

Thriving or surviving? Evaluating active microbial guilds in Baltic Sea sediment

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Summary

Microbial life in the deep subsurface biosphere is taxonomically and metabolically diverse, but it is vigorously debated whether the resident organisms are thriving (metabolizing, maintaining cellular integrity and expressing division genes) or just surviving. As part of Integrated Ocean Drilling Program Expedition 347: Baltic Sea Paleoenvironment, we extracted and sequenced RNA from organic carbon-rich, nutrient-replete and permanently anoxic sediment. In stark contrast to the oligotrophic subsurface biosphere, Baltic Sea Basin samples provided a unique opportunity to understand the balance between metabolism and other cellular processes. Targeted sequencing of 16S rRNA transcripts showed *Atribacteria* (an uncultured phylum) and *Chloroflexi* to be among the dominant and the active members of the community. Metatranscriptomic analysis identified methane cycling, sulfur cycling and halogenated compound utilization as active *in situ* respiratory metabolisms. Genes for cellular maintenance, cellular division, motility and antimicrobial production were also transcribed. This indicates that microbial life in deep subsurface Baltic Sea Basin sediments was not only alive, but thriving.

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Introduction

Marine sediments are the largest reservoir for organic matter (Hedges and Keil, 1995; Arndt *et al.*, 2013) that may harbour as many microbial cells as the global ocean (Kallmeyer *et al.*, 2012; Parkes *et al.*, 2014). Over the past several decades, numerous studies have described the microbial communities of marine sediments using 16S gene sequencing, and more recently, metagenomics and single-cell genomics (Parkes *et al.*, 1994; Reed *et al.*, 2002; Biddle *et al.*, 2006; Inagaki *et al.*, 2006; Lloyd *et al.*, 2013; Orcutt *et al.*, 2013). However, far fewer analyses have demonstrated *in situ* activities of subsurface microbes, and many of these are from basalt (Lever *et al.*, 2013; Robador *et al.*, 2014) or based on modelling (Roy *et al.*, 2012). These studies indicate that diverse metabolic strategies support microbes in various environments, including aerobic respiration in sediments underlying gyres, sulfate reduction in subsurface fluids and methanogenesis in organic-rich sediments.

In 2014, sediments were recovered from the Baltic Sea Basin (BSB) via the Integrated Ocean Drilling Program (IODP) and the European Consortium for Ocean Research Drilling (ECORD) in order to understand the effect of depositional conditions (e.g., glacial lacustrine versus non-glacial) on current microbial communities. Often, organic matter availability limits growth in the oligotrophic subsurface biosphere (D'Hondt, 2004; Jorgensen and Boetius, 2007; Hoehler and Jorgensen, 2013). However, compared to many other subsurface sites, the BSB sediments are extremely rich in organic matter and are potentially more representative of coastal organic-rich sites. In BSB surface sediments (less than 1 meter below seafloor), diverse microbial assemblages with activities related to methane cycling, nitrate reduction and sulfate reduction have been observed (Thureborn *et al.*, 2013; 2016; Reyes *et al.*, 2017). However, the deeper, anoxic sediments, where many canonical electron acceptors (e.g., O₂, NO_x, SO₄²⁻) are depleted, have not been similarly characterized.

Microbial activity in the deep biosphere has been inferred from cultivation-based approaches, stable isotope fractionations, laboratory incubations and metagenomic analyses. Long doubling times, 'bottle effects' and resistance to isolation complicate cultivation-based and

