RUNNING TITLE: Mercury, dissolved organic matter, and microbiomes

Trends in dissolved organic matter cycling, sediment microbiomes, and methylmercury production across vegetation heterogeneity in a Great Lakes wetland

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Abstract.

Recent advances have allowed for greater investigation into microbial regulation of mercury toxicity in the environment. In wetlands in particular, dissolved organic matter (DOM) may influence methylmercury (MeHg) production both through chemical interactions and through substrate effects on microbiomes. We conducted microcosm experiments in two disparate wetland environments (unvegetated and vegetated sediments) to examine the impacts of plant leachate and inorganic mercury loadings on microbiomes, DOM cycling, and MeHg production in the St. Louis River Estuary, which has a legacy of mercury contamination. Overall, our research reveals the greater relative capacity for mercury methylation in vegetated over unvegetated sediments in this environment. Further, oligotrophic unvegetated sediments receiving leachate produced more MeHg than unamended microcosms, pointing to the role of organic matter and vegetation patterns as an important control on MeHg production in these sediments.We also show that while leachate influenced the microbiome in both environment types, sediment with high organic carbon content was more resistant to change than oligotrophic sediment. Our work supports emerging research suggesting that *Clostridia* may be important methylators in oligotrophic environments. We demonstrate changes in community structure towards *Clostridia* and metagenomic shifts toward fermentation as well as degradation of complex DOM and MeHg production in unvegetated microcosms receiving leachate. Together, our work shows the importance of wetland vegetation in driving MeHg production in the Great Lakes region and provides evidence that this may be due to both enhanced microbial activity as well as differences in the composition of microbiomes associated with higher DOM levels.

Keywords. St. Louis River, Clostridia, oligotrophic, fermentation, mercury methylation, carbon

Introduction.

Mercury methylation in anoxic sediments is central to the bioaccumulation of mercury in plant and animal tissue (Benoit et al., 2003; Morel et al., 1998; Ullrich et al., 2001) and poses a significant environmental and human health concern in the freshwater wetlands of the Great Lakes region (Branfireun et al., 1999; Harmon et al., 2005; Jeremiason et al., 2006). Dissolved organic matter (DOM) has been a focus of geochemical investigations for decades, and both positive and negative interactions between DOM and mercury methylation – principally, a microbial transformation (Hsu-Kim et al., 2013) – have been demonstrated under contrasting environmental conditions (Graham et al., 2013; Hsu-Kim et al., 2013; Ravichandran, 2004). Yet, the role of sediment microbiomes that directly mediate mercury methylation have not been examined in the context of DOM quantity and quality.

Dissolved organic matter is comprised of various classes of organic compounds (primarily organic acids) with a wide range of molecular weights and aromaticities (Lambertsson and Nilsson, 2006; Wetzel, 1992). DOM concentrations are elevated in wetlands relative to other freshwater systems (>10 mg/L), and the humic fraction derived from plant leachate predominates. With respect to mercury cycling in wetlands, mercury methylation is impacted both by binding properties of the humic DOM fraction, resulting either in increased dissolution of inorganic mercury complexes or in physical inhibition of mercury bioavailability (Drexel et al., 2002; Haitzer et al., 2002; Waples et al., 2005), and by provisioning organic substrate for microbial activity (Hsu-Kim et al., 2013; King et al., 2000; Lambertsson and Nilsson, 2006). These effects may also vary with ambient geochemistry, as Graham *et al.* (2013) have demonstrated that sulfide concentrations and DOM aromaticity interact to influence MeHg production. Further, interactive effects of sediment microbiomes and DOM biogeochemistry are

less well-resolved than other aspects of linkages between environmental geochemistry and mercury toxicity. Since mercury methylation is so strongly impacted by DOM, environments such as the St. Louis River estuary, which contains areas of both vegetated and unvegetated sediments, may also show differences in the capacity for MeHg production across vegetation graidents. Numerous studies have shown regulation of freshwater microbial communities by DOM quantity or quality (Docherty et al., 2006; Forsström et al., 2013; Pernthaler, 2013), and such changes in environmental microbiomes may alter ecosystem biogeochemical cycling (Graham et al., 2016). The character of humic DOM (putatively most influential to mercury methylation) can be assessed at scales relevant to microbial activity with fluorescence spectroscopy, which correlates changes in humic fluorescence relative to other portions of the optically-active DOM pool (Fellman et al., 2010).

