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Assessing Microbial Communities Related to Mercury Transformations in Contaminated Streambank Soils

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Abstract In nature, the bioaccumulative potent neurotoxin methylmercury (MeHg) is produced from inorganic mercury (Hg) predominantly by anaerobic microorganisms. Hg-contaminated soils are a potential source of MeHg due to microbial activity. We examine streambank soils collected from the contaminated East Fork Poplar Creek (EFPC) in Tennessee, USA, where seasonal variations in MeHg levels have been observed throughout the year, suggesting active microbial Hg methylation. In this study, we characterized the microbial community in contaminated bank soil samples collected from two locations over a period of one year and compared the results to soil samples from an uncontaminated reference site with similar geochemistry ($n = 12$). Microbial community composition and diversity were assessed by 16S rRNA gene amplicon sequencing. Furthermore, to isolate potential methylators from soils, enrichment cultures were prepared using selective media. A set of three clade-specific primers targeting the gene *hgcA* were used to detect Hg methylators among the δ-Proteobacteria in EFPC bank soils across all seasons. Two families among the δ-Proteobacteria that have been previously associated with Hg methylation, Geobacteraceae and Syntrophobacteraceae, were found to be predominant with relative abundances of 0.13% and 4.0%, respectively. However, in soil enrichment cultures, Firmicutes were predominant among families associated with Hg methylation. Specifically, Clostridiaceae and Peptococcaceae and their genera *Clostridium* and *Desulfosporosinus* were among the ten most abundant genera with relative abundances of 2.6% and 1.7%, respectively. These results offer insights into the role of microbial communities on Hg transformation processes in contaminated bank soils in EFPC. Identifying the biogeochemical drivers of MeHg production is critical for future remediation efforts.

Keywords Methylmercury · Mercury methylation · Geochemistry · Deltaproteobacteria · Clostridia

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1 Introduction

The Oak Ridge Reservation (ORR) of the US Department of Energy in East Tennessee, USA, was established during World War II for nuclear weapons

development. During the 1950s and 1960s, the Y-12 National Security Complex (Y-12) located at the headwaters of East Fork Poplar Creek (EFPC) used elemental mercury (Hg) to separate lithium isotopes using a Li-Hg amalgamation process at industrial scales. EFPC originates within the Hg-contaminated subsurface storm drain network of Y-12 and discharges process water directly into the stream (Brooks and Southworth 2011; Turner and Southworth 1999; Donovan et al. 2014). Historical records indicate that a total of approximately 350 tons of elemental Hg were lost to the environment during the mid of the last century, contaminating soils, groundwater, air, and EFPC (Brooks and Southworth 2011). Also, Hg migration from the source areas via atmospheric deposition, surface water runoff, sediment, and groundwater transport contaminated the upper (U) and lower (L) EFPC ecosystem (Donovan et al. 2014). The bank and floodplain soils of EFPC were found to contain high concentrations of Hg, reaching up to 3000 μg/g in various physicochemical forms (Barnett et al. 1997; Barnett and Turner 1995; Harris et al. 1996), with insoluble mercuric sulfide as the predominant species (Revis et al. 1989).

Despite extensive remediation efforts in UEFPC and around the Y-12 plant that led to an 85% decrease in total Hg (THg) flux, more recent studies identified soils with high Hg concentrations downstream along the banks of LEFPC (Southworth et al. 2010; Southworth et al. 2013). Contaminated bank soils at sites with little to no past remediation activities represent new point sources of Hg entering EFPC locally and downstream (Southworth et al. 2010; Southworth et al. 2013). Methylmercury (MeHg) concentrations increase from upstream to downstream locations as a result of instream methylation by periphyton and other biological activity (Watson et al. 2016). Recent modeling studies linked the highest Hg fluxes into the EFPC ecosystem with streambank erosion and streambed sediment resuspension during storm events (Watson et al. 2016; Ketelle et al. 2017). The estimated annual fluxes of Hg and MeHg due to streambank erosion for all LEFPC are estimated to be 38.6 kg and 0.0056 kg, respectively (Ketelle et al. 2017), while 5.4 km downstream from Y-12 the estimated annual fluxes were 98 kg of Hg and 0.085 kg of MeHg (Riscassi et al. 2016). Therefore, MeHg concentrations in water and especially in fish have not declined in response to improvements in water quality and have shown increasing trends in some cases (Brooks and Southworth 2011).

Inorganic Hg species can be transformed into the more toxic organic MeHg predominantly by anaerobic microorganisms (Hintelmann 2010; Gilmour et al. 2013). Mercury methylation has been attributed mainly to sulfate-reducing bacteria (SRB) (Pak and Bartha 1998; Achá et al. 2011; Achá et al. 2012; Bravo et al. 2016), iron-reducing bacteria (IRB) (Fleming et al. 2006; Bravo et al. 2015), methanogens (Hamelin et al. 2011), and syntrophs (Bae et al. 2014). Two genes, *hgcA* and *hgcB*, were found to be essential for microbial Hg methylation in bacteria and archaea. They encode a putative corrinoid protein, HgcA, and a 2[4Fe-4S] ferredoxin HgcB, each of which was proposed to act as methyl and electron donor, respectively, which are required for the conversion of Hg^{2+} to $CH3Hg^{+}$ (Parks et al. 2013). Sequence analysis of HgcA orthologs in all confirmed and predicted methylators revealed a highly conserved motif characterized by the amino acid sequence, N(V/I)WCA(A/G)GK, and a set of four to five C-terminal transmembrane helices (Parks et al. 2013). Degenerate PCR primers were developed to detect microorganisms carrying *hgcAB* genes in diverse environments. These primers showed reliable results for most strains carrying *hgcAB* among the three major Hg-methylating clades, Deltaproteobacteria, Clostridia, and Euryarchaeota (Christensen et al. 2016).

While several studies have been conducted to characterize the microbial community structure in EFPC sediments (Christensen et al. 2018; Vishnivetskaya et al. 2011), none has examined the influence of Hg contamination on the prevalence of Hg methylators in streambank soils. In the present study, we collected EFPC streambank soils at two locations with different Hg levels and determined microbial community compositions using 16S rRNA gene amplicon sequencing. Furthermore, we used three sets of clade-specific primers targeting *hgcA* as a biomarker for Hg methylation and compared the results to an uncontaminated reference site with similar geochemistry.