incubation approaches (Orcutt *et al.*, 2013). Abiotic isotopic fractionation can overprint biological fractionation (Shanks, 2001; Sim *et al.*, 2011). Traditional metagenomic analyses are often hindered by the presence of dead or dormant microbes, unexpressed genes and extracellular DNA preserved in organic-rich sediments (Corinaldesi *et al.*, 2011; Torti *et al.*, 2015). However, DNA transcription has been shown to directly correlate with cellular activity (Jansson *et al.*, 2012). As such, RNA-based metatranscriptomic approaches have been employed as an activity proxy in several natural and medical environments (Gilbert *et al.*, 2008; Gosalbes *et al.*, 2011; Mason *et al.*, 2012), and more recently also in marine sediments (Orsi *et al.*, 2013; Urich *et al.*, 2013; Pachiadaki *et al.*, 2016). Here, we used metatranscriptomics and targeted quantification of 16S rRNA to examine the active microbial community abundance, structure and function of deeply buried sediments from IODP Expedition 347: Baltic Sea Paleoenvironment. Compared to other deep marine sediments studied, these Baltic Sea sediments are young (Holocene in age), sulfate-depleted and organic matter rich. These sediments represent an opportunity to characterize active microbial community function in a nutrient replete deep biosphere setting.

Results and discussions

Site description

The BSB is relatively shallow (average water depth 54 m), with a halocline due to density-driven stratification from freshwater runoff present at a depth of 60–80 m. This causes sub-basins to be seasonally or permanently hypoxic or anoxic (< 0.2 mg O₂ L⁻¹) (Carstensen *et al.*, 2014). The combination of eutrophic conditions in the surface water, shallow average water depth and regional dysoxia have resulted in high sedimentation rates (0.1–0.5 cm year⁻¹) across the BSB over the past ~11.7 kyr (Andrén *et al.*, 2015). In the present study, we focus on two BSB locations, the Landsort Deep and Little Belt (hereafter referred to as Sites M59 and M63 respectively) (Fig. 1A).

Site M59 (55°0.29'N, 10°6.49'E) is located at an entry-way where marine waters from the North Sea flow into the Baltic Sea (Fig. 1A). The top 46.2 m of sediment at this site was classified as biosiliceous clay deposited during marine to brackish phases of the Baltic Sea Basin (Andrén *et al.*, 2015), with TOC levels at 4.3–7.4 percent dry weight (wt%). Chloride-based salinity in the porewater is consistently high (23.75–24.57) in the top 15 m, and then decreases to a minimum of 7.0 by 65.38 meters below seafloor (mbsf). The shape of the

