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Mercury methylation genes identified across diverse anaerobic microbial guilds in a eutrophic sulfate-enriched lake

Benjamin D. Peterson[1,*], **Elizabeth A. McDaniel**[2], **Anna G. Schmidt**[2], **Ryan F. Lepak**[1,3,4], **Sarah E. Janssen**[3], **Patricia Q. Tran**[2,5], **Robert A. Marick**[6], **Jacob M. Ogorek**[3], **John F. DeWild**[3], **David P. Krabbenhoft**[3], **Katherine D. McMahon**[2,7]

[1]Environmental Science & Technology Program, University of Wisconsin - Madison, 660 N. Park Street, Madison, WI 53706, USA

[2]Department of Bacteriology, University of Wisconsin - Madison, 1550 Linden Drive, Madison, WI 53706, USA

[3]U.S. Geological Survey, Upper Midwest Water Science Center, Mercury Research Laboratory, 8505 Research Way, Middleton, WI 53562, USA

[4]U.S. Environmental Protection Agency Office of Research and Development, Center for Computational Toxicology and Exposure, Great Lakes Toxicology and Ecology Division, 6201 Congdon Blvd, Duluth, MN 55804, USA

[5]Department of Integrative Biology, University of Wisconsin - Madison, 250 N. Mills St.Madison, WI 53706, USA

[6]Department of Biochemistry, University of Wisconsin - Madison, 433 Babcock Drive, Madison, WI 53706, USA

[7]Department of Civil and Environmental Engineering, University of Wisconsin – Madison, 1415 Engineering Drive, Madison WI 53706, USA

Abstract

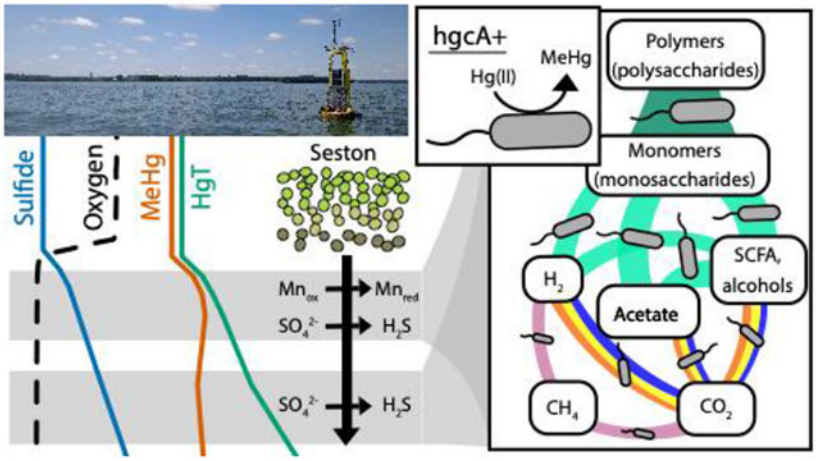
Mercury (Hg) methylation is a microbially mediated process that converts inorganic Hg into bioaccumulative, neurotoxic methylmercury (MeHg). The metabolic activity of methylating organisms is highly dependent on biogeochemical conditions, which subsequently influences MeHg production. However, our understanding of the ecophysiology of methylators in natural ecosystems is still limited. Here we identified potential locations of MeHg production in the anoxic, sulfidic hypolimnion of a freshwater lake. At these sites, we used shotgun metagenomics to characterize microorganisms with the Hg-methylation gene *hgcA*. Putative methylators were dominated by *hgcA* sequences divergent from those in well-studied, confirmed methylators. Using genome-resolved metagenomics, we identified organisms with *hgcA* (hgcA+) within the Bacteroidetes and the recently described Kiritimatiellaeota phyla. We identified hgcA+ genomes

*Corresponding author: bpeterson26@wisc.edu.

Supplementary Information (PDF):
Supplementary Methods includes additional details on sampling efforts, Hg analyses, DNA extractions, and bioinformatics workflows. Supplementary Figures include additional geochemical profiles, *hgcAB* alignments, detailed phylogenetic trees, and figures detailing metabolic pathways of methylators.

derived from sulfate-reducing bacteria, but these accounted for only 22% of hgcA+ genome coverage. The most abundant hgcA+ genomes were from fermenters, accounting for over half of the *hgcA* gene coverage. Many of these organisms also mediate hydrolysis of polysaccharides, likely from cyanobacterial blooms. This work highlights the distribution of the Hg-methylation genes across microbial metabolic guilds and suggests that primary degradation of polysaccharides and fermentation may play an important but unrecognized role in MeHg production in the anoxic hypolimnion of freshwater lakes.

Graphical Abstract



Keywords

hgcAB; mercury methylation; methylmercury; freshwater; metagenomics

Introduction

Environmental Hg levels have increased 3-4 times compared to pre-development times, largely due to human activity.[1] Much of this anthropogenically released Hg is gaseous-elemental Hg, which can later be oxidized to Hg(II) and deposit to terrestrial and aquatic ecosystems.[2] Microorganisms can then convert Hg(II) to methylmercury (MeHg) in various low redox environments, including sediments, periphyton, rice paddy soils, and sub- or anoxic regions of freshwater and marine water columns.[3–9] MeHg bioaccumulates and biomagnifies up the food web, making Hg-methylation an important process in food web Hg accumulation.[10] In freshwater lakes, MeHg accumulation has historically been attributed to production in sediments followed by diffusion across the sediment-water interface.[5,11,12] However, Hg-methylation also occurs in the water column, under both anoxic and oxic conditions, and can account for the bulk of water column MeHg accumulation in some lakes.[4,6,9,13–15]

Biogeochemical conditions, like redox status and carbon bioavailability, can indirectly drive MeHg production by fueling metabolic activity of Hg-methylating microorganisms.[12,16] Inhibition of sulfate reduction in cultured isolates and *in situ* assays have linked sulfate-

reducing bacteria (SRBs) activity to MeHg production.[11,12] Many subsequent studies have linked sulfate reduction to Hg-methylation across many ecosystems, suggesting that SRBs are the primary drivers of MeHg production *in situ*.[17–20] Later studies identified iron-reducing bacteria (FeRB) and methanogenic archaea that can also produce MeHg, expanding the terminal electron-accepting processes (TEAPs) associated with MeHg production.[21,22] We know little about how primary degradation of organic molecules, syntrophy, or fermentation influence MeHg production, but some fermentative and syntrophic microbes, such as Clostridia, are known to methylate.[23,24] On the community level, Hg-methylation rates increase with increasing overall heterotrophic activity, suggesting that simply increasing carbon and energy flux through microbial communities can promote MeHg production.[9,25] To date, most of our understanding of microbial Hg-methylation relies on reductionist monoculture experiments or on amendment/inhibition studies with environmental samples that lack information about the microbial community supporting MeHg production.