2 Materials and Methods

2.1 Soil Sampling and Analysis

Streambank soil samples were collected from EFPC at two locations over a period of one year (fall 2016–summer 2017). The sampling locations were designated as EFK 18.2, and EFK 11.2, where the number indicates

the stream distance in kilometers (East Fork kilometer), from the mouth of the creek (Fig. 1). Bank soils sampled at EFK 18.2 (36.00438 N, 84.28246 W) were collected from a dark-colored soil layer containing high Hg concentrations associated with the presence of coal fines, which has been designated as the historical release deposit (HRD) (Southworth et al. 2010; Southworth et al. 2013). EFK 11.2 (35.982243 N, 84.32755 W) is located further downstream and represents a section of streambank soils with lower Hg contamination levels. Hg-contaminated bank soil samples collected in all seasons were compared to bank soil samples from an uncontaminated reference site, Hinds Creek (HC), which is located 25 km northeast of Y-12 near Clinton, TN, USA (36.140842 N, 84.051302 W). Bank soil samples were placed into sterile plastic sampling bags (Whirl-Pak, Nasco, Fort Atkinson, WI) and transported on dry ice to Alabama State University within 24 h, homogenized, and kept at − 20 °C until analysis. Soil temperature, pH, and other geochemical parameters, including C, S, Fe, and soil particle size analysis, were determined according to standard methods and are reported elsewhere (Egbo et al. 2017; Dickson et al. 2019). A 10-g (wet weight) subsample was taken from all samples ($n = 12$), placed in acid-washed and sterilized glass vials, and shipped overnight on dry ice to Brooks Applied Labs (Bothell, WA) for THg and MeHg analyses. Samples were prepared and analyzed following USEPA Method 1631 for THg and a modified USEPA Method 1630 for MeHg.

2.2 Bacterial Community Composition

Community genomic DNA (gDNA) was extracted in triplicate from 1 g (dry weight) of soil using a PowerSoil DNA isolation kit (QIAGEN, Germantown, MD) following the manufacturer's instructions. The gDNA was purified ($n = 36$) using a DNA purification kit (Promega, Madison, WI), and the quality and concentration were measured with a NanoDrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA). Extracted DNA from streambank soils and enrichment cultures were shipped in a dry ice container to the University of Alabama at Birmingham Microbiome Resource (UAB, Birmingham, AL) for 16S rRNA amplicon sequencing, as previously described in detail (Kumar et al. 2014). Briefly, the barcoded primers 515F and 806R targeting the V4 region of the 16S rRNA gene were used for PCR amplification. The PCR protocol used was as follows: 1 cycle of preincubation at 94 °C for 1 min, followed by 32 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 1 min, and extension at 65 °C for 1 min, then 1 cycle of final incubation for 3 min at 65 °C. PCR products were resolved on agarose gels, isolated, and purified using Qiagen kits and quantitated prior to sequencing at the UAB Genomic Core Facility. Each sample was run in duplicate, and the amplicons were sequenced on the Illumina MiSeq platform. 16S rRNA gene amplicon data were processed using the microbiome analysis pipeline (QWRAP) at UAB

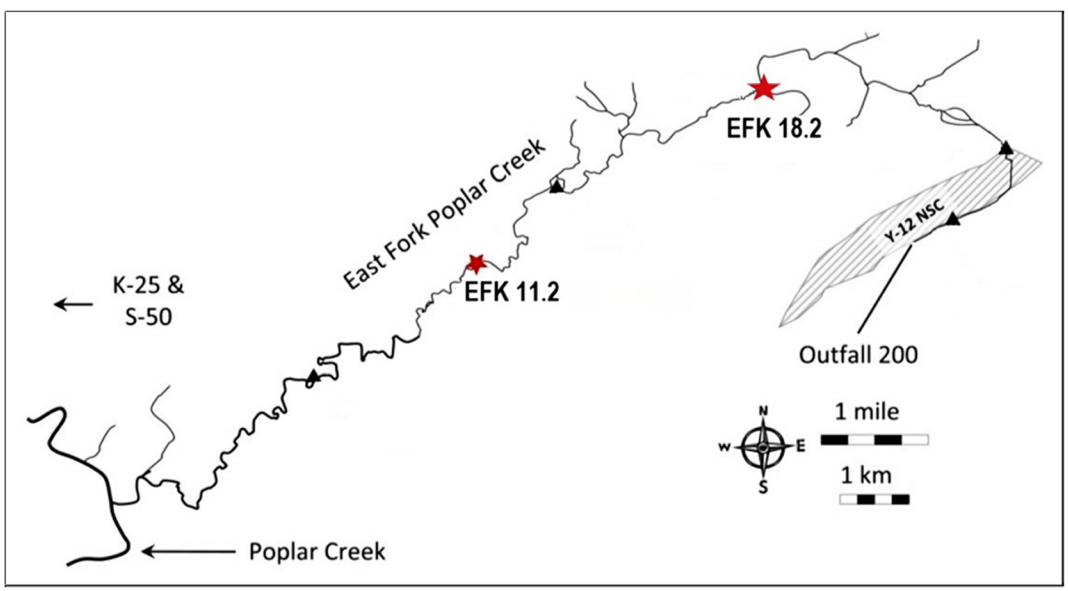

Fig. 1 EFPC streambank soil sampling locations (ELSEVIER LICENSE NUMBER: 4864400393068)

(Kozich et al. 2013; Kumar et al. 2014). A combination of tools within the QIIME pipeline were utilized for clustering reads into operational taxonomic units (uclust), taxon assignment (RDP classifier using the Greengenes 16S rDNA database (McDonald et al. 2012; Wang et al. 2007). Operational taxonomic units (OTUs) were clustered at 97% pairwise identity. For result reporting, QWRAP generates a static HTML report. The output includes the percentages of OTUs down to genus and species level and alpha and beta diversity using different metrics. Filtered lists of top 10, 25, and 100 OTUs and taxa were also generated.

2.3 Enrichment Cultures

Here, the intent was to compare the Hg methylation-relevant microbial abundance in the soil samples of the studied sites in all seasons under similar growth conditions based on their existence and persistence in these samples. All soil samples were grown in triplicate under optimum conditions, and the extracted DNAs were pooled and assigned identification codes based on site and season (108 total). One gram of streambank soils ($n = 12$) was used for enrichment cultures in three different selective media (described below). Soils were placed in 10 ml of media in Hungate tubes and incubated at 30 °C or 37 °C for two weeks under anoxic conditions. The bottom 2 ml of the media and the settled soil samples were taken separately for DNA extraction. The UltraClean Microbial DNA and the PowerSoil DNA extraction kits (QIAGEN, Germantown, MD) were used for DNA extraction from the enrichment culture media and settled soil, respectively, following the manufacturer's instructions. Extracted DNAs were used for 16S rRNA gene amplicon sequencing, as described above, and termed enrichment cultures.

