and consumption of those organic and inorganic (H_2) intermediates depend on factors such as sediment pH, temperature, quality of the deposited organic matter, and availability of inorganic electron acceptors. These factors are expected to vary depending on seasonality, external input variability, bioturbation, and sedimentation rates. Thus, the presence of gene biomarkers only represents the potential of the system for the analyzed processes and not the actual metabolite flows (the analyzed gene biomarkers are listed in Supplementary Material).

In marine and brackish sediments where the pH is usually between 7 and 7.5, major fermentation products comprise acetate, formate, ethanol, propionate, butyrate, lactate, and H_2 (Shaw and McIntosh 1990; Glombitza et al. 2019). Most enzymes involved in transformations of these metabolites can catalyze reactions in both directions which will depend on environmental conditions and the employed metabolism by the organism.

In general, most MAGs contained the genes encoding either one or several enzymes involved in acetate metabolism.

(ADP-forming) acetate-CoA ligase (Acd) and acetyl-CoA synthetase (Acs) were the most widespread biomarkers among both bacterial and archaeal MAGs including *Thorarchaeota*, *Bathyarchaeota*, and *Lokiarchaeota*. Thus, those archaea could contribute to fermentative acetate production or assimilation in methanic sediments below SMTZ.

The potential for formate metabolism was assessed by the presence of pyruvate-formate lyase (Pfl) which catalyzes formate formation from pyruvate, and formate dehydrogenase (Fdh/Fdo) for formate oxidation. Both genes were widely distributed among bacterial and archaeal MAGs. Many putative fermenters including *Spirochaetales*, *Bacteroidales*, *Aminicenantes*, and *Atribacteria* contained both. Among archaeal MAGs, the potential for acetate and formate turnover was widespread corroborating previous results for *Bathyarchaeota* and *Thorarchaeota* being potentially involved in fermentative production of acetate and formate (Lazar et al. 2016; Seitz et al. 2016).

The potential for ethanol metabolism which was assessed by the presence of aldehyde and alcohol dehydrogenases (Aldh/Adh/Exa/Frm/Yia/Eut) appeared to be one of the most widespread traits among the retrieved bacterial MAGs. This was an indication for ethanol being next to acetate and formate an important metabolite in the analyzed sediment system. Among archaea, however, it was only detected in *Bathyarchaeota*, *Thaumarchaeota*, and *Methanosaeta*.

Lactate metabolism was assessed by the presence of cytochrome- (Lldh/Ldhd) and NAD(P)-dependent (Ldh) lactate dehydrogenase encoding genes. Several bacterial MAGs contained lactate utilization biomarkers. In archaea, only the highly incomplete MAG assigned to ANME contained an Lldh-like gene. The rest of archaeal bins did not seem to possess capacity for lactate metabolism.

The capacity for H_2 metabolism was assessed by the presence of genes encoding for subunits of various types of hydrogenases. Overall, genes encoding hydrogenase subunits or hydrogenase maturation pathways were detected in most of the analyzed bacterial and archaeal MAGs indicating the central importance of H_2 in the analyzed sediment ecosystem. Putative bacterial fermenters including *Spirochaetales*, *Bacteroidales*, *Anaerolineales*, *Aminicenantes*, and SRB contained genes encoding for several hydrogenase systems indicating the capacity for adaptation to ambient fluctuations in metabolite concentrations. One *Gemmatimonadales* MAG which encoded the whole SO_4^{2-} reduction pathway also contained genes encoding two types of hydrogenases indicating a possibility for H_2 being an electron donor for SO_4^{2-} reduction. Similarly, also *Syntrophobacterales* which encoded the full SO_4^{2-} reduction pathway revealed a wide H_2 utilization potential via different hydrogenases. In general, all genomes with a Dsr encoded for one or several hydrogenases. Interestingly, also most archaeal MAGs contained genes encoding several hydrogenases indicating their important role in H_2 metabolism in Bothnian Sea sediments.