The identification of the *hgcAB* gene cluster has provided a molecular marker for MeHg production, allowing for the in-depth examination of microbial communities and conditions that promote Hg-methylation in the environment.[23,26] Surveys of publicly available genomes, metagenomes and metagenome-assembled genomes have expanded the known phylogenetic and metabolic diversity of organisms with *hgcA* (hgcA+).[23,27–32] Using polymerase chain reaction (PCR)-based amplicon sequencing, several studies have demonstrated that hgcA+ communities are phylogenetically distinct and linked to geochemical conditions across different environments.[33–37] While this approach generally captures the deep diversity of *hgcA* sequences in complex communities, PCR primers are subject to amplification bias and do not provide additional phylogenetic or metabolic information.[14,29,30] Shotgun metagenomics, which involves sequencing random small strands of DNA from a sample, reduces amplification biases and enables assembly of longer DNA segments that provide additional genetic context for identified genes. This method also offers a more robust identification of novel *hgcA* sequences from environmental samples, since computational tools such as Basic Local Alignment Search Tool (BLAST) and Hidden Markov Models (HMMs) are better equipped to identify divergent sequences.[31,38,39] To provide further genetic context for *hgcA*, genome-resolved metagenomics can be used to generate population genomes (bins) from the assembled DNA, enabling phylogenetic identification using conserved genes and metabolic characterization.[14,40,41] This approach has been used to identify prominent novel Hg-methylators from the Aminicenantes and Kiritimatiellaeota phyla in the water column of a sulfate-enriched lake.[14] The ability to not only identify Hg-methylators but also describe their metabolic potential *in situ* makes genome-resolved metagenomics an important tool in closing the gap between culture work and *in situ* assays or observations and in understanding how nutrient and biogeochemical conditions influence Hg-methylation.

In this study we applied genome-resolved metagenomics to identify the metabolic pathways linking biogeochemical cycling to MeHg production in the hypolimnion of Lake Mendota, Wisconsin, USA, a large, well-studied, freshwater eutrophic lake. During stratification, >50% of the total Hg (HgT) in the hypolimnion is MeHg, yet it is unclear which microbial communities are contributing to these high MeHg levels. Mendota has elevated sulfate

concentrations due to watershed geology, which supports sulfate reduction in the anoxic hypolimnion; thus, we hypothesized that the hgcA+ community is dominated by sulfate-reducing organisms.[42,43] We used Hg speciation and redox profiles to identify sites with suspected *in situ* MeHg production and selected a subset of these for shotgun metagenomic sequencing. This approach allowed us to identify novel methylators and examine their metabolic pathways, place the methylators in the context of the broader microbial community, and ultimately provide insight into how biogeochemical cycles may influence MeHg production. Historically, TEAPs such as sulfate reduction and methanogenesis have been identified as drivers of MeHg production, but this work suggests that primary degradation and fermentation may drive *in situ* MeHg production in this system.

Materials and Methods

Field sampling.

Lake Mendota is a large dimictic lake located in Madison, Wisconsin, USA. Sampling was conducted at the deepest part of the lake, near the North Temperate Lakes Long-Term Ecological Research (NTL-LTER) buoy. The lake is approximately 24 meters deep at this site. Samples were collected approximately monthly in 2017 from the onset of hypolimnetic anoxia in June until stratification broke down and the water column mixed. Profiles of temperature, dissolved oxygen, and turbidity were collected with a multi-parameter sonde (YSI Incorporated, Yellow Springs, OH). Samples were collected through acid-washed Teflon sampling line using a peristaltic pump. Samples for total sulfide analysis were preserved in 1% zinc acetate. Water samples for dissolved metal (non-Hg) analysis were filtered through a 0.45 μm PES Acrodisc filter (Pall Corp., Port Washington, NY) and acidified to 1% hydrochloric acid (HCl). Hg samples were collected using trace-metal-clean methods in 2.5L bottles.[44] These bottles were allowed to overflow before capping to minimize oxygen exposure within the sample, then were double-bagged and stored in a cooler. Water was filtered through ashed quartz fiber filter (QFF) within 24 hours and preserved to 1% HCl for filter-passing Hg and MeHg analysis.[44] The QFF filters were frozen for particulate Hg analysis.[44] DNA samples were collected by filtering 300-400 ml of sample water onto 0.22 μm pore-size PES filters (Pall Corp., Port Washington, NY) and were flash-frozen on liquid nitrogen within 90 seconds of collection.

Geochemical analyses.

Sulfide was quantified spectrophotometrically using the Cline method.[45] Iron and manganese were quantified by inductively coupled plasma optical emission spectrometry. Processing and analysis of Hg samples was done at the U.S. Geological Survey (USGS) Mercury Research Laboratory (MRL), Middleton, WI. Filter-passing and particulate HgT samples were oxidized using bromine monochloride (BrCl) and quantified using tin reduction coupled to cold vapor atomic fluorescence spectrometry.[46,47] Filter-passing and particulate MeHg samples were distilled to remove matrix interferences and then quantified by inductively coupled plasma mass spectrometry following US EPA method 1630 modified by quantification via isotope dilution.[48–50] All HgT and MeHg analyses passed required quality assurance and control standards. Geochemical data can be found in Table S1a.

DNA extraction, sequencing, and assembly.

We selected five samples for shotgun metagenomic DNA sequencing and analysis. Three of these samples were selected to coincide with the chemocline and are referred to as CHE1, CHE2, and CHE3 (Table S2). The other two samples are from the deep euxinic hypolimnion and are referred to as EUX1 and EUX2 (Table S2). DNA was extracted by enzymatic and physical lysis followed by phenol-chloroform extraction and isopropanol precipitation.[51] DNA library preparation was done at the Functional Genomics Lab and sequencing was done in the Vincent J. Coates Genomics Sequencing Lab (QB3-Berkeley, Berkeley, CA). Library preparation was done with a Kapa Biosystem Library Prep kit, targeting inserts ~600bp in length (Roche Sequencing and Life Science, Kapa Biosystems, Wilmington, MA). The five libraries were pooled and run on a single lane on an Illumina HiSeq4000 with 150bp paired-end sequencing (Illumina, Inc., San Diego, CA). Raw reads were trimmed using Sickle (v1.33) and assembled using metaSPADes (v3.12) (Table S3).[52,53] Assembly-based analyses were run on all scaffolds >500bp long. Open reading frames (ORFs) were predicted using Prodigal (v2.6.2).[54] Reads were mapped to the scaffolds of each assembly using BBMap (v35) with default settings.[55] Scaffold abundance is defined as the mean value of the read coverage at each nucleic acid residue in a scaffold. Gene abundances are defined as the abundance of the corresponding scaffold. Scaffold abundances in each metagenome were normalized by calculating the ratio of reads in the smallest metagenome to the number of reads in that metagenome, then multiplying the abundance of each scaffold by this ratio.