Three different media were used for soil enrichment; mineral salt medium (DCB-1 medium for syntrophs), nutrient broth (NB) basal salt medium (PCA medium for IRB), and sulfate-free modified minimal organic yeast (MOY) medium (Zane et al. 2010) (SRB medium). The mineral salt medium was previously described by Mohn and Tiedje (1991) and DeWeerd et al. (1990). Briefly, it contained 40 mM pyruvate, 0.2 mM $Na_2S{\cdot}9H_2O$, 0.2 mM l-cysteine, 1 g yeast extract/l, and 10 ml/l of a vitamin solution as described by Wolin et al. (1963) plus 5 ml/l special vitamin solution prepared by adding 4 mg 1,4-naphthoquinone, 10 mg nicotinamide, 1 mg thiamine, 1 mg lipoic acid, 1 mg hemin, to 100 ml of 10 mM HEPES. The gas-phase was N_2:CO_2 (80:20%), and the pH was adjusted with CO_2 to 7.3. The NB basal salt medium was prepared as previously described (Coppi et al. 2001; Lin et al. 2014) containing 20 mM acetate as an electron donor, and 40 mM fumarate as an electron acceptor at a final pH 6.8. The sulfate-free MOY medium was described by Zane et al. (2010), with the following modifications; 2 mM NaH_2PO_4, 0.1 μM nickel chloride instead of nickel sulfate, 40 mM sodium pyruvate, and 40 mM sodium fumarate. The pH was adjusted with 2 N NaOH to 7.2.

All cultures were maintained under strictly anoxic conditions in an anaerobic workstation (Don Whitley Scientific Ltd., West Yorkshire, UK) using a gas mixture of 80% N_2 and 10% each for H_2 and CO_2. Tubes with the mineral salt medium were incubated at 37 °C, while tubes with NB or modified MOY media were incubated at 30 °C for two weeks before DNA extraction.

2.4 Identification of Hg-Methylating Bacteria

Three pairs of strain-specific *hgcA* primers for potential methylators developed at Oak Ridge National Laboratory (ORNL) were used to amplify the *hgcA* target gene (Christensen et al. 2016). Primer pairs for *hgcA* from two confirmed and one predicted methylator were purchased from Integrated DNA Technologies (Coralville, IA). For each primer pair of the target strains, gDNA obtained from the American Type Culture Collection (ATCC, Old Town Manassas, VA) was used as a positive control. These primers were developed according to the nucleotide sequences in conserved regions, including a highly conserved sequence motif common to all known *hgcA* homologs with some level of degeneracy, which allows them to amplify multiple *hgcA* variants. Forward and reverse primers were constructed based on *hgcA* sequence information from *Desulfovibrio desulfuricans* ND132; F: 5′-GCCA ACTACAAGCTGACCTTC-3′ R: 5′-CCCG CCGCGCACCAGACGT T-3′, *Geobacter sulfurreducens* PCA; F: 5′-GCCAACTACAAGAT GAGCTAC-3′ R: 5′-CCGG CGGCGCACCAGACA TT-3′ and *Desulfomonile tiedjei* DCB-1; F: 5′-GCGA ATTACAAGATGA GTTTC-3′ R: 5′-CCCGC TGCACACCAGACATT-3′. The GC-content of δ-Proteobacteria is typically high and consequently higher melting temperatures are needed for amplification. Multiple experiments were performed evaluating different

strategies to optimize PCR parameters, which included variation of annealing temperature, extension time, and primer concentration, to achieve amplification resulting in a single distinct band of PCR product at the expected size. Two PCR kits were used: Premix Taq (Ex Taq™ version 2.0 -TaKaRa-Clontech, Mountain View, CA) and Phusion High-Fidelity master mix (New England BioLabs, Ipswich, MA). The TaKaRa kit produced the expected product (~950 bp) with ND132-primers only in 25-μl reaction tubes containing 12.5 μl premix, 10.5 μl primers mix (0.5 μl of each 10 μM primer plus 9.5 μl PCR-grade water), and 2 μl template DNA (25–30 ng total). The amplification was initiated in a Bio-Rad T100 thermal cycler (Bio-Rad, Hercules, CA) with 1 cycle of preincubation at 95 °C for 2 min, followed by 5 cycles of denaturation twice as follows; 30 s at 95 °C, 30 s at 68 °C or 70 °C for each five-cycle run, then 1 min at 72 °C, followed by 30 cycles of denaturation (95 °C for 30 s), annealing (70 °C for 30 s), and extension (72 °C for 1 min) followed by a final incubation step at 72 °C for 10 min. The Phusion kit produced the expected product with PCA and DCB-1 primers in 25-μl reaction tubes containing 6.5 μl premix, 16.5 μl primers mix (0.5 μM final conc.), and 2 μl template DNA (25 ng total). The amplification started with preincubation at 98 °C for 5 min, followed by 30 cycles of denaturation at 98 °C for 30 s, annealing for 30 s at 58 °C (PCA) or 60 °C (DCB-1), then extension at 72 °C for 1 min, and then final incubation at 72 °C for 10 min. PCR products were resolved in 1% agarose gel at 80 V for 1 h. Bands at the expected size (~950 bp) and corresponding amplicons were recovered using a QIAquick Gel Extraction Kit (QIAGEN, Germantown, MD) and sent for Sanger sequencing with the primers for both strands at the Heflin Genomics Center, University of Alabama at Birmingham or the Genomics & Sequencing Laboratory, Auburn University. The software MacVector version 16.0.1 (MacVector Inc., Apex NC, USA) and the NCBI BLASTN tools (https://blast.ncbi.nlm. nih.gov/Blast.cgi) were used to perform a 6-frame translation and sequence alignment with known *hgcA* sequence for each strain.

2.5 Statistical Analysis

The objective of the statistical analysis was to compare microbial abundance percentages to evaluate seasonal- or site-related differences and whether the differences among levels of one factor were similar across levels of the other factor. Thus, the study was analyzed as a 4 × 3 factorial design with analysis of variance. This design requires that the residuals be normally distributed with equal variance. Typically, response variables that are measured as percentages violate one or both assumptions. Initial analyses explored the use of square root and arcsine transformations. Because the results of these tests were not drastically different from untransformed data, only results from the untransformed data are presented here.

The MIXED procedure of SAS 9.4 (SAS Institute, Inc., Cary, NC) was used to perform the analysis of variance. Least square means and standard errors were calculated by the level of the two factors and their interactions. When the interaction was declared significant from the omnibus F test, the SLICE option was used to perform a partitioned analysis of the interaction least square means or, in other words, an analysis of simple effects was performed. When no interaction was detected, and the omnibus F test indicated differences among levels of a factor, a comparison of mean differences was conducted using Tukey's (Tukey 1949) adjustment. Letter groupings were facilitated using a macro provided by Arnold Saxton (Saxton 1998). The level of significance for all analyses was set at 0.05.