CO_2 fixation and acetogenesis

Next to the organic matter input which is eventually turned over into CO_2, energy and new biomass by heterotrophic organisms, CO_2 fixation by autotrophs represents another organic carbon input route into the sediment ecosystem. In deep anoxic sediments, many autotrophs gain energy from the oxidation of inorganic electron donors such as H_2S, PRSS, H_2, and reduced metals. In natural systems, CO_2 can be fixed via several pathways which are summarized by Berg (2011).

Here, the Wood–Ljungdahl (WL) pathway is not only indicative of autotrophy, but can also be used for acetate production by acetogens or assimilation of acetate, CO, or methylamines (Berg 2011). Biomarker genes encoding the CO dehydrogenase (Cdh/Coo), the key enzyme of WL pathway, were widespread among both bacterial and archaeal MAGs. As expected, the putative *Syntrophobacterales* SRB and other δ-proteobacterial MAGs contained Cdh/Coo biomarkers. Previous research showed that SRB utilize the WL pathway in both reductive and oxidative directions (Schauder et al. 1988). One of the bins classified as *Lentisphaerales* contained Cdh/Coo biomarkers. So far, no reports on the presence of the WL pathway in these organisms are available. Thus, they might represent novel acetate producers/scavengers in methanic sediments.

Also putative fermenters including *Spirochaetales*, *Anaerolineales*, *Aminicenantes*, and *Atribacteria* contained Cdh/Coo-encoding biomarkers. In fermenters, the WL pathway was discussed to function as an electron sink by reduction of CO_2 to acetate (Berg 2011). Thus, those organism groups might be producing acetate via this route during fermentation. In fermentative *Chloroflexi*, the WL pathway was suggested to be operating either under heterotrophic conditions to reduce the intracellular CO_2 by simultaneous oxidation of reduced ferredoxin and NADH, or under autotrophic conditions for CO_2 fixation (Ragsdale and Pierce 2008; Hug et al. 2013; Fullerton and Moyer 2016). In many of the same MAGs which contained Cdh/Coo biomarkers, we detected genes encoding for pyruvate-ferredoxin oxidoreductase (Pfor) which might point to a link between autotrophic CO_2 fixation via WL pathway and TCA cycle via acetyl-CoA (Furdui and Ragsdale 2000).

Cdh/Coo biomarkers were widespread among archaeal MAGs including methanogens, *Bathyarchaeota* and *Thaumarchaeota*. Methanogens use an archaeal variant of the WL pathway which is employed in hydrogenotrophic and acetotrophic methanogenesis (Berg 2011; Borrel et al. 2016). Interestingly, we also detected Cdh/Coo in the retrieved *Methanomassiliicoccales* MAG. Recent research has shown that in contrast to most methanogens *Methanomassiliicoccales* possess a bacterial type CO dehydrogenase which might function in the oxidative direction (Adam et al. 2018).

The detection of Cdh/Coo and other enzymes of the archaeal-type methylotrophic branch of WL pathway in the retrieved *Bathyarchaeota* MAGs further corroborated previous findings for this group of archaea (He et al. 2016; Lazar et al.

2016). *Bathyarchaeota* have been discussed to employ WL pathway for acetogenesis (He et al. 2016; Lazar et al. 2016). Similarly, Cdh/Coo and other genes encoding for the archaeal variant of the WL pathway were present in *Thorarchaeota* MAGs which hints to their involvement in acetogenesis in the analyzed sediment system.