Metagenomic binning and annotation.

Automatic binning was done for each assembly on scaffolds >1000bp in length. Bins were generated using Metabat2 (v2.12.1), MaxBin (v2.1.1), and CONCOCT (v0.4.1), then aggregated using Das Tool.[56–59] Bins across assemblies were clustered into “high matching sets” (HMSs) if they shared at least 98% ANI over at least 50% of the genome. CheckM (v1.1.2) was used to estimate the completeness and redundancy of each bin.[60] One bin from each HMS was selected for further analysis. We retrieved 228 medium quality bins that were more than 75% complete and less than 10% redundant (Table S6).[61] These bins accounted for only 33% of the total number of reads. Bins were then decontaminated using Anvi’o (v5.2), reassembled with SPADes, and re-binned in Anvi’o.[53,62] Taxonomy of each bin was estimated using GTDB-TK (v0.3.2).[63] Preliminary metabolic annotations were done using MetaPathways.[64] Annotations of metabolic genes of interest were confirmed using Hidden Markov Models (HMMs) from TIGRFAM and PFAM, gene neighborhoods, and phylogenies.[38]

hgcA identification.

A custom HMM for HgcA amino acid sequences was built with hmmbuild from hmmer (v3.1b2) using experimentally verified HgcA amino acid sequences (Table S4).[23,65] Putative HgcA sequences were identified using hmmsearch (v3.1b2) (Table S5).[65] Each hit was manually screened for the cap helix domain and at least 4 transmembrane domains.[26] HgcA sequences were dereplicated across assemblies using CD-HIT (v4.8.1) with a 97% identity cut-off.[66]

Phylogenetic analyses.

Bin phylogenies were based on 16 ribosomal protein (rp16) sequences.[67] For rp16 and HgcA phylogenies, amino acid sequences were aligned using MUSCLE (v3.8.31).[68] Each rp16 gene was aligned individually, then all alignments were concatenated. Sequences with less than half of the aligned residues were manually removed. Alignments were inspected in Geneious and trimmed using BMGE1.1 with the BLOSUM30 substitution matrix.[69] The final HgcA alignment included 181 residues and the final rp16 alignment included 2217 residues. RAxML (v8.2.11) was used to generate a maximum likelihood (ML) tree under the GAMMA distribution with the LG model.[70] Branch support was generated by rapid bootstrapping. For HgcA phylogenies, RogueNaRok (v1.0) was used to remove “rogue taxa” interfering with proper tree generation.[71] Rogue taxa were classified using pplacer and included in the analysis.[72] The best-scoring ML tree for HgcA was mid-point rooted using the Phangorn R package and visualized using ggtree.[73,74] For unbinned HgcA sequences, taxonomy was assigned to each HgcA sequence based on phylogenetic clustering with HgcA reference sequences from NCBI and bin phylogenies of binned HgcA sequences. HgcA sequences that did not fall into a monophyletic cluster are marked as “unknown”. The rp16 ML tree was rooted using an archaeal outgroup.

Data availability.

Trimmed metagenomes and metagenomic assemblies can be found under BioProject PRJNA646991. The scaffolds and the ORFs for the bins, can be found at the project page on the Open Science Framework (OSF), here: https://osf.io/9vwgt/. The nucleic acid and amino acid sequence files for the confirmed *hgcA*/HgcA sequences and the HgcA HMM used in this study can be found on the same OSF page.

Results and Discussion

Redox and Hg biogeochemistry in Lake Mendota.

Microbial anaerobic respiration is regulated by terminal electron acceptor availability, which continually evolves vertically in Mendota’s hypolimnion throughout the summer-fall season as negative redox conditions strengthen due to high biochemical oxygen demand (Figures 1, S1). We monitored limnological and biogeochemical conditions in the hypolimnion to identify likely TEAPs at play (Table S1). Anoxia developed in the hypolimnion as early as June, likely due to senescence and decomposition of biomass from spring phytoplankton blooms (Figure S1). Nitrate/nitrite levels reached 6 μM at the oxic/anoxic interface in August, but by September were nearly undetectable (Figure S1). Dissolved iron (Fe) transiently accumulated (5 μM) in the hypolimnion immediately following anoxia, but was quickly precipitated out by sulfide and was unlikely to serve as an electron acceptor in the water column (Figure S1).[75]

Manganese (Mn) also accumulated shortly after anoxia developed and remained in the hypolimnion throughout the anoxic period, ranging from 4-6 μM. In June and August, the near-bottom hypolimnetic accumulation of Mn and linear profile suggests that Mn was being reduced in the surficial sediments and diffusing into the hypolimnion.[76] During September and October, there was a peak in dissolved Mn near the oxic/anoxic interface (Figures S1,

S2). Particulate Mn was detected (1.4 μM) just above the peak in dissolved Mn in late September (Figure S2). While this peak was not detected in October, this could be due to insufficient sampling resolution, since the profile from September suggests that particulate Mn is localized to a thin band in the water column (Figure S2). Together, these data suggest localized reduction, just below the oxic-anoxic interface, of settling Mn oxides that were produced by the downward migration of the thermocline, as previously shown to occur in Lake Mendota and other lakes.[75,76] This indicates that Mn reduction could be an important TEAP near the oxic/anoxic interface during late anoxia.[77]

Sulfate reduction, commonly implicated in MeHg production, has been documented in the water column of Lake Mendota.[43] During early stratification, under relatively high redox conditions, sulfate levels were approximately 180 μM throughout the water column (Figures S1, S2). Sulfide was first detected in August and accumulated to over 150 μM by October (Figures 1, S1, S2). Sulfate depletion mirrored sulfide accumulation, with sulfate levels falling to 21 μM in the deep hypolimnion by October. Sulfate levels have previously been shown to be limiting below 100 μM in Lake Mendota sediments, and other work has shown that SRB require >60 μM sulfate to outcompete methanogens.[43,78] Taken together, these data suggest that sulfate reduction is likely an important TEAP in driving anaerobic metabolism throughout the hypolimnion during late fall, but may slow in the deep hypolimnetic waters during late stratification.