While the microbial mechanisms generating methylmercury are poorly understood, the recent discovery of the *hgcAB* gene cluster has allowed investigations into the microbial ecology of mercury cycling (Gilmour et al., 2013; Parks et al., 2013; Poulain and Barkay, 2013; Smith et al., 2015). In particular, interactions between environmental microbiomes, DOM quantity and quality, and mercury methylation in natural systems remain an uncertainty in predicting hotspots of mercury toxicity in the environment (Hsu-Kim et al., 2013; Podar et al., 2015). Nonetheless, recent work has increased knowledge on the microbiology of mercury methylation, expanding potential microorganisms mediating methylation beyond sulfate-reducing bacteria (Compeau and Bartha, 1985; Hsu-Kim et al., 2013), iron-reducing bacteria (Kerin et al., 2006) and methanogens (Hamelin et al., 2011). To date, all tested microorganisms containing the *hgcAB* gene cluster have been confirmed as methylators, and the gene appears to be highly conserved allowing it to serve as a genetic marker for methylating organisms (Gilmour et al., 2013; Hsu-Kim et al.,

2013). Gilmour *et al.*[12] have identified five clades of putative methylators, including new clades of syntrophic and *Clostridial* organisms. While research has provided insight into the abundance of these new organisms in mercury-contaminated landscapes (Bae et al., 2014; Hamelin et al., 2015; Liu et al., 2014a), many studies have continued to focus on the involvement of sulfate- (Liu et al., 2014b; Lu et al., 2016) and iron-reducing bacteria (Si et al., 2015) as well as methanogens (Yu et al., 2013) in mercury methylation. As such, the importance of organisms with alternative metabolisms in mercury methylation remain relatively unexplored. Resolving interactions between sediment microbiomes, environmental chemistry, and inorganic mercury complexes is thought to be central in understanding variation in methylation rates among natural systems (Gilmour et al., 2013; Hintelmann et al., 2000; Hsu-Kim et al., 2013).

Here, we examine the influence of DOM from plant leachate on net methylmercury (MeHg) production in a contaminated freshwater estuary at the base of Lake Superior. First, we describe MeHg production in environments associated with high (vegetated sediments) and low (unvegetated sediments) ambient DOM in building in understanding of conditions that underlie mercury methylation in the St. Louis River Estuary. We hypothesize that environmental biogeochemistry (in particular, DOM quantity and quality) influences mercury methylation both by regulating microbial activity and by shifting the abundance and metabolic diversity of mercury methylators. We test this hypothesis across chemically distinct sediments associated with unvegetated (oligotrophic) and vegetated (high-C) environments, using a microcosm experiment to monitor changes in sediment microbiomes, DOM chemical quality, and net MeHg production in response to additions of leachate from overlying plant material. In total, this work delineates a broad view of how vegetated vs. unvegetated sediments in the St. Louis River Estuary may have different capacities for the cycling of mercury. Our results show the