Next to the WL pathway which is mainly found in anaerobic organisms operating close to the thermodynamic limit, Calvin–Benson–Bassham (CBB) cycle is employed by a variety of chemolithoautotrophic organisms and can operate under higher redox potentials (Berg 2011). We analyzed the presence of two biomarkers which are unique to the CBB cycle: phosphoribulokinase (Prk) and *ribulose*-1,5-*bisphosphate carboxylase* (Cbb). Among bacterial MAGs, both Cbb and Prk were only detected in *Xanthomonadales/Chromatiales* and *Nitrosomonadales*. The ability for CO_2 fixation via the CBB cycle has been recently reported to be widespread among γ-proteobacterial lineages *Woeseiaceae*/JTB255 which belong to the core community in diverse marine sediments (Mußmann et al. 2017) and to which Bothnian Sea *Xanthomonadales/Chromatiales* were closely related. Several genomes have been shown to encode biomarkers of CBB cycle, truncated denitrification pathway to N_2O, and PRSS oxidation to SO_4^{2-} (Dyksma et al. 2016; Mußmann et al. 2017). Similarly, also *Xanthomonadales/Chromatiales* retrieved within this study contained biomarkers for PRSS transformations, denitrification, and CBB cycle. Thus, these ubiquitous γ-Proteobacteria could be involved in chemolithoautotrophic PRSS oxidation coupled to denitrification to N_2O in shallow brackish sediments. The detection of CBB biomarkers in the obtained *Nitrosomonadales* MAG was in accordance with characterized chemolithoautotrophic metabolism of this organism group (Utåker et al. 2002). However, as *Nitrosomonadales* might be involved in an alternative anaerobic metabolism in the analyzed sediment, it remains unknown whether their CBB cycle is functional. This could be elucidated by future transcriptomic studies on this ecosystem.

Among archaea, Cbb encoding genes were detected in methanogens. Various methanogens have been shown previously to possess Cbb biomarkers, however their functionality remained debated. Recently, a functional pathway involving Cbb and Prk, similar to CBB cycle in autotrophic organisms, was proposed for methanogens (Kono et al. 2017). However, the ability for autotrophy based on this pathway among methanogens remains unclear (Kono et al. 2017).

Nitrospirales was the only retrieved lineage which contained *ATP citrate lyase* (Acl), the biomarker for the *reductive citric acid cycle* (rTCA). NO_2^--oxidizing *Nitrospira* have been reported to employ rTCA cycle for CO_2 fixation (Lücker et al. 2010). Thus, this autotrophic pathway seems to be restricted to only one dominant group of bacteria retrieved from the analyzed sediment.

Methanogenesis/-trophy

Three of the obtained archaeal MAGs could be classified as methanogens: *Methanosaeta*, *Methanosarcina*, and *Methanomassiliicoccales*. Methyl-coenzyme M reductase (Mcr) was either partially or fully encoded in all three MAGs. Members from these groups represent different functional groups within methanogens. Despite high incompleteness of the obtained *Methanosarcina* MAG, we identified genes encoding several complexes involved in methylotrophic metabolism. Both, *Methanosarcina* and *Methanosaeta* are abundant core community members in anaerobic methanic sediments (Webster et al. 2015; Carr et al. 2017). In contrast, the distribution of *Methanomassiliicoccales* methanogens in natural methanic sediments is underexplored. Originally, all described *Methanomassiliicoccales* were isolated or enriched from intestinal tracts of animals (Dridi et al. 2012). Since then, biomarkers of *Methanomassiliicoccales* have been detected in various sediment ecosystems and their distribution was investigated in more detail recently (Becker et al. 2016; Speth and Orphan 2018). All physiological and genomic information available so far point to a strictly H_2-dependent methylotrophic methanogenesis (Borrel et al. 2016). Interestingly, one archaeal genome (metabat2.27) obtained from the Bothnian Sea sediment was closely related to *Methanomassiliicoccales* but lacked Mcr and other several methanogenic biomarkers. It is related to several other genomic bins obtained from an aquifer (Anantharaman et al. 2016) and together forms a separate lineage basal to the *Methanomassiliicoccales* lineage (Borrel et al. 2019).

Our amplicon sequencing data revealed that methanotrophic ANME-2 archaea were among the most abundant groups of archaea in the analyzed depth intervals. However, only one highly incomplete (22.6%) MAG classified as ANME-2a could be retrieved from the analyzed sediment. The MAG did not contain Mcr biomarkers so we analyzed the metagenome for the total *mcrA* inventory by blastx analysis (Supplementary Fig. S2). The result revealed ANME-like and *Methanosarcina*-like *mcrA* gene reads to make up the majority of the total *mcrA* pool.