Once oxygen was depleted, both HgT and MeHg began accumulating in the hypolimnion (Figures 1, S1). We discuss here total MeHg and HgT levels, calculated by summing the dissolved and particulate fractions, but the dissolved and particulate fractions are shown in Figure S1. The lower hypolimnetic buildup of MeHg and HgT during June and August suggests that diffusion of Hg from sediments is important. This persists in September and October for HgT, which reached 1.86 ng/L in the deep hypolimnion. However, during this time, MeHg increased in the metalimnion up to 0.63 ng/L, while hypolimnetic MeHg remained between 0.4 and 0.5 ng/L. Correspondingly, the percent MeHg (the MeHg:HgT ratio) peaked at the oxic/anoxic interface (52%). The late fall peak in MeHg and percent MeHg near the oxic-anoxic interface is likely due to elevated MeHg production in the metalimnion relative to the hypolimnion. Other potential explanations of this MeHg enrichment include increased demethylation of MeHg in the deep hypolimnion, which to our knowledge has not been shown to occur in lakes; or MeHg diffusion from above, which is unlikely because MeHg levels are low in the epilimnion. Elevated MeHg production just beneath the oxycline has been shown in other freshwater lakes[9,13] and in other redox transition zones, such as hyporheic zones and Sphagnum moss mats.[19] There are likely two concurrent reasons for this elevated MeHg production in this region. First, high sulfide levels can strongly inhibit MeHg production by reducing the bioavailability of Hg to methylators.[79] While sulfide concentrations are high enough in the deep hypolimnion to inhibit Hg-methylation, sulfide levels near the oxycline are relatively low. However, some of the highest MeHg levels recorded were at 17.8 m in October, when dissolved MeHg was 0.63 ng/L and sulfide was over 100 μM (Figure 1). Second, overall microbial metabolism is often elevated near strong redox gradients.[9] During late stratification, the percent MeHg maxima also coincided with peaks in turbidity, which has been previously shown to co-localize with elevated microbial activity and MeHg production.[15] It is most

likely that a combination of these two factors (abiotic speciation and Hg-methylator activity) led to elevated MeHg production near the oxic-anoxic interface.

HgcA identification.

Metagenomic approaches were used to identify *hgcA* genes that corresponded with metalimnetic peaks of MeHg and hypolimnetic euxinic regions. We identified 108 unique *hgcA* genes on assembled scaffolds recovered from the five samples (Figure S3). While we used *hgcA* as our marker for Hg-methylation, the *hgcB* gene is also required for methylation activity.[26] Ninety of the identified *hgcA* genes also had a downstream *hgcB* gene, confirming that these are likely functional Hg-methylation genes. Seven of the 18 hgcA+ scaffolds lacking *hgcB* ended just downstream of *hgcA*, and it is possible that *hgcB* did not assemble into the scaffold. The remaining 11 *hgcA* genes with no *hgcB* partner had a similar phylogenetic and coverage distribution to those with a downstream *hgcB* (Figure S4, Table S5). Notably, Hg-methylation has been experimentally verified in Desulfovibrio africanus sp. Walvis Bay and Desulfovibrio inopinatus, in which hgcA is separated from hgcB by a single gene and 29kbp, respectively.[26,80,81] Since we cannot rule out that the corresponding *hgcB* gene is elsewhere in the genome for these 11 sequences, we included all 108 *hgcA* genes in our analysis.

While our biogeochemical data shows greater MeHg accumulation in the metalimnion than in the hypolimnion, *hgcA* abundance did not vary substantially between the metalimnion and hypolimnion (Figure 2b). This is consistent with a lack of correlation between *hgcA* abundance and Hg-methylation activity or MeHg levels in the literature.[79] Relating overall abundance of hgcA genes from metagenomes to MeHg levels is problematic due to the fact that metagenomic data is compositional rather than absolute.[82] In addition, culture experiments show there is a wide range of Hg-methylation activity by different hgcA+ organisms.[23] Finally, as discussed above, abiotic factors such as sulfide complexion also likely play a large role in determining MeHg production in the water column.[79]

We then searched for the hgcA gene in the 228 reconstructed metagenomic bins. We identified 41 hgcA+ bins that were representative of the overall hgcA genetic diversity. All but three of these genomes had an hgcB sequence paired with hgcA, and no bins were found with hgcB but no hgcA. One of these bins (LEN_0031) included two copies of the *hgcA* gene. However, bins represent composite population genomes rather than individual genomes.[83] Thus, we cannot confirm that the two *hgcA* sequences were present together in a single organism, which, to our knowledge, has not been demonstrated. These 41 bins accounted for 51% of the total *hgcA* coverage in our assemblies. This limited coverage highlights an inability to recover genomes for 13 out of the 30 most abundant *hgcA* sequences, including the most abundant *hgcA* gene (Figure S5). Efforts to recover these abundant hgcA+ bins through read subsampling, contig curation using assembly graphs, reassembly, and manual binning and curation were unsuccessful. Many of these scaffolds had highly abundant sequence nucleotide variants, suggesting there were multiple closely related strains, which can interfere with the binning process. While this means our view of the metabolic diversity of hgcA+ bins in these metagenomes is incomplete, we did successfully bin hgcA+ scaffolds corresponding to most of the HgcA phylogenetic clusters,

suggesting that most of the methylator diversity is represented in our bins (Figure 2). The hgcA+ bins accounted for 17% of the total read coverage from all bins and included some of the most abundant bins from our metagenomes (Figure S6a). The hgcA+ bins were slightly less abundant than bins without *hgcA* (hgcA-) bins, but this could be due to the greater degree of manual curation of the hgcA+ bins (Figure S6b). Overall, the hgcA+ bins recruited 6% of the total number of reads from our metagenomic datasets. Because the hgcA+ bins accounted for only 51% of the total coverage of all recovered *hgcA* sequences, we estimate that hgcA+ genomes account for ~12% of the total metagenomic reads across our five samples, which is consistent with previous work applying this technique in similar systems.[14]

Phylogenetic diversity of hgcA+ community.