92 importance of DOM availability as a control the production of MeHg in Lake Superior's St.
93 Louis River Estuary, an integral environment to human society and industries of the region.
94 Further, our work provides evidence for the involvement of metabolisms that ferment recalcitrant
95 organic matter in mercury methylation, particularly within oligotrophic unvegetated
96 environments, an effect that may be imperative to understanding and mitigating human exposure
97 to MeHg with increasing DOM deposition into aquatic environments (Regnier et al., 2013).
98
99 **Methods.**
100 *Field site.*
101 The St. Louis River Estuary is home to the largest U.S. port on the Great Lakes and
102 covers roughly 12,000 acres of wetland habitat directly emptying into Lake Superior. Mining in
103 the headwaters, industrial discharge in the port, and atmospheric deposition have left a legacy of
104 mercury contamination in the sediment. We obtained sediment samples from vegetated
105 (*Zizania palustris (*wild rice), 46^{o} 40.855' N, 91^{o} 59.048'W) and unvegetated (46^{o} 41.918' N, 92^{o}
106 0.123' W) patches in Allouez Bay and wild rice plant matter from nearby Pokegama Bay
107 (46.683448^{o}N, 92.159261^{o}W) to minimize sampling impacts. Both habitats are clay influenced
108 embayments that drain an alluvial clay plain created by deposition during the retreat of the last
109 glaciation approximately 10,000 years BP.
110
111 *Experimental design.*
112 A total of 20 anoxic microcosms were constructed in September 2013 to investigate
113 relationships between sediment microbiomes, DOM chemical quality, and mercury methylation.
114 Sediment was obtained in 250-mL amber Nalgene bottles from the top 10 cm of sediment using a

115 block sampling design described in the Supplemental Material. Leachate was extracted using 1 g
116 dried, ground plant matter:20 mL of Nanopure water, filtered through Whatman 0.7 μm GFF
117 filters (Whatman Incorporated, Florham Park, NJ, USA). Microcosms were constructed in 500-
118 mL airtight glass mason jars and stored at room temperature in the dark in Mylar bags with
119 oxygen-absorbing packets between subsampling. Our experiment was designed to promote
120 microbial MeHg production by minimizing abiotic photo-methylation and -demethylation (Morel
121 et al., 1998) and sustaining a low redox environment to inhibit demethylation (Compeau and
122 Bartha, 1984). All experimental set up and sample processing was conducted in an anaerobic
123 glovebox containing 85% N_2, 5% CO_2, and 10% H_2 gas mix at the USGS in Boulder, CO. Jars
124 were degassed in the glovebox for 48hr prior to experimentation to remove oxygen.
125 A full-factorial design was employed with two environments (vegetated and unvegetated
126 sediment) and two treatments (plant leachate and Nanopure water). Sediments were
127 homogenized via mixing but unsieved to maintain environment characteristics. Large roots (>1
128 cm) were infrequent and removed to lessen heterogeneity among replicates. Each microcosm
129 received 100 g wet sediment, and 250 mL solution consisting either of leachate at 100 mg/L (~5x
130 natural concentrations to mimic a loading event) and $HgCl_2$ at 20 mg/L (50 μg/g wet sediment) in
131 Nanopure water (leachate replicates) or solely of $HgCl_2$ at 20 mg/L in nanopure water (no
132 leachate replicates). The purpose of $HgCl_2$ addition at high concentration was to negate initial
133 differences in mercury, overcome $HgCl_2$ inaccessibility due to abiotic organo-metal interactions,
134 and provide substrate for the duration of the experiment. $HgCl_2$ concentrations were comparable
135 to microcosm experiments of similar design (Harris-Hellal et al., 2009; Poulain et al., 2006;
136 Ruggiero et al., 2011; Zhou et al., 2012), and we estimate minimal dosage effects as
137 communities without leachate did not change through time in unvegetated microcosms and only

slightly changed through time in vegetated microcosms (R^2 = 0.19, see results and Fig S1). Microcosms were incubated for 28 days, and subsamples of sediment and water were taken every seven days for analysis of sediment microbiomes and DOM characteristics.

Sediment chemistry, extracellular enzyme activity, and mercury methylation.