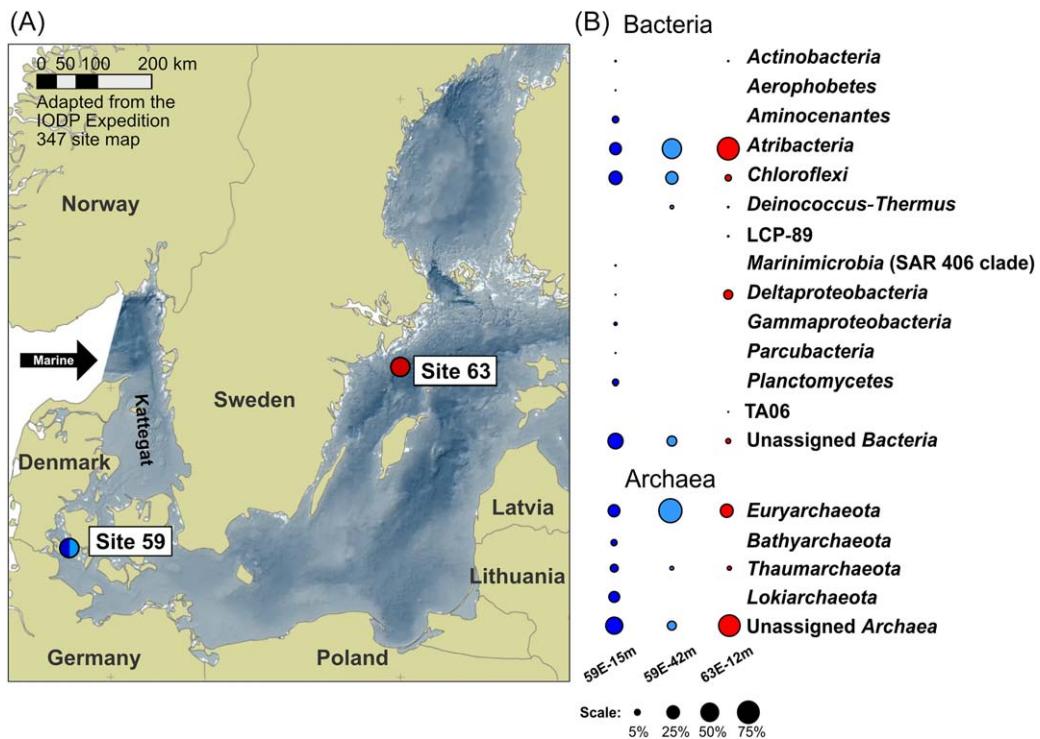


Fig. 1. A. Location of samples taken during IODP X347 and used in this study. B. Active microbial community structures based on 16S rRNA transcript sequencing. Bacterial and archaeal 16S rRNA transcripts were sequenced using domain-specific primers. Percentages represent the relative abundance of each phylum with respect to the total within each domain. The sample names are indicated below the Archaea.

porewater salinity profile indicated post-depositional diffusion of ions from the marine-influenced Holocene sediments to the underlying lacustrine sediments (Supporting Information Table S1).

At 459 m water depth, the Site 63E ($58^{\circ}37.34'N$, $18^{\circ}15.25'E$) is in the deepest part of the BSB. It experiences periodic anoxia that occasionally results in ferruginous or euxinic conditions (Hardisty *et al.*, 2016). The sediments at Site M63 were characterized by organic-rich clays. Nanofossils and porewater salinity indicate that sediment deposition in the top 25 m occurred during a marine-brackish phase (Andrén *et al.*, 2015). The highest salinity (12.5) was measured at 14.95 mbsf and decreased to < 1 in the glaciolacustrine sediments below 50 mbsf. The total organic carbon was 6.41 wt% in the marine sediment, and < 1 wt% in the glaciolacustrine sediments (Supporting Information Table S1).

Bacteria to Archaea ratios

Using droplet digital PCR, we determined the relative abundance of archaeal and bacterial 16S rRNA transcript copy numbers. Archaea comprised 2.7–4.7% of the total rRNA quantified and, correspondingly, Bacteria comprised 97.3–95.3%, with an average Archaea to Bacteria ratio of 1:26 (Supporting Information Table S2). A separate study on nearby sediment cores using quantitative PCR of 16S rRNA genes also showed a bacterial dominance, but, compared to our findings, noted higher percentages of Archaea (26–47% of rRNA gene copies) at one site (M59E) (Buongiorno *et al.*, 2017) and a recent study on sediments from nearby Aarhus Bay found higher percentages of Archaea in deeper sediments as well (Chen *et al.*, 2017). These data suggest that Bacteria dominate the active community, or contain more ribosomes per cell, despite numerical abundance of Archaea in DNA-based analyses.

Microbial diversity

RNA was extracted from two depths (15 and 42 mbsf) at Site M59 and one depth (12 mbsf) at Site M63, hereafter referred to as 59E-15m, 59E-42m and 63E-12m respectively. The 16S rRNA gene transcripts were sequenced using archaeal and bacterial specific primers, and subsequently classified at the 80% confidence interval against the Silva 123 database (Pruesse *et al.*, 2007). The relative abundances of the gene transcripts that could be classified are reported (Fig. 1B).