Most of the identified *hgcA* genes from this study are not representative of well-characterized and experimentally verified methylating organisms. Of the 108 HgcA sequences, only 43 clustered with HgcA sequences from experimentally verified methylators (Figures 2, S4). The majority of these sequences are associated with the Deltaproteobacteria class. The most abundant of these, accounting for 12% of the hgcA+ coverage, belong to the Syntrophobacterales order, which includes both syntrophic and sulfate-reducing organisms (Figures S4, S7). The two other Deltaproteobacteria orders are Geobacterales and Desulfobacterales, both of which are metabolically diverse but commonly associated with iron reduction and sulfate reduction, respectively. Notably, no sequences associated with Desulfovibrionales or Pseudodesulfovibrionales, two well-studied orders that include the model sulfate-reducing methylator *Desulfovibrio desulfuricans* ND132, were detected.[84] We also detected *hgcA* genes from the other two common groups of confirmed methylators, the phylum Firmicutes and methanogenic Archaea. However, both of these also constituted a small percentage of the total *hgcA* coverage (<7% each). Overall, *hgcA* sequences associated with confirmed methylators only accounted for about 27% of the total *hgcA* coverage. While abundance does not necessarily correlate to activity, this suggests that novel unconfirmed methylators may play a larger than expected role in MeHg production in Lake Mendota.

The majority of *hgcA* read coverage was accounted for by two large *hgcA* clusters, neither of which are associated with experimentally verified methylators. Fourteen of these sequences, accounting for 13% of the total *hgcA* coverage, formed a monophyletic cluster with substantial bootstrap support (Figure S4). Taxonomic and phylogenetic analysis of the four bins with *hgcA* genes from this cluster placed them in the Bacteroidales order (phylum Bacteroidetes) (Table S6, Figures S7, S8). The other large cluster of HgcA sequences included 33 sequences and accounted for 50% of the total *hgcA* coverage. We could only recover a few genes from the NCBI NR database that clustered with these sequences, and none from reference isolate genomes (Figure S4). Phylogenetic analysis of the 15 bins with these *hgcA* genes identified them as members of the Planctomycetes-Verrucomicrobia-Chlamydia (PVC) superphylum, 11 of which are members of the recently proposed Kiritimatiellaeota phylum (Figures S7, S9).[85] The PVC superphylum dominates the overall read coverage of our bins as well, with 79 PVC bins accounting for 42% of total bin coverage, with 30% coming from Kiritimatiellaeota alone (Figure S9, Table

S6). There are very few publicly available Kiritimatiellaeota genomes and only three cultured representatives.[85,86] Notably, a recent paper also identified several hgcA+ bins associated with the Kiritimatiellaeota phylum in a sulfate-enriched lake, but neither the HgcA sequences nor the rp16 sequences from those bins clustered closely with those from the current study (Figure S4).[14] This suggests that the Kiritimatiellaeota phylum is far more diverse than our current reference databases indicate and that the *hgcA* gene could be widely distributed throughout it. For both the Kiritimatiellaeota and the Bacteroidales, the presence of *hgcA* within bins was not phylogenetically conserved (Figures S8, S9). We also identified several other novel putative methylators that were lower in number and abundance, including Margulisbacteria, Firestonebacteria, and Actinobacteria. The dominance of highly diverse and novel hgcA+ organisms in these samples highlights the value of using genome-resolved shotgun metagenomics (as compared to amplicon sequencing) to identify methylators in a new study system, as it allows for the identification of divergent *hgcA* lineages and taxonomic classification of the associated bins.

Metabolic potential of methylating bins.

Many of the hgcA+ lineages we identified can employ a wide variety of metabolic strategies, while others have few closely related reference genomes; thus, it was vital to examine their metabolic pathways to determine which TEAPs and other biogeochemical cycles could be potentially linked to Hg-methylation. While most of the literature has focused on both SRBs and methanogens as the dominant methylators, due to the sulfate/sulfide abundance in Lake Mendota and documentation of sulfate reduction in the water column,[43] we hypothesized that most hgcA+ genomes in Lake Mendota harbor genes enabling sulfate reduction. The ability to respire sulfate to sulfide is encoded by the *dsrABD*, *aprAB*, *sat*, and *qmoABC* genes; bins with this complete set of genes were termed SR+.[87,88] Three of the four Desulfobacterales hgcA+ bins and both of the Syntrophobacterales hgcA+ bins, including the most abundant hgcA+ bin (SYN_0007), were SR+ (Figures S10, S11). SR+ methylators are slightly more abundant in the euxinic samples (25-26% of hgcA+ bin coverage) than the chemocline samples (14-21%) (Figure 3). Overall, SR+ bins (hgcA+ and hgcA-) account for only 7% of the total bin coverage. Sulfate reduction is not the only respiratory pathway reliant on sulfur redox reactions, though. Three hgcA+ bins that are not SR+ contained polysulfide reductase (*psr*) homologues, which provide the ability to respire partially reduced sulfur compounds such as tetrathionate or thiosulfate (Figures S10, S12).[89] However, these three bins were relatively low in abundance and may also rely on other TEAPs for respiration (Figure S10). Methanogenic methylators were even less abundant, with only a single bin (MET_0028) accounting for 2% of the hgcA+ bin coverage, mostly in the deep euxinic sites where MeHg production is suspected to be lower (Figure 3). No hgcA- methanogens were identified. MET_0028 is a member of the hydrogenotrophic Methanomicrobiales order.[90] Overall, the fraction of methylators relying on metabolic pathways historically associated with Hg-methylation (sulfate reduction and methanogenesis) was far lower than we expected.