Percent carbon and nitrogen, NO_3^-/NO_2^-, NH_4^+, total particulate organic carbon (TPOC), total dissolved nitrogen (TDN), pH, and extracellular enzyme activities of β-1,4-glucosidase, β -1,4-N-acetylglucosaminidase, and acid phosphatase were determined on pre-incubation sediments, as described in the Supplemental Material. For total- and methylmercury analysis, initial (day 0) and final (day 28) subsamples were frozen at -70^{o}C, freeze-dried, and sent on dry ice to the USGS Mercury Lab in Middleton, WI for analysis by aqueous phase ethylation, followed by gas chromatographic separation with cold vapor atomic fluorescence detection (Method 5A-8), acid digestion (Method 5A-7), and QA/QC. Mercury analyses were performed on 3 of 5 replicates for each environment and microcosm type. All other analyses were performed on 5 replicates, except for no unvegetated microcosms without leachate beyond day 0 (n = 4, one replicate destroyed during experiment).

Dissolved organic matter characteristics.

Water subsamples were collected at 7-day intervals (days 0, 7, 14, 21, and 28) to determine non-purgeable organic carbon (NPOC) concentration and specific UV absorbance at 254 nm ($SUVA_{254}$) as well characteristics of the optically active DOM pool (mostly associated with humic DOM fraction), as described in the Supplemental Material. We calculated the fluorescence index (FI) to determine the relative contribution of microbial vs. terrestrial matter to

the DOM pool, the humic index (HIX) to identify large aromatic compounds consistent with humic material, and the freshness index to determine the availability of labile carbon (Fellman et al., 2010; Gabor et al., 2014a) using MATLAB software (2013a, The MathWorks, Natick, MA) according to Gabor *et al.* (2014b).

Microbial DNA extraction, 16S rRNA amplicon, and metagenomic shotgun sequencing.

DNA from each sediment subsample was extracted using the MO Bio Power Soil DNA Extraction kit (MO BIO Laboratories, Carlsbad, CA, USA), as described in Knelman *et al.* (2012). The region encoding the V4 fragment of the 16S rRNA gene was amplified with the primers 515F/806R, using the PCR protocol described by the Earth Microbiome Project (Caporaso et al., 2012) (Supplemental Material). The final multiplexed DNA samples were sequenced at CU-Boulder (BioFrontiers Institute, Boulder, CO) on an Illumina MiSeq with the MiSeq Reagent Kit v2, 300 cycles (Illumina, Cat. # MS-102-2002) to generate 2 x 150-bp paired-end reads. Sequences are available at XXXXXX. In addition, 3 unvegetated leachate replicates at day 0 (before leachate addition) and day 28 were sent to the Joint Genome Institute (JGI) for shotgun metagenomic sequencing on the Illumina HiSeq platform. Sequences are available at XXXXX.

Sequence analysis.

Partial 16S rRNA gene were filtered for sequence length and minimum quality score in the UPARSE pipeline (Edgar, 2013) and OTUs were assigned using QIIME (Caporaso et al., 2010) (Supplemental Material). Metagenomic shotgun sequences were assembled and classified against the protein families database (Pfam) (Finn, 2012), Clusters of Orthologous Groups of

proteins (COG) (Tatusov et al., 2003), and Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa and Goto, 2000) by JGI via the IMG database pipeline (Markowitz et al., 2012).

In addition, 46 of 52 genomes identified by Parks *et al.* (2013) were represented by complete or partial 16S rRNA gene sequences in the NCBI GenBank database (Benson et al., 2013), spanning all clades of methylators. We used two different approaches to determine methylator relative abundance and community structure. To determine relative abundance, we combined available methylating sequences with generated sequences and re-preformed *de novo* OTU-picking. We then identified OTUs containing known methylator sequences as potential methylators. Because of high *Deltaproteobacteria* abundance, many closely-related methylator sequences may have clustered with non-methylating *Deltaproteobacteria* in this approach. Thus, to examine methylator community structure at finer resolution, we created a database of known methylator sequences and performed closed-reference OTU-picking in QIIME against this database. In addition, a BLAST database was constructed from all *hgcA* and *hgcB* gene sequences available in GenBank. A BLASTX search was conducted against this database to identify taxonomic affiliation of methylators in our samples; however, our query resulted in no matches, likely due to inadequate sequencing depth.