Atribacteria or *Chloroflexi*, two groups that are ubiquitous in anoxic marine sediments (Parkes *et al.*, 2014), were the dominant active bacterial phyla in all three samples (Fig. 1B). *Atribacteria* dominated at 63E-12m (76.6% of classifiable rRNA transcript sequences) and at 59E-42m (59.1%), but they were less abundant at

59E-15m (21.7%) (Fig. 1B). The *Chloroflexi* were also abundant at 59E-15m (26.9%) and at 59E-42m (23.9%), but less abundant at 63E-12m (6.1%). Most of the *Chloroflexi* were further assigned to the genus *Dehalococcoides*, which has cultivated isolates proven capable of reductive dehalogenation (Müller *et al.*, 2004; Loffler *et al.*, 2013) but other *Chloroflexi* taxa were present as well. Other bacterial phyla identified in the 16S rRNA transcripts included, for example, *Aminicenantes* (6.7% at 59E-15m), *Planctomycetes* (5.9% at 59E-15m) and *Proteobacteria*. The *Atribacteria* and *Aminicenantes* are bacterial phyla that have no cultured species.

Within the Archaea, the transcripts annotated as *Euryarchaeota* were 22% at 59E-15m, 26.3% at 63E-12m and 87.5% at 59E-42m. Most of these sequences were further classified as ANME-1b or as unclassified families within the order *Methanomicrobiales*. Note that the ANME-1b group, often observed co-occurring with sulfate reducing bacteria (Girguis *et al.*, 2003), are capable of the anaerobic oxidation of methane (AOM), but also are shown to perform methanogenesis (Lloyd *et al.*, 2006; 2011). The *Methanomicrobiales*, an order of methanogens with members that use H₂/CO or formate as substrates (Garcia *et al.*, 2006), were observed in all our samples and corresponded with relatively high methane concentrations.

In sample 59E-15m, 18% of the archaeal sequences were related to *Lokiarchaeota*, previously referred to as the Marine Benthic Group B (MBG-B) (Spang *et al.*, 2015). Sequences attributed to this candidate phylum has been found in marine sediments, and *Lokiarchaeota* specifically have been described as a key microbial group in the sedimentary deep biosphere (Starnawski *et al.*, 2017). The remaining archaeal rRNA sequences were assigned to *Thaumarchaeota*, *Bathyarchaeota* or could not be assigned at the phylum level. Overall, the active microbes identified via 16S rRNA sequencing agree well with those identified as deep biosphere persisting microbes (Starnawski *et al.*, 2017).

Active metabolisms

Metabolic pathways transcribed by the microbial communities were determined from annotated sequences using the KEGG database (Supporting Information Fig. S1 and Table S2 and Methods). An average of 89.9% of reads were not assigned to any protein in this database. Of the remaining reads, more were assigned to the 'metabolism' category (3.72–4.9% of all reads) than to 'genetic information processing' (0.87–1.32%), 'environmental information processing' (0.67–1.04%) or 'cellular processes' (0.19–0.24%) (Supporting Information Fig. S1). It should be noted that the relative abundance of transcripts represents the number of transcripts

assigned to these processes relative to the whole of the metatranscriptome, and is not equivalent to the total number of transcripts *in situ*. Within the 'metabolism' category, the active pathways with the most gene expression included carbohydrate, energy, and amino acid metabolisms (Supporting Information Fig. S1). These categories are broad, but after subsampling the metatranscriptomes to the smallest metatranscriptome *in silico* and normalizing to the abundance of assigned transcripts, samples show notable differences. The deepest and presumably the oldest sample, 59E-42m, contained the highest relative percentages of transcripts assigned to amino acid metabolism (1.59% of all transcripts), energy metabolism (1.26%) and nucleotide metabolism (0.91%). We interpret this to mean that the deeper community allocates more transcriptional activity on maintenance and energy conservation than do the shallower communities, which have younger and presumably more labile substrates to respire. This agrees with a recent study by Kempes *et al.* (2017) in which smaller, more energy starved Bacteria focus cellular energy on protein and nucleotide metabolisms, and Orsi *et al.* (2015) which found that protein cycling accounts for a large percent of cellular activity at low metabolic rates.

Methanogenesis. Methane concentrations ranged from 2.92 mM near the surface to 2.02 mM at 47.88 m mbsf at site M59A (Andrén *et al.*, 2015). At site M63, methane was detected at all depths down to 72 m. Significant methane outgassed from the sediment cores during sampling, so the reported concentrations represent minimum values (Egger *et al.*, 2017).