We also identified several hgcA+ bins corresponding to potential Mn-reducing organisms. While reduced Mn has been correlated to MeHg levels,[91] Mn reduction has not, to our knowledge, been linked directly to MeHg production *in situ* and has even been

proposed as a method for limiting MeHg production in sediments.[92] Organisms that respire insoluble metal oxides often use porin-cytochrome C complexes (PCCs) to mediate extracellular electron transfer (EET).[93] We recovered genomes for several Verrucomicrobia, Bacteroidetes, and Kiritimatiellaeota with PCC-like gene clusters, but they were not closely related to PCCs experimentally verified to conduct EET (Figures S10, S13). However, both hgcA+ Geobacterales bins had a PCC operon homologous to the *extEFG* operon from *Geobacter sulfurreducens*, which has been shown to mediate both Fe and Mn oxide reduction (Figures S10, S13).[94] These bins both had low read coverage, but were most abundant in CHE3, where we saw evidence for enhanced Mn cycling and peaks in fraction MeHg (Figure 3). Notably, there is little evidence for Fe redox cycling, suggesting that these organisms were unlikely to rely on Fe reduction (Figure S2). While Mn levels are low in the water column relative to sulfate (<5 μM), previous work has shown that low Fe levels can drive substantial carbon oxidation in regions with a steep redox gradient.[95] Combined with observations that Geobacterales methylators often produce MeHg at a high rates in culture, this suggests that Mn cycling at the oxic-anoxic interface may play a role in MeHg production in Lake Mendota.[22,23,96]

We also detected genes for nitrogen species reduction in hgcA+ bins (Figure S10). While nitrite-oxidizing *Nitrospinae* have been identified as potential methylators,[39] reduction of nitrogen species has not, to our knowledge, been linked to MeHg production. In fact, nitrate amendment has been shown to reduce MeHg levels in lakes.[97] While many bins, both hgcA+ and hgcA-, encoded genes required for dissimilatory nitrate/nitrite reduction to ammonia (DNRA), these proteins can detoxify nitrite or disperse reducing equivalents during fermentation in addition to respiration..[98,99] This, in combination with low nitrate/nitrite levels in the water column during this time of year and the presence of other respiratory pathways in these bins suggest that nitrogen-based respiration does not play a major role in overall community metabolism or MeHg production in this system. However, we cannot rule out the potential role of cryptic N cycling, especially near the chemocline.

The remaining 27 hgcA+ bins are likely to be derived from fermentative or syntrophic organisms, based on their lack of canonical genes for TEAPs. Bins linked to obligate fermentation were also common in the total microbial community, as they represent 106 of the 228 bins, accounting for almost 50% of the bin coverage. This is likely an underestimate of the organisms relying on fermentation, as it does not include the many bins containing genes for dissimilatory nitrate/nitrite reduction or oxidases that were likely maintaining fermentative metabolism at these anoxic depths. These bins possess an array of genes for pyruvate fermentation and aldehyde and alcohol dehydrogenases for fermentative production of short chain fatty acids (Figure S14). They also had genes that could facilitate syntrophy through hydrogen or formate evolution.[100] Hydrogenases used for H2 uptake and formate dehydrogenases were present in many respiratory bins (hgcA+ and hgcA-), further suggesting that this community may rely on syntrophic metabolism. Many of these fermentative/syntrophic bins correspond to organisms specialized in polysaccharide degradation, with 13 hgcA+ bins having at least 40 glycoside hydrolases (GHs). The highly abundant Kiritimatiellaeota appear particularly suited to polysaccharide degradation, with bins carrying up to 468 GHs. In fact, 100 total bins carried over 40 GH genes each, suggesting that primary polysaccharide degradation is a common metabolic strategy in the

anoxic water column in Lake Mendota. Of these, 49 represent obligate fermenters, while 50 are thought to represent facultative aerobes. Together, these data indicate that fermentative and syntrophic processes may play a much larger role in MeHg production than we had hypothesized.

Our data show that hgcA is widely distributed throughout members of the anaerobic microbial food web. Overall, typical metabolic pathways associated with Hg-methylation such as sulfate reduction, methanogenesis, and iron reduction are outnumbered by fermentative, polysaccharide-degrading hgcA+ organisms, which predominated at both meta- and hypolimnetic sites. The dissolved organic carbon pool in Lake Mendota is dominated by autochthonous inputs and primary production at this time of year is controlled by cyanobacteria, which have a high proportion of exopolysaccharides in their biomass.[42,101,102] Together, this suggests that the supply of large organic molecules, particularly polysaccharides from cyanobacterial blooms in the epilimnion, may contribute directly to MeHg production. A previous study in a eutrophic lake reported abundant polysaccharide-degrading, fermentative hgcA+ Kiritimatiellaeota, suggesting these organisms may link polysaccharide degradation to Hg-methylation in many eutrophic systems.[14] Similar *hgcA* sequences were also identified in the Baltic sea on marine snow in oxygen-depleted waters, although at lower relative abundances.[27] It is unclear whether these novel *hgcA* sequences will be amplified by existing primers so we cannot comment on their presence/absence in other systems where PCR-based amplicon sequencing methods are used. On the other hand, respiratory hgcA+ organisms are much less abundant in Lake Mendota and are dominated by SRB in the meta- and hypolimnion during late stratification, likely due to the elevated levels of sulfate in the lake. Sulfate reduction in general appears to be the dominant form of anaerobic respiration in the hypolimnion. At the onset of stratification, sulfate levels are approximately 160 μM, well above what they need to outcompete methanogens [Loveley and Klug, 1983]. Interestingly, Jones et al reported similar levels of hgcA+ SRB in the water column of two lakes heavily enriched in sulfate (~3mM and ~0.5mM), suggesting that in water columns both heavily and moderately impacted by sulfate loading, SRBs still account for a relatively small portion of the hgcA+ community.[14] Other TEAPs, such as Mn reduction, may be linked to Hg-methylation under certain conditions in Lake Mendota as well, since the hgcA+ Geobacterales appeared in the metalimnion during late stratification where we saw evidence for enhanced Mn cycling. However, we still do not know which of these hgcA+ organisms are active methylators or how rapidly they produce MeHg. Additional work using more functional measurements such as metatranscriptomics or metaproteomics will help identify which of these hgcA+ organisms are metabolically active and expressing *hgcA* under in situ conditions.

It is also important to consider that each of these *hgcA*-carrying organisms is a member of the anaerobic microbial food web and is thus influenced by the overall levels of community metabolism. For example, while there is little information on mass flux constraints on carbon degradation in freshwater anoxic water columns, in other anoxic environments such as marine sediments, hydrolysis and primary fermentation are the rate-limiting steps in community metabolism.[103–105] Additionally, syntrophic organisms require that respiratory partners consume their metabolic end-products, such as hydrogen.[100] Thus, the supply of terminal electron acceptors and/or carbon substrates and the corresponding activity of

flanking microbial community members controls the flux of carbon and energy through the anaerobic microbial food web, influencing the metabolism of individual hgcA+ organisms and presumably their methylation rates. This is supported by data that show that overall levels of heterotrophic activity correlate to MeHg production.[9,25] This highlights the need for further research on complex natural communities to probe not only which organisms have and express the *hgcAB* genes or what metabolic pathways they have, but also how biogeochemical conditions and the overall flux of carbon and energy through different levels of the microbial anaerobic food web can directly and indirectly influence MeHg production *in situ*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Synopsis:

Fermentative microorganisms dominate the mercury-methylating community in a sulfate-enriched lake.