Our results show that methanogenesis in the coastal methanic sediments of Bothnian Sea can potentially proceed via acetoclastic, hydrogenotrophic, and methylotrophic pathways. However, previous studies have shown that the hydrogenotrophic activity prevails. At the same time, putatively methanotrophic ANME-2a could scavenge the produced methane by reduction of PRSS or Fe oxides, either alone or in partnerships with abundant SRB.

Conclusions

The obtained MAGs and total functional gene analysis of dominant organisms from the coastal methanic sediments in the Bothnian Sea indicated a wide genetic potential for a respiratory S cycle via PRSS transformations and diverse fermentation metabolisms with acetate, alcohols, and H_2. The potential for the respiratory N cycle was dominated by denitrification over DNRA. There was also potential for methanogenesis through acetoclastic, hydrogenotrophic, and methylotrophic pathways. Methanotrophic archaeal lineage ANME-2a dominated the Euryarchaeal population and variations in their

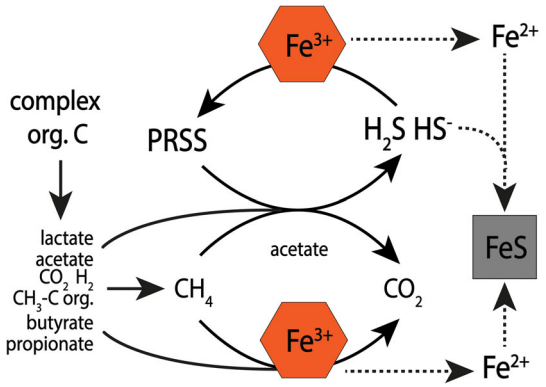

Fig. 9. Simplified overview of predicted processes involving Fe and sulfur to take place in the analyzed coastal anaerobic sediments in the Bothnian Sea at site NB8. Ferrihydrite would represent the major electron acceptor in the analyzed sediments and be involved in the biotic and abiotic oxidation of sulfide and fermentation products. Anaerobic oxidation of methane could potentially be fueled by the reduction of PRSS formed by ferrihydrite or ferrihydrite directly. Acetate would be one of the key metabolites formed during the primary and secondary fermentations. The reduced Fe would react with the free sulfide and form insoluble iron sulfides.

abundance correlated with solid phase Fe contents. Despite similar pore-water profiles of CH_4, Fe, and SO_4^{2-}, the bacterial and archaeal populations in the sediments changed with distance from the shoreline, which we attribute to an offshore increase in organic matter input, sedimentation, and degradation rates. With the PRSS, Fe oxides are thought to play a critical role in the observed redox transformations in the Bothnian Sea methanic sediment system (Fig. 9). For future studies, enrichments and physiological characterizations of dominant communities would improve our understanding of their physiology, in situ biological function, and biogeochemical impact. Furthermore, transcriptomic analysis of in situ sediment would add more information about the active communities and functions in these sediments.

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Acknowledgments

We would like to thank Theo van Alen and Geert Cremers for sequencing the metagenomes. The captain, crew, and scientific participants aboard R/V *Lotty* are thanked for their assistance during sampling in August 2015. This work was carried out on the Dutch national e-infrastructure with the support of SURF Cooperative. LABGeM (Genoscope, Institute of Genomics, CEA Sciences) and the National infrastructure "France Génomique" are acknowledged for support within the MicroScope annotation platform. O.R., M.S.M.J., and C.P.S. were supported by NESSC (Netherlands Organization for Scientific Research, grant number 024.002.001), J.F. and M.S.M.J were supported by the SIAM Gravitation Grant on Anaerobic Microbiology (Netherlands Organization for Scientific Research, grant number 024.002.002) and European Research Council (ERC AG, grant number 339880), C.P.S., W.L., and N.A.G.M.v.H. were supported by NWO (grant number 865.13.005) and the European Union and FORMAS through BONUS COCOA (grant number 2112932-1).

Conflict of interest

None declared.

Submitted 20 February 2019
Revised 14 June 2019
Accepted 25 September 2019

Associate editor: Hans-Peter Grossart