Transcripts for genes involved in methanogenesis were found in our metatranscriptomes. These include genes that encode for methyl-CoM reductase (*mcr*), heterodisulfide reductases (*hdr*), formylmethanofuran reductases (*fwa*), methylenetetrahydromethanopterin dehydrogenase (*mtd*) and 5,10-methylenetetrahydromethanopterin reductase (*mtr*) (Fig. 2; Supporting Information Table S3). It

has been proposed that these genes are used in AOM, but only when running the methanogenesis pathway in reverse (Wang *et al.*, 2013). The expression of these genes and the presence of ANME ribosomal sequences could indicate an active AOM community in these sediments, but with an unknown electron acceptor. It has been suggested that labile iron oxides may play this role in Baltic sediments, including at site 63E (Egger *et al.*, 2017).

The absence of the *fwd*, *ftr*, *mch*, *mtd* and *hmd* genes in the deepest sample (59E-42m) indicates that hydrogenotrophic methanogenesis was not fully expressed there or that the methanogenic community involved in this process was small and below detection. However, transcripts for acetoclastic methanogenesis (*acds*) and methylotrophic methanogenesis (*mta*, *mtb*, *mtt*), as well as subunits for *mcr* and other steps of methanogenesis were found in all three sediment samples, including in 59E-42m. This indicates that methylated compound breakdown and acetate utilization were important methanogenesis pathways in the BSB sediments (Fig. 2). Methylotrophic methanogenesis is recognized as an important metabolic pathway in estuarine sediments and in sulfate-reducing zones of marine sediments (Valentine, 2011). Additionally, this process has been reported in several deep subseafloor environments, including the Peru margin (Orsi *et al.*, 2013). The combination of expressed methanogenesis protein-encoding genes with millimolar concentrations of methane points to biological methane production as the main terminal process of the microbial food chain at these sites.

Sulfate reduction. Sulfate in sediment porewater may serve as a terminal electron acceptor in the oxidation of organic matter by sulfate reducing bacteria. The porewater sulfate concentration at site M59 was at 1.44 mM at 0.73 m mbsf, but dropped to low micromolar levels at most depths down to 80 m mbsf, including in samples studied here. At site M63, sulfate was < 0.01 mM at most depths down to 74 m mbsf (Andrén *et al.*, 2015). In an

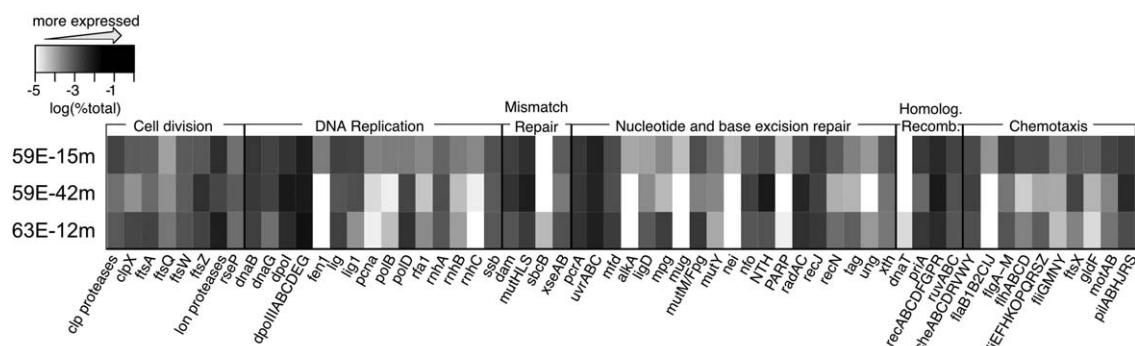


Fig. 2. Heatmap showing log transformed relative expression of genes related to cell division, DNA replication and repair and chemotaxis. Darker colours indicate higher numbers of reads assigned to that gene.

environment deposited under marine water conditions, such low concentrations are the result of microbial sulfate reduction, which is sometimes accompanied by detectable sulfide (Andrén *et al.*, 2015). However, low or undetectable levels of sulfide – as was the case in our sediments – may be due to precipitation with iron, biological oxidation or chemical oxidation (Reese *et al.*, 2013).