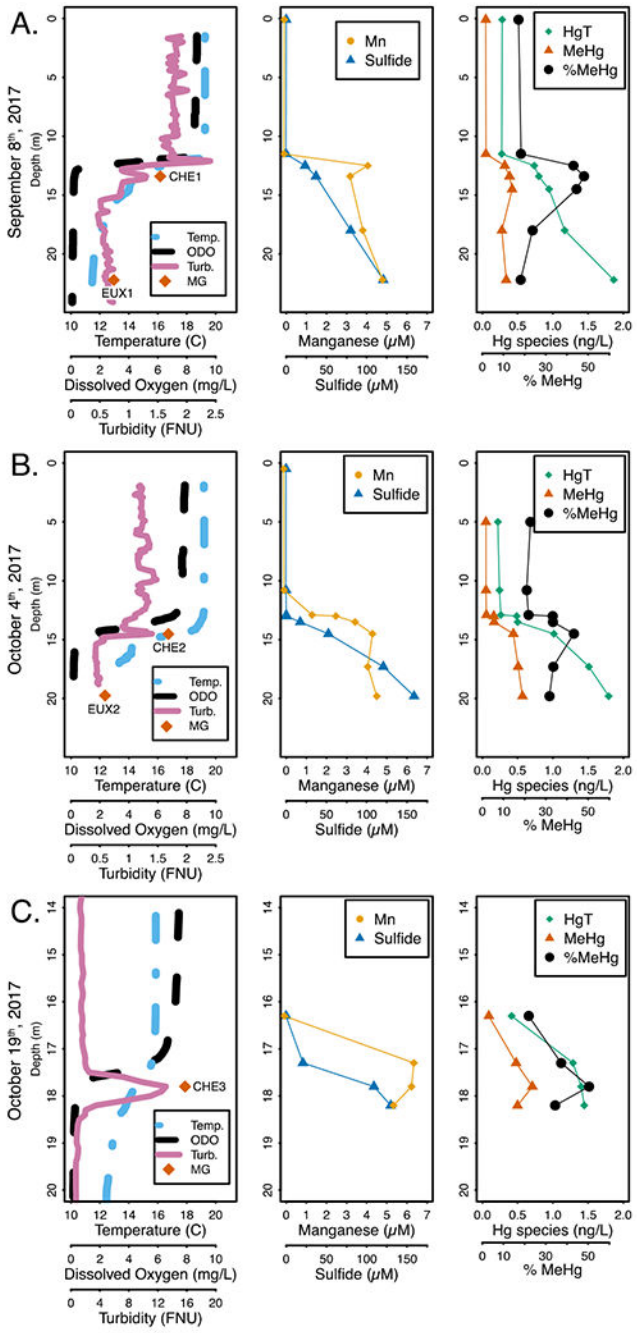

Figure 1:

Physical and geochemical profiles of Lake Mendota from 2017 on September 8th (A), October 4th (B) and October 19th (C). Column 1: Parameters measured continuously with a sonde and includes orange diamonds where samples for metagenomic sequencing were collected, names denoted next to symbol. Column 2: Total sulfide and filter-passing manganese values at discrete depths. Column 3: HgT and MeHg values, as sum total of the dissolved and particulate fractions. Dissolved and particulate fractions are plotted individually in Figure S1. Note the changed scale for depth on the y-axis and for turbidity

on the x-axis in the October 19th profiles (C). The metagenomic samples collected near the metalimnion for October 4th and October 19th were both collected coincident with the observed spike in turbidity. Abbreviations: Temp. - Temperature (°C), ODO - Optical dissolved oxygen in mg/L, Turb. - Turbidity in Formazin Nephelometric Units (FNU), MG - metagenome sample