3 Results

3.1 Variability of Hg and MeHg Between Sampling Sites

The relationships between total Hg, MeHg, geochemistry, and seasonal variations were explored. Temperature, redox conditions, and pH influence the activity of potential methylators and Hg methylation potential. The physicochemical characteristics of EFPC streambank soils and Hg levels vary between upstream and downstream locations. A previous study reported the soil composition and geochemistry of the streambank soils at the sampling sites (Dickson et al. 2017). An analysis of the soil particle size distributions showed that EFPC bank soils can be classified as silty clay loam with more silt and clay contents at EFK 11.2 (Table 1). Elemental analyses showed Cu, Mn, Mg, Zn, S, and others were at levels of less than 1% of the dried soils, while Ca, K, Al, Si, C, and Fe were at levels higher than 1%. Fe levels were high in EFK 18.2 (3.49%) and EFK 11.2 (1.97%) (Egbo et al. 2017; Dickson et al. 2019), which might

influence Hg bioavailability and the potential role of Hg methylators among the iron-reducing bacteria (IRB) in these sites. Targeted sampling of the HRD layer was conducted at EFK 18.2, which is known to contain high THg concentrations. Overall, THg levels were much higher in HRD soil at EFK 18.2 (232.9–695.9 μg/g) compared to EFK 11.2 (3.3–54.5 μg/g) and the reference site (HC) (0.03–0.04 μg/g). The HRD represents a distinct layer in streambank soils, which is dark gray to black in appearance due to the presence of coal fines and ash resulting in higher total carbon (TC) and organic carbon (OC) contents compared to EFK 11.2 and the reference site (Table 1), which may impact microbial community composition and the bioavailability of inorganic Hg for methylation.

Seasonal variations in THg levels were observed for both EFPC sampling sites. For site EFK 18.2, THg was highest in the fall followed by the summer, while in EFK 11.2, the highest THg was in the winter followed by the spring (Fig. 2a). MeHg in EFK 18.2 was highest in the winter sample (12.7 ± 0.6 ng/g) and lowest in the fall (4.7 ± 0.2 ng/g). In contrast, MeHg in EFK 11.2 was highest in the spring (3.4 ± 0.1 ng/g), and the lowest in the fall (0.15 ± 0.008 ng/g) (Fig. 2b). Generally, MeHg levels did not correlate with THg levels. The observed seasonal variations in THg and MeHg at each site are likely a result of heterogeneities within the soil layers and the area sampled.

3.2 Microbial Community Characterization

3.2.1 Soil Microbiome

Microorganisms can have a significant impact on net MeHg concentrations. Microbial community composition, as well as bioavailability of inorganic and organic Hg species, influences the relative contributions of microbial methylation and demethylation processes to net Hg methylation, which also depend on environmental conditions, such as geochemistry, temperature, and pH (Bratkič et al. 2017; Han et al. 2007; Bigham et al. 2017; Hsu-Kim et al. 2013). Methylation of mercury has been observed across a wide range of microbial clades, mainly within the δ-Proteobacteria and Clostridia, in addition to a few members among the Chloroflexi, Bacteroidetes, methanogenic archaea, and others (Parks et al. 2013; Podar et al. 2015). Some strains within these clades were confirmed as Hg methylators (Gilmour et al. 2013; Gilmour et al. 2018), while others are predicted to methylate Hg based on harboring *hgcAB* genes and are awaiting experimental confirmation. Overall, 16S rRNA sequence analysis showed that the soil microbial community across all samples consisted of 22 bacterial and 3 archaeal phyla, including Proteobacteria (ranging from 15.6 to 41.8% per sample), Acidobacteria (17.4–35.6%), Nitrospirae (4.2 to 14%), Actinobacteria (2.7 to 13.3%), Chloroflexi (3.6 to 10.1), Verrucomicrobia (0.3 to 8.3%), Cyanobacteria (0.1–7.1%), Bacteroidetes (0.1 to 6.2%), Firmicutes (0.1 to 1.5%), Euryarchaeota (0.0 to 0.6%), and few unclassified bacteria. Among those associated with Hg methylation, five bacterial clades have been identified across different seasons and sites. Figure 3a and Table 2 show predominant clades and associated with Hg methylation present at the studied sites. Throughout the year, Proteobacteria were the dominant clade (25.6%), followed by Nitrospirae (10.1%), Chloroflexi (6.5%), Bacteroidetes (1.9%), and Firmicutes (0.4%). Three classes of bacteria associated with Hg methylation were predominant at the contaminated sites with relative abundances of 7.2% for the δ-Proteobacteria (28.1% of total Proteobacteria), Nitrospira 5.9% (58.4% of Nitrospirae), and Anaerolinea 1.8% (27.7% of Chloroflexi). Among the δ-Proteobacteria, two families, the Syntrophobacteraceae (SRB) and Geobacteraceae (IRB), were predominant particularly during the fall and spring with relative abundances of 4.0% and 0.13% (55.6% and 1.8% of total δ-Proteobacteria), respectively. Furthermore, the 16S rRNA gene amplicon sequencing data confirmed seasonal- and site-dependent abundance variations in clades associated with Hg methylation. The abundance of Chloroflexi was significantly ($P < 0.05$) higher during the summer, while no site-specific differences were observed. However, throughout the year, both Firmicutes and Proteobacteria were significantly ($P < 0.05$) higher in EFK 18.2 compared to EFK 11.2 and the control

Table 1 Contaminated soil physicochemical characteristics; *EFK*, East Fork kilometer; *gdw*, grams dry weight

Site ID	% gravel	% sand	% silt	% clay	% moisture	THg (μg/gdw)	MeHg (ng/gdw)	pH
EFK 18.2	1.6	31.4	50.1	16.9	22.50	232.9–689.5	4.72–12.7	7.8
EFK 11.2	0.0	22.1	56.1	21.8	21.61	3.32–54.50	0.1–3.38	7.4

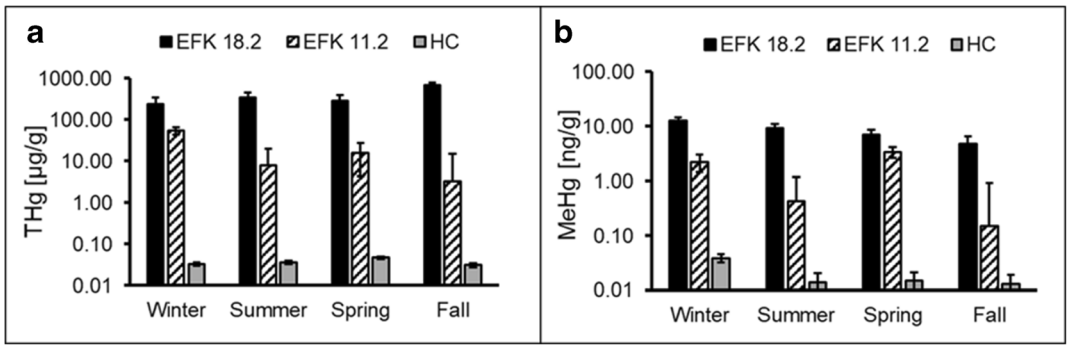

Fig. 2 Seasonal variation of **a** THg and **b** MeHg in EFPC streambank soils. EFK, East Fork kilometer; HC, Hinds Creek

site, while Bacteroidetes were significantly more abundant in EFK 11.2 (Fig. 3b). Furthermore, the Firmicutes were significantly ($P < 0.05$) higher in fall and winter relative to the other seasons, while the Proteobacteria showed no seasonal variation ($P > 0.05$). At the class level, the Anaerolinea class among the Chloroflexi was significantly more abundant ($P < 0.05$) in EFK 11.2 compared to EFK 18.2 and the control site and during the fall compared to other seasons, while the δ-Proteobacteria were significantly more abundant ($P < 0.05$) in EFK 18.2 without seasonal variation. At the family level, the Geobacteraceae were significantly more abundant ($P < 0.05$) in the fall in both Hg-contaminated sites compared to the uncontaminated control site. In contrast, the Syntrophobacteraceae were not statistically ($P > 0.05$) different among sites and seasons (Fig. 3c, d). Two genera, *Geobacter* and *Nitrospira*,