Most of the genes related to sulfur cycling were classified as assimilatory (e.g., *cys*, *paps*), but a few dissimilatory transcripts were noted as well. Genes encoding proteins which mediate the first steps of dissimilatory sulfate reduction to sulfite were expressed, including sulfate adenylyltransferase (*sat*) and adenosine-5'-phosphosulfate reductase (*apr*). These were only found in sample 59E-15m (Fig. 3), which had the most potential seawater intrusion during sampling. None of the transcripts from any of the samples were annotated as dissimilatory sulfite reductase (*dsr*) against the KEGG database. However, when assigning function against the SEED database, three transcripts from 59E-15m were annotated as *dsrAB*. In addition to mediating sulfate reduction, the *dsrAB* protein has been posited to produce sulfate through sulfur oxidation (Loy *et al.*, 2009; Muller *et al.*, 2015).

Polysulfide reductase genes (*psr*), which confer to microbes the ability to use polysulfides as electron

acceptors (Jormakka *et al.*, 2008), were found in 59E-15m. In sediments where sulfides are produced during diagenesis, expression of the *psr* gene could indicate some microbes are polysulfide reducers. These data provide evidence that a subset of the active microbial community could be using polysulfide reduction for energy in sulfate-depleted environment.

Reductive dehalogenation. Halogenated organic compounds are produced naturally in marine environments and some are subsequently buried in sediment (Häggblom and Bossert, 2003). Under anoxic conditions, they can serve as terminal electron acceptors in biological reductive dehalogenation. Labile carbon compounds are released as byproducts, which can serve as carbon and energy sources for non-dehalogenating microbes (Futagami *et al.*, 2009; Jorgensen and Marshall, 2016). Elevated porewater concentrations of bromide relative to chloride indicate reductive dehalogenation of brominated organic compounds (Berg and Solomon, 2016). The Br/Cl molar ratio within the BSB sediment porewater was greater than that in seawater and increased with depth at both sites. At site M59, this ratio was 1.67×10^{-3} at the surface and increased to 4.06×10^{-3} at 42 mbsf, and at site M63, the ratio was 1.78×10^{-3} at the surface increasing to 2.08×10^{-3} at 12.3 mbsf (Andrén