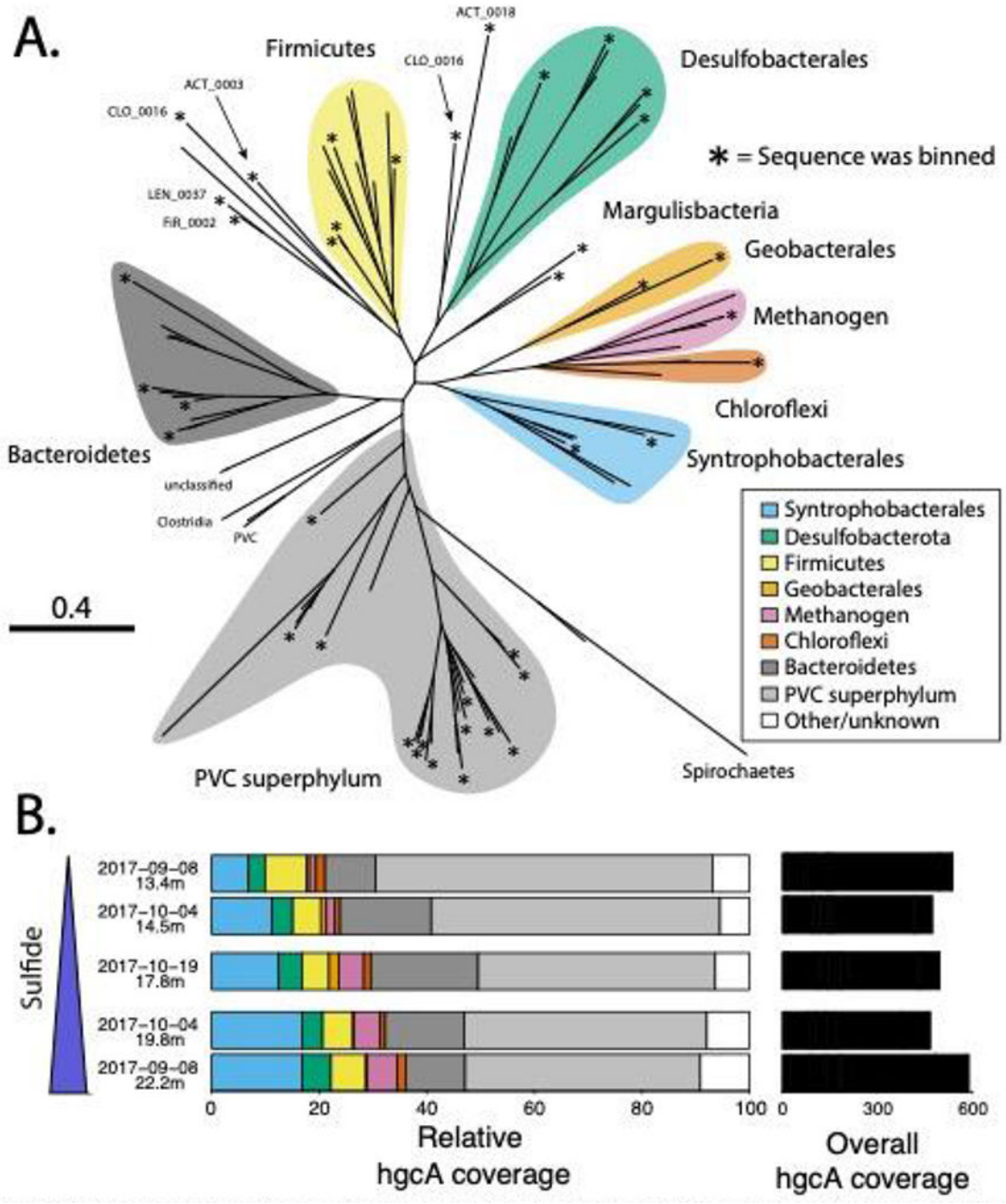

Figure 2.

A) Phylogenetic tree of HgcA sequences front this study and B) fractional coverage of *hgcA* genes within predirted taxonomic groups. Unconfirmed methylators dominate hgcA sequence diversity in Lake Mendota, both numerically (A) and by coverage (B). For panel A, asterisks at the end of branches indicate sequence was binned, white all other branches are uncinned HgcA sequences from this study. Sequences were assigned a predicted taxonomic group based on phylogenetic clustery with HgcA reference sequences from NCBI and bin phytogenies of binned HgcA sequences (for delated tree with reference sequences,

see Figure S3). Binned sequences outside of a monophyletic cluster are labeled with their bin name. In panel B, the back bars on the refer to the overall coverage of all *hgcA* sequences in each metagenome. Samples are arranged in order of increasing sulfide levels.

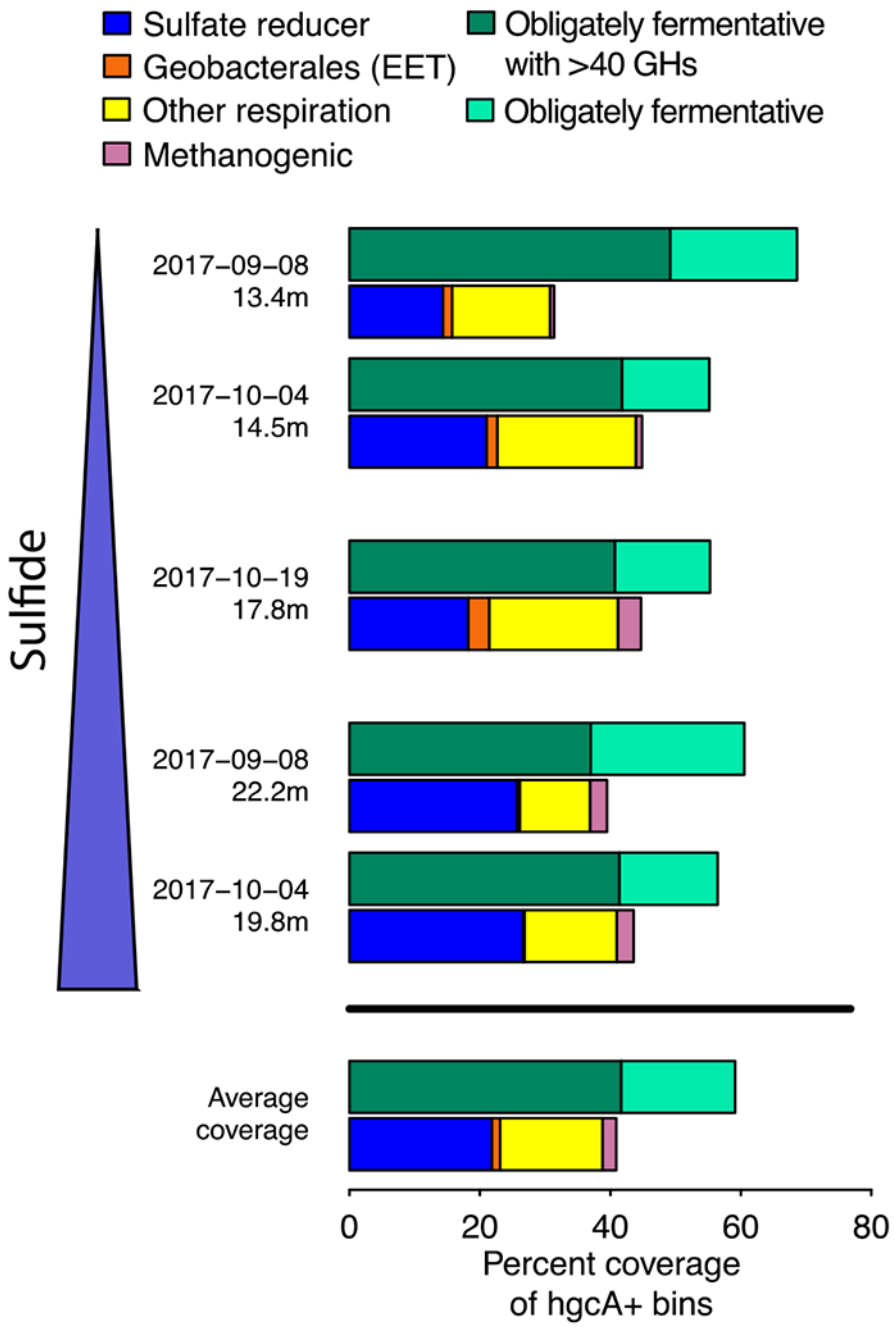

Figure 3.

Fractional coverage of metabolic functional groups within hgcA+ community. Fermentative organisms are the most abundant hgcA+ organisms in Lake Mendota. Coverage of each functional group has been normalized to the coverage of all hgcA+ bins in each metagenome. Plots of coverage in the different metagenomes are arranged by decreasing redox potential, which corresponds to increasing sulfide concentrations. Abbreviations: GHs - glucoside hydrolases, EET - external electron transfer.