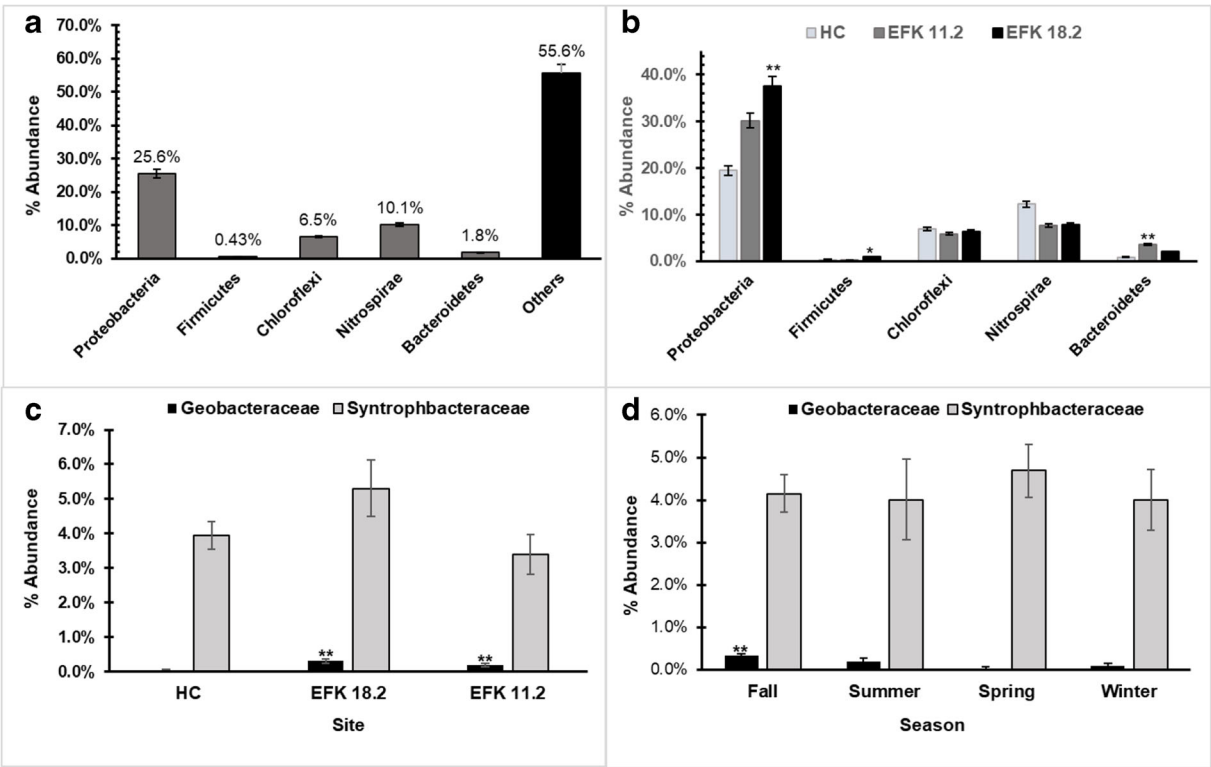

Fig. 3 Percentage abundances of microbial clades associated with Hg methylation in bank soil samples. **a** Average abundances of major bacterial clades across all sites and seasons. **b** Differences between clade abundances associated with Hg methylation across sites. **c** Site and **d** seasonal abundance variations of bacterial families in soil, EFK (East Fork kilometer). Percentage values represent the number of reads assigned to these OTUs relative to the number of reads across all OTUs in 26 samples from all sites and seasons

were detected in the soils with relative abundances of 0.2% and 1.4%, respectively. The genus *Geobacter* was detected in the fall samples only. It was significantly more abundant ($P < 0.05$) in Hg-contaminated sites compared to the uncontaminated control site. In contrast, *Nitrospira* were detected in all samples and were significantly more abundant in EFK 11.2 and during the fall season compared to the other sites and seasons (Table 2).

3.2.2 Enrichment Cultures

Soil enrichment cultures favored the growth of Firmicutes over Proteobacteria for samples collected across all seasons and sites except for the winter and summer samples collected from EFK 18.2. Out of the 11 bacterial and 2 archaeal phyla found in enrichment cultures, four bacterial and one methanogenic archaeal phyla were associated with Hg methylation with seasonal- and site-specific variations. Firmicutes dominated in the soil enrichment cultures with a relative abundance of 73%, followed by Proteobacteria to a level almost equal to that found in soil samples (24.2%), followed by Bacteroidetes (0.28%), and Chloroflexi by 0.1% (Fig. 4a). When comparing seasonal differences in enrichment cultures, Firmicutes were significantly more abundant ($P < 0.05$) in the soil enrichments of summer and winter samples from the HC reference (control) site, and the spring and fall samples from EFK 18.2, while no seasonal variation was observed for samples from EFK 11.2. Both Bacteroidetes and Chloroflexi were significantly more abundant ($P < 0.05$) in soil enrichments of the winter and the spring samples from EFK 11.2 compared to all other sites and seasons. The Proteobacteria were significantly more abundant in enrichment cultures of summer and winter samples from EFK 18.2 and the fall samples from the control site when compared to the other seasons across the different sites.

At the class level, the Clostridia dominated by 43.0% in soil enrichments compared to 0.27% for each δ-Proteobacteria and Bacteroidia. A comparison among sites showed that Clostridia were significantly more abundant ($P < 0.05$) in the enrichment cultures using soils from the control site with a relative abundance of 61.4% compared to 36.2% and 25.9% for soil samples from EFK 11.2 and EFK 18.2, respectively, while the abundance of the δ-Proteobacteria was higher in samples collected from EFK 11.2 (0.47%), compared to 0.2% in soils from both the control and EFK 18.2 site, but the difference was statistically insignificant.

For those associated with Hg methylation, five families among the Clostridia and two among the δ-Proteobacteria were found in soil enrichments across different seasons and sites. Clostridiaceae, Peptococcaceae, Ruminococcaceae, Syntrophomonadaceae, and Veillonellaceae were predominant among the Clostridia with relative abundances of 6.3%, 1.8%, 2.0%, 0.13%, and 15.2%, respectively. Geobacteraceae and Syntrophobacteraceae were predominant among the δ-Proteobacteria with a relative abundance of 0.1% each (Fig. 4b). The family of Desulfovibrionaceae among the δ-Proteobacteria was detected with a relative abundance of 0.4% in the EFK 11.2 fall sample enrichment culture only, while it was not identified in soil isolates.