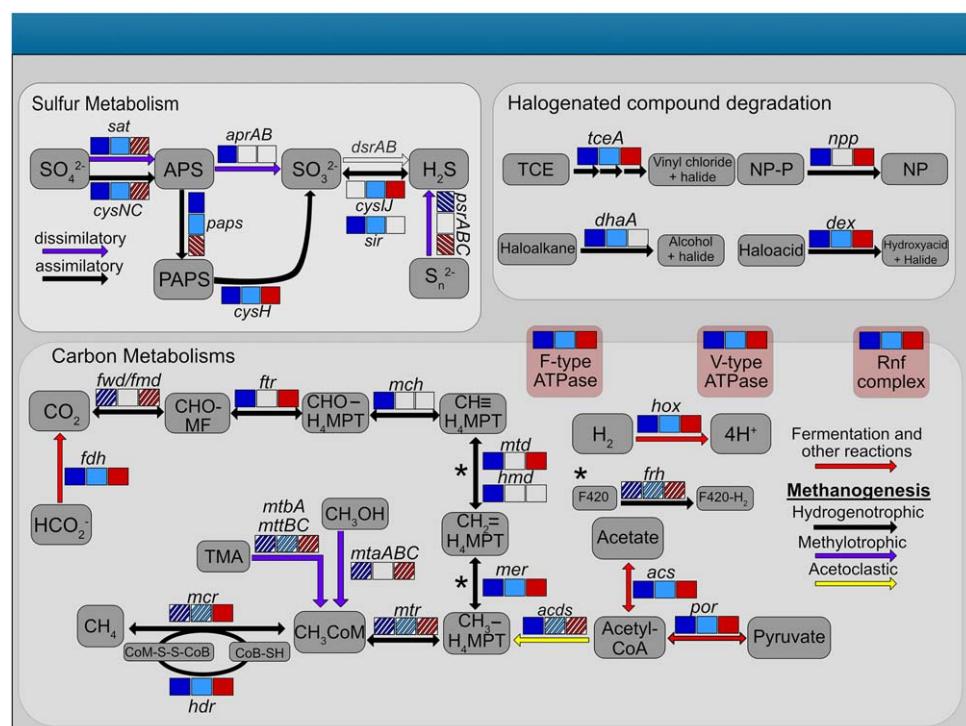


Fig. 3. Expression of key metabolic genes mapped onto biochemical pathways. Hashed boxes represent genes where some but not all necessary subunits were expressed. Asterisks in boxes represent samples with the highest percent of reads assigned. NP-P is nitrophenyl phosphate, NP is nitrophenyl. See Supporting Information Table S3 for a detailed list of gene subunits, abbreviations and number of assigned transcripts.

et al., 2015). This points to reductive dehalogenation as a plausible source of energy within the BSB sediment.

Consistent with this, a canonical reductive dehalogenation gene, trichloroethylene reductive dehalogenase (*tceA*), was expressed in all three samples. However, the relative proportion of transcripts for this gene was higher at site M59E, which featured higher seawater-influenced chloride concentrations than site M63E. Expressed transcripts were observed for genes in pathways for degradation of other chloroalkanes, including carboxymethylenebutanolidase (*CMBL*) and nitrophenyl phosphatase (*NPPase*) in 63E-12m and haloacid dehalogenase (*DEX*) in both 63E-12m and 59E-15m (Fig. 2), though complete pathways were not found. Reductive dehalogenation has been widely observed in subsurface sediment from the Peru Margin, eastern equatorial Pacific, Juan de Fuca Ridge flank and northwest Pacific near Japan (Futagami *et al.*, 2009). At those locations and in the BSB, this metabolic process may play a significant role in biogeochemical cycles of chlorine, iodine, bromine and halogenated carbon substrates and facilitate organic matter degradation.

Active cellular processes

Motility. In static deep marine sediment that lack noteworthy lateral or vertical fluid flow, the ability of a microbe to move through pore spaces to find nutrients might confer a useful, yet costly advantage (Parkes *et al.*, 2000). Motility and chemotaxis were recently estimated to be perhaps too costly in the deep biosphere (Hoehler and Jorgensen, 2013), but the high nutrient concentrations in BSB sediments could potentially support such activities. Additionally, motility would be beneficial in accessing solid substrates such as particulate organic matter and mineral-bound phosphates. In the three samples examined in the current study, there was evidence of motility gene transcription (KEGG category 'Flagellar assembly'). In addition, transcripts of flagellar biosynthesis genes (e.g., *fla*, *flg*, *flh* and *fli*) were present in all samples (Fig. 3; Supporting Information Table S4). Genes associated with gliding and twitching motility (*pil*, *gld*, *fts*) were also transcribed in the sediments in all depths. Reads assigned to *mot* and *che* proteins, which effect and regulate flagellar movement, were identified in all samples, with a higher percentage at site 59E than at 63E-12m. Other subsurface studies have also found these genes expressed, suggesting their presence in deep marine sediments (Orsi *et al.*, 2013). In brief, it appears that at least some deep subsurface microbes retain the capacity to move toward food or other substrates.