Statistical analysis.

All analyses, unless otherwise noted, were conducted using the *R* software platform. Shapiro-Wilk tests were used to verify normality and assess the appropriateness of parametric vs. non-parametric tests. Multivariate sediment properties (*e.g.*, sediment geochemistry, extracellular enzyme activity, and DNA quantity) were compared across environments at day 0 with Hotelling's T-square Test and post hoc Student's *t*-tests. MeHg production was calculated by

subtracting day 0 from day 28 MeHg concentrations; values below detection limit were assigned the detection limit as a value for a conservative estimate of change. MeHg production was compared across groups using ANOVA. Changes in DOM indices (FI, freshness, HIX) through time (days 0, 7, 14, 21, and 28) in each sample group were assessed with linear and quadratic regressions. DOM samples with $SUVA_{254}$ >7 were removed due to fluorescence interference from inorganic molecules. Comparisons of DOM indices between data subsets were conducted with ANOVA and post hoc Tukey HSD.

Microbial community dissimilarity matrices based on 16S rRNA sequences were constructed using the weighted UniFrac method (Lozupone et al., 2011) in QIIME. We preformed analysis using the full community and within the methylating community. To examine the relative abundance of our methylating OTUs, we removed OTUs with less than eight total occurrences (bottom quartile) in our 91 subsamples to limit artifacts from sequencing errors among rare organisms (methylating OTUs were <1% of sequences). Alpha diversity for each sample was assessed using the PD whole tree metric in QIIME. The relative abundance of methylators was compared within each environment at days 0 and 28 (leachate vs. no leachate) using unpaired one-way Student's *t*-tests.

Changes in community structure through time (days 0, 7, 14, 21, 28) were assessed with ANOSIM in QIIME. Differences in alpha diversity at day 0 were assessed using unpaired one-way Student's *t*-tests. Relative abundances of major clades were assessed between vegetated and unvegetated environments at day 0 and changes in clades through time (days 0, 7, 14, 21, 28) were assessed using non-parametric Kruskal-Wallis tests with FDR-correct *P* values. SIMPER analysis was conducted using the 'vegan package' to identify OTUs associated with community dissimilarity between days 0 and 28 in microcosms receiving leachate. Correlations between

methylating clades that exhibited significant changes (Kruskal-Wallis, days 0, 7, 14, 21, 28), HIX, and MeHg production were assessed at day 28 using the Pearson product-momentum correlation coefficient, grouping leachate and no leachate microcosms within each environment in a single analysis to provide sufficient variation.

Increases in the frequency of COGs, Pfams, and KEGG pathways at day 28 relative to day 0 were evaluated using binomial tests. Targets more abundant at day 28 (FDR-corrected $P < 0.01$) were examined for correlations with HIX and MeHg production at day 28 with the Pearson product-momentum correlation coefficient.

Results.

Ambient geochemistry and microbiology.

Physicochemical and biological properties of vegetated and unvegetated environments significantly differed (Hotelling $P = 0.004$, Table 1). The unvegetated environment was extremely oligotrophic, with low concentrations of sediment C and N, and both vegetated and unvegetated environments appeared to be N-limited (C:N 16.43 and 20.06). DNA concentration, enzyme activities, and mercury concentrations were an order of magnitude higher within the vegetated environment (Table 1). In addition, methylmercury production in sediments without leachate addition was significantly higher in vegetated sediment than unvegetated sediment, by nearly two orders of magnitude (Figure 1).