Table 2 Average percent abundances for 26 bank soil samples across all seasons and study sites

Clade	% EFK 18.2	% EFK 11.2	% Hinds Creek
Proteobacteria	36.5 ± 1.48^{A}	29.6 ± 1.04^{B}	19.0 ± 0.74^{C}
Firmicutes	0.9 ± 0.10^{A}	0.3 ± 0.07^{B}	0.3 ± 0.05^{B}
Bacteroidetes	2.1 ± 0.67^{B}	3.5 ± 0.61^{A}	0.9 ± 0.09^{C}
Chloroflexi	6.8 ± 0.48^{A}	6.8 ± 0.33^{A}	6.8 ± 0.24^{A}
Nitrospirae	7.9 ± 1.33^{B}	7.6 ± 0.73^{B}	12.2 ± 0.32^{A}
δ-Proteobacteria	8.5 ± 0.76^{A}	7.4 ± 0.54^{AB}	6.4 ± 0.38^{B}
Anaerolinea	1.6 ± 0.31^{AB}	2.4 ± 0.22^{A}	1.2 ± 0.15^{B}
Geobacteraceae	0.3 ± 0.07^{A}	0.2 ± 0.05^{A}	0.03 ± 0.03^{B}
Syntrophobacteraceae	5.3 ± 0.82^{A}	3.4 ± 0.57^{A}	3.9 ± 0.41^{A}
Geobacter	0.53 ± 0.03^{A}	0.50 ± 0.06^{A}	0.1 ± 0.02^{B}
Nitrospira	1.3 ± 0.28^{AB}	1.6 ± 0.74^{A}	1.2 ± 0.10^{B}

The letters A, B, and C indicate statistical significance among sites with A is the most abundant and C is the least abundant

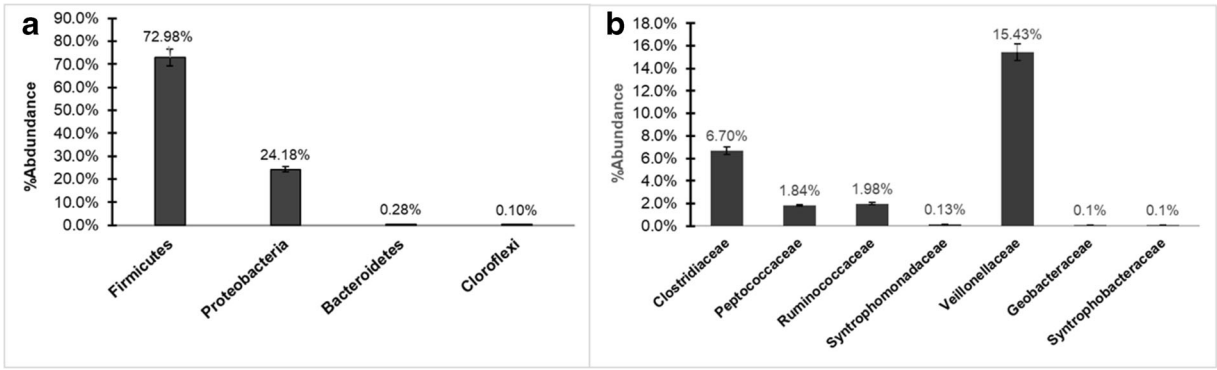

Fig. 4 Microbial community composition in enrichment cultures as average percentage abundances at **a** the phylum and **b** the family level

At the genus level, five genera, *Geobacter*, *Clostridium*, *Alkaliphilus*, *Acetonema*, and *Desulfosporosinus*, were detected in different soil enrichments. Variation in abundance between sampling sites was observed for *Clostridium* and *Desulfosporosinus* only, and both genera were significantly more abundant ($P < 0.05$) in enrichments from spring samples. Among soil enrichments *Clostridium* and *Desulfosporosinus* were among the top ten genera in soil enrichments from the HC reference site and EFK 18.2 with relative abundances of 2.6% and 1.7%, respectively.

3.2.3 Methanomicrobia in Enrichment Cultures

The Methanomicrobia are a class of microorganisms among the Euryarchaeota associated with Hg methylation. Representatives of this class were detected in enriched soil cultures from Hg-contaminated sites in winter and spring samples, with a relative abundance of 0.6%. Specifically, the Methanomicrobiaceae were identified with an abundance of 5.8% in the soil enrichment from EFK 11.2 collected in the spring, while the Methanocellaceae were found with an abundance of 0.6% in the soil enrichment of the winter sample from EFK 18.2. The Methanosarcinaceae were also identified in enrichments of the winter sample from EFK 18.2 (4.2%), the winter and the spring samples from EFK 11.2 (5.6 and 0.1%, respectively) and the fall sample from the HC reference site (2.3%) (Fig. 5a). Two genera, *Methanocella* and *Methanosarcina*, were identified in enrichment cultures with the winter samples from the Hg-contaminated sites. The genus *Methanocella* was identified in the winter samples from EFK 18.2 with a relative abundance of 0.6%. In comparison, the genus *Methanosarcina* was found in both EFK 18.2 and EFK 11.2 in the winter samples with relative abundances of 3.2% and 5.6%, respectively. In addition, *Methanosarcina* was also present in enrichment of the fall sample from the HC reference site. (Fig. 5b).

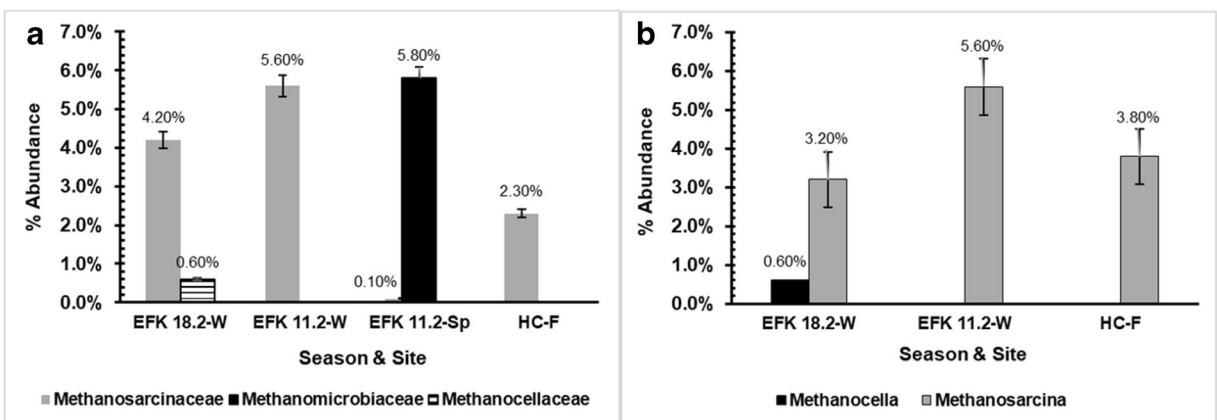

Fig. 5 Percentage abundances in enrichment cultures for **a** families and **b** genera among the Euryarchaeota; W, Winter; F, Fall; Sp, Spring; HC, control