Division and replication. In the sedimentary biosphere, cell numbers decrease exponentially with depth, and it is

unclear if the resident communities are multiplying or only turning over biomass without division (Lomstein *et al.*, 2012). Extremely long doubling times of over 1000 years have been estimated for deep subsurface microbial communities (Hoehler and Jorgensen, 2013). However, the cell counts in BSB sediments are higher than those predicted from global regression lines (Kallmeyer *et al.*, 2012; Parkes *et al.*, 2014; Buongiorno *et al.*, 2017). Due to the high sedimentation rate in the BSB, even deeply buried sediment can be relatively young (less than < 10,000 years old) and the sediment organic matter is likely more labile than at other deep biosphere sites. This not only appears to support microbial life, but also active cellular division.

Transcripts were assigned to functional genes within the KEGG categories 'cell growth and death' and 'nucleotide metabolism' (Supporting Information Fig. S1), and multiple genes involved in cell division were found within the metatranscriptomes of all three samples (Fig. 3). In addition, transcripts for the protein responsible for initiating cell division and recruiting other proteins to produce a new cell wall (*fts*) were identified, as were ATP-dependent *lon* and *cip* proteases, which are indicative of replicative DNA helicases, DNA primases and DNA polymerases (Fig. 3). The active gene expression indicates that the cells in BSB sediment were active and dividing, and what we define as thriving and not just dying slowly.

To survive in the deep biosphere, cells must also maintain structural and DNA integrity (Lomstein *et al.*, 2012). The percent of reads assigned to DNA repair in the deepest sample (59E-42m) was double the percent of reads assigned to DNA repair in the shallower samples. This agrees with a recent study that calculated genetic mutation rates in the deep biosphere and concluded that DNA mutation repair is maintained in subsurface sediments (Starnawski *et al.*, 2017), and further demonstrates the importance of maintaining current cellular integrity in the deep biosphere.

Antimicrobial production. To survive, subseafloor microorganisms likely engage in a wide variety of interactions with both the environment and each other. With increasing depth, metabolisms slow and growth rates decrease, suggesting that cell maintenance becomes an increasingly important factor in survival. To respond to changing environmental conditions, microbial populations can (i) outcompete their neighbours by adapting their metabolisms to the available resources, (ii) enter into a mutualistic cooperation with their neighbours and/or (iii) inhibit or kill their neighbours through the production of secondary metabolites (i.e., antimicrobials) that confer a competitive advantage. The two most common mechanisms of antimicrobial production are through polyketide synthase (PKS) and nonribosomal polyketide synthase

pathways (Winter *et al.*, 2011), both of which form specific peptides with conserved and variable domains, which account for the diversity of natural antimicrobials (Clardy and Walsh, 2004; Yin *et al.*, 2007). In all of our BSB samples, gene transcripts for the PKS pathway were expressed, including for ansamycin biosynthesis (e.g., *tktAB*), tetracycline biosynthesis (e.g., *accABCD* and *bccP*), polyketide sugar unit biosynthesis (e.g., *rffACD*, *rffGH*, *rmlCD*) and vancomycin biosynthesis (e.g., *rfbB* and *rffG*) (Supporting Information Table S4). Antimicrobial production genes have been isolated from a diverse set of microbial lineages and environments, but most research to date has focused on *Actinomycetes* and terrestrial soil environments. Given the increasing prevalence of antimicrobial resistance, further exploration into natural products is warranted, especially in poorly studied environments such as the deep subsurface.

Summary

The microbial communities within the organic-rich, deeply-buried sediment of the Baltic Sea are diverse and active. RNA-based analyses revealed that these communities carry out a wide range of metabolisms, including methanogenesis, sulfate reduction and reductive dehalogenation. We found gene expression associated with cellular maintenance, division and motility, which could imply that microbes in the active community not only manage to survive in these sediments, but are actually thriving long after burial.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Assignment of transcripts to KEGG categories as a percent of all sequences per sample.

Table S1. Sample characteristics, from Andrén et al. (2015).

Table S2. Sequencing and droplet digital PCR results.

Table S3. Genes examined in characterizing active metabolic pathways.

Table S4. Gene transcript assignments for active cellular processes.