3.3 Detection of *hgcA*-Biomarkers in Streambank Soils Using Primers

PCR amplification with the primers designed to target homologs of the *hgcA* gene produced a product of ~ 950 bp. The three target strains were selected based on the families' abundance determined in the soil sequencing and to represent major groups of potential Hg methylators. Their primers are expected to hybridize with *hgcA* variants from multiple species due to a high degree of sequence conservation among *hgcA* genes. The product was successfully amplified from a total of 13 soils and enriched soils (54.2%) from the 24 tested samples (Table 3). Amplicons corresponding to the expected size were recovered, purified, and sequenced. When *hgcA* primers for *D. desulfuricans* ND132 were used, two soils and four enriched soils were found to be positive for *hgcA* (EFK 18.2—winter and summer enriched soils; EFK 11.2—spring and fall for both soil and enriched soil samples) (Fig. 6a). Using primers targeting *hgcA* from *G. sulfurreducens* PCA, homologs were detected in soil samples from EFK 18.2 during winter, fall, and summer. With primers targeting *hgcA* from *D. tiedjei* DCB-1, four samples were found positive, two in EFK 18.2 soils (winter and summer), and two in enriched soils from EFK 11.2 (summer and spring) (Fig. 6b). The recovered and sequenced amplicons were aligned to the *hgcA* sequence of the corresponding target strain using the NCBI BLASTN tools and matched with significant *e*-values (Table 3).

4 Discussion

The goal of this study was to evaluate potential impacts of THg levels on the microbial community structures and to detect clades associated with Hg methylation in bank soils of a Hg-contaminated creek. Two contaminated bank soils, one with high THg levels and another one with lower levels, were compared to evaluate differences in the abundance of microbial clades associated with Hg methylation. In addition, samples collected across four seasons were compared to assess seasonal variations in the microbial soil community. In general, our results indicated that EFPC streambank soils contain several microbial clades that have been associated with Hg methylation, and their abundances in most cases were significant in one or both Hg-contaminated sites compared to the control in different seasons (Table 2). A previous study conducted with EFPC stream sediments has shown significant correlations between the bacterial community and seasonal as well as geochemical factors, where some community members including SRB were associated positively with THg and MeHg concentrations (Vishnivetskaya et al. 2011). Other studies have shown seasonal effects on the biotic production of

Table 3 Sanger sequencing result of recovered bands and amplicons of expected positive soil and enriched soil samples. The first letter of the sample ID indicates culture (C) or soil (S); followed by season Spring (Sp.); Fall (F.); Winter (W.); Summer (S.); followed by sampling site location EFK (East Fork kilometer). The letters at the end of the sample ID indicate the strain-specific primers used (ND132, PCA or DCB-1)

#	Sample ID	Sanger sequence	Translation/motif	Seq. position	Blast position	*E*-value
1	C.Sp.EFK11.2. ND	AACGTCTGGTGCGCGGCGGGAAAG	NVWCAAGK	328–352	267–287	9e−07
2	S.Sp.EFK11.2. ND	AACGTCTGGTGCGCGGCGGGA	NVWCAAG	305–325	268–276	1e−08
3	C.F.EFK11.2. ND	ACGTCTGGTGCGCGGCGGGA	NVWCAAG	230–250	268–287	5e−08
4	S.F.EFK11.2. ND	AACGTCTGGTGCGCGGCG	NVWCAA	70–91	268–285	2e−07
5	C.W.EFK18.2. ND	AACGTCTGGTGCGCGGCGGGA	NVWCAAG	109–129	268–287	1e−08
6	C.S.EFK18.2. ND	AACGTCTGGTGCGCGGCCGGAAAG	NVWCAAGK	327–350	268–284	9e−07
7	S.S.EFK18.2. PCA	AATGTCTGGTGCGCCGCCGGA	NVWCAAG	65–85	235–254	1e−08
8	S.W.EFK18.2. PCA	AATGTCTGGTGCGCCGCCGGG	NVWCAAG	761–781	235–254	1e−08
9	S.F.EFK18.2. PCA	AATGTCTGGTGCGCCGCCGGA	NVWCAAG	54–74	235–254	1e−08
10	S.S.EFK18.2. DCB	AATGTCTGGTGTGCCAGCGGGA	NVWCAAG	763–783	256–269	5e−05
11	C.S.EFK11.2. DCB	AATGTCTGGTGTGCAGCGGGC	NVWCAAG	59–79	256–275	1e−08
12	C.Sp.EFK11.2. DCB	AATGTCTGGTGTGCAGCGGGC	NVWCAAG	220–240	256–275	1e−08
13	S.W.EFK18.2. DCB	AATGTCTGGTGTGCAGCTGGTAAA	NVWCAAGK	453–476	256–272	9e−07

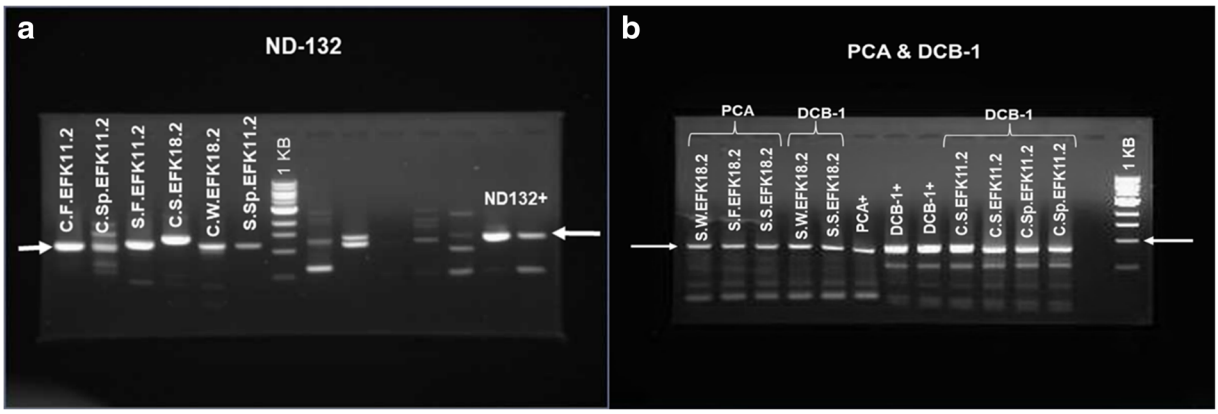

Fig. 6 PCR results using *hgcA* primers, **a** *D. desulfuricans* (ND-132) primers, **b** *G. sulfurreducens* (PCA), and *D. tiedjei* (DCB-1) primers. The first letter of the sample ID indicates culture (C) or soil (S), followed by season Spring (Sp.); Fall (F.); Winter (W.); Summer (S.); followed by sampling site location EFK (East Fork kilometer)

MeHg, with the highest levels linked to increased microbial activities during the summer months (Gilmour and Henry 1991; Stoichev et al. 2004).

Overall, the average fraction of MeHg was lower in EFK 18.2 compared to EFK 11.2 (2.2% vs. 7.6% MeHg/THg) and in all seasons except winter, which could be attributed to Hg bioavailability and differences in the microbial activity. In addition to microbial activity, soil constituents and geochemistry determine Hg speciation and its fate in the environment under various conditions (Bratkič et al. 2017; Han et al. 2007; Bigham et al. 2017; Ma et al. 2019). In aquatic systems containing low levels of sulfide, such as EFPC, Hg speciation is controlled to a large extend by complexation with natural organic matter (NOM) (Aiken et al. 2011). In addition, pH, soil particle distributions, and redox conditions exert a substantial influence on Hg speciation, release, and bioavailability in EFPC (Egbo et al. 2017; Dickson et al. 2019). For example, the soil texture of the streambank soils along EFPC has been classified as loam and silty loam with the silt content decreasing with EFK distance (Dickson et al. 2017). The soil texture of the soils used in this study is silty clay loam (Egbo et al. 2017; Dickson et al. 2019), with a higher silt and clay content in EFK 11.2. Generally, higher Hg levels in the soil are associated with the finest size fractions, which can be explained by high specific surface area of clays to sorb Hg and NOM (Coufalík et al. 2012). Also, clays can form stable complexes with Hg depending on soil geochemistry (Liao et al. 2009; Biester et al. 2002; Boszke et al. 2008), which may decrease Hg leachability, bioavailability, and consequently MeHg levels. While the clay content in bank soils at EFK 18.2 is lower compared to EFK 11.2, the Hg level is higher, which has attributed to the inclusion of Hg in coal fines and ash in the HRD layer at this site. Due to the higher carbon and NOM contents, as demonstrated by other studies (Bigham et al. 2017; Hsu-Kim et al. 2013; Różański et al. 2016; Beckers and Rinklebe 2017), the interaction of the Hg species with these soil constituents may control solubility and bioavailability for methylation. A study showed that elevated THg is often associated with organic-rich soil as Hg has a high affinity to thiol functional groups present in NOM (Beckers and Rinklebe 2017). Also, it was shown that soil organic carbon might increase the fraction of mobile Hg. In contrast, clay reduces the mobile fraction (Różański et al. 2016). Therefore, variations of the factors mentioned above in our study sites may play a critical role and contribute to differences in THg and MeHg levels.

Other geochemical factors can also exert a strong influence on Hg bioavailability and microbial activity. The growth and the activity of the potential methylators as well as any other microorganisms are influenced by the pH. A high pH also reduces methylation potential by reducing Hg bioavailability. It was shown that a high pH increased Hg bound to Fe-Mn-oxides, thus lowering bioavailability and reducing methylation potential (Chen et al. 2016). Other studies demonstrated that Hg(II) leachability and bioavailability are highly affected by pH and increase under acidic conditions (Miretzky et al. 2005) (Coufalík et al. 2012; Gilmour and Henry 1991). On the other hand, the high level of Fe in EFK 18.2 soils (3.49%) and to a lesser extent in EFK 11.2 soils (1.97%) may create favorable redox conditions for IRB as evidenced by the presence of IRB at these sites.

The elevated MeHg concentrations at EFK 18.2 may be a result of the biogeochemical soil characteristics leading to higher MeHg levels.

Several microbial community members, including SRB in EFPC sediments, were positively associated with THg and MeHg (Vishnivetskaya et al. 2011). In our results, we observed that about 44.4% of the microbial phyla in the EFPC streambank soils were from those being associated with Hg methylation (see Fig. 3a), and some of them were significantly more abundant in the Hg-contaminated sites compared to the control (Table 2). A comparison of the present data with a previously published study shows that the microbial communities in EFPC bank soils and creek sediments are almost identical (Vishnivetskaya et al. 2011). Similar relative abundances suggest a frequent exchange between bank soils and sediments due to bank erosion, flooding events, and runoff. A comparison of samples over four seasons did not show significant changes among the most abundant microbial clades. The Proteobacteria showed no seasonal variation, while Firmicutes were more abundant during the fall and winter and the Chloroflexi were the only clade that showed higher abundance in the summer compared to other seasons with no site preference. However, Proteobacteria and Firmicutes were higher in EFK 18.2. The large variability in the MeHg concentrations over different seasons and across sites was most likely a result of geochemical factors specific to each site that determine Hg speciation, bioavailability, and microbial activity. Interestingly, soil enrichment cultures promoted the growth of only 52% of the microbes identified in the respective soil. Firmicutes were enriched preferentially up to an abundance of 73%.

Detection of the *hgcAB* genes and determining their abundance in Hg-contaminated environments is crucial for predicting active microbial Hg methylation. Using primers of strains that represent three major methylating clades, we were able to detect homologs of the key *hgcA* gene in 54.2% of the EFPC streambank soil samples across all seasons. Amplicon sequencing and alignments showed that all positive samples could be matched to target strains closely related to Hg-methylating species. Moreover, the abundance of the Hg-methylating clades correlated with higher MeHg levels, and in samples where *hgcA* was detected. Using ND132 *hgcA* primers, homologs were detected at EFK 18.2 in winter and summer samples. With the PCA *hgcA* primers, homologs were detected at EFK 18.2 only and in samples collected during the winter, fall, and summer. In contrast, with DCB-1 *hgcA* primers, homologs were detected at EFK 18.2 in winter and summer samples and EFK 11.2 in spring and summer samples. These results suggest a potential role for IRB and syntrophs in EFPC bank soils.

5 Conclusions

Microbial communities play an essential role in the transformation of Hg in contaminated watersheds. Net MeHg production is controlled by a complex interplay between biotic and abiotic factors. In this study, we investigated the role of the microbial community composition in contaminated bank soils in light of other biogeochemical factors, such as Hg concentration, soil composition, and chemistry. Overall, microbial clades associated with Hg methylation were found to be more abundant in Hg-contaminated soils, which is also in agreement with the distribution of detectable *hgcA* genes in the soil. Thus, determining the microbial community structure and targeting genes linked to Hg methylation to identify potential methylators is crucial for informing mitigation efforts for Hg-contaminated sites.

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Authors' Contributions All authors read and approved the final manuscript.Data AvailabilityThe authors confirm that the data supporting the findings of this study are available within the article.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethics Approval Not applicable—no animal or human participants.

Consent to Participate Not applicable—no animal or client participants.

Consent for Publication Not applicable—no individual person's data in any form.

Code Availability Not applicable—no custom code required.

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