Microbial community structure and alpha diversity were significantly different between the two environments (ANOSIM, $P = 0.001$, R= 1.00, *t*-test, P = 0.01), though major phyla were similar (Table 1) and both environments were bacteria (vs. archaea) dominated. The relative abundance of methylators was higher in the vegetated environment (*t*-test, $P = 0.005$), and the

253 structure of potential methylators within microbial communities also differed between
254 environments (ANOSIM, P = 0.001, R = 0.99).
255
256 *Microbiome response to $HgCl_2$ and leachate addition.*
257 Over the course of the incubation, microcosms with vegetated, high-C sediment produced
258 over ten times more MeHg than unvegetated sediment microcosms, regardless of leachate
259 amendment (ANOVA P = 0.002, Figure 1). Mercury methylation was enhanced by leachate
260 within the unvegetated nutrient-poor environment with roughly two to four times more
261 production in microcosms receiving leachate as compared to those without leachate. However,
262 leachate did not stimulate MeHg production in the vegetated environment.
263 Community structure changed through time in vegetated and unvegetated environments
264 with leachate (ANOSIM across days 0, 7, 14, 21, 28, veg.: P = 0.001 R = 0.40, unveg.: P =
265 0.001 R = 0.43, Figure S1A and B), but not without leachate (veg.: P = 0.02, R = 0.19, unveg.: P
266 > 0.05, Figure S1A and B), indicating no substantial effect from high concentrations of added
267 mercury. At day 28, communities in unvegetated microcosms with leachate were different than
268 those without leachate (ANOSIM, P = 0.01, R = 0.54), while structure in vegetated sediment
269 microcosms only weakly differed between leachate and no leachate groups (P = 0.04, R = 0.22).
270 When examining only potential methylators, the relative abundance of methylators was
271 significantly greater in lechate microcosms versus no leachate for each environment at day 28
272 (Figure 2A, *t*-test, veg.: P = 0.04, unveg.: P = 0.04). However, for both environments, there
273 were no significant changes in community structure within methylating clades through time
274 (ANOSIM across days 0, 7, 14, 21, 28, P > 0.05). This result was not unexpected given our small
275 sample sizes (methylator OTUs contained less than 1% of sequences).

Changes in community structure in response to leachate was partially generated by an increase in *Clostridia* in both environments (Kruskal-Wallis, veg.: FDR-corrected $P = 0.003$, unveg.: $P = 0.018$, Figure 2B, Table S3) and a decrease in *Deltaproteobacteria* in unvegetated sediment (Kruskal-Wallis, veg.: FDR-corrected $P = 0.36$, unveg.: FDR-corrected $P = 0.015$, Figure 2B). In particular, *Clostridia* abundances increased by 3-fold (1.1% to 3.8% of the microbiome) and 10-fold (1.5% to 10.5% of the microbiome), respectively in vegetated and unvegetated environments, driven by increases in nearly all families of *Clostridia*. These shifts were mirrored within our subset of data containing only suspected methylators (Figure 2C), which showed distinct (non-significant) trends for increases in *Clostridia* and decreases in *Deltaproteobacteria* in response to leachate in both environments.

Changes in the methylating community were more evident at finer taxonomic levels. One family of *Clostridia* (*Peptococcaceae*), sharply increased with leachate in unvegetated sediment and displayed a similar trend in vegetated sediment (Kruskal-Wallis, veg.: FDR-corrected $P = 0.18$, unveg.: FDR-corrected $P = 0.04$, Figure 2D). These changes were due to increases in two closely related methylating OTUs (Kruskal-Wallis, *Dehalobacter restrictus* veg.: FDR-corrected $P = 0.24$ (uncorrected $P = 0.04$), and *Syntrophobotulus glycolicus*, unveg.: FDR-corrected $P = 0.006$) grouped in a single genus by our classification system (*Dehalobacter_Syntrophobotulus*, Kruskal-Wallis, veg.: FDR-corrected $P = 0.09$, unveg.: FDR-corrected $P = 0.0027$, Figure S2). Increases in *Clostridia* (*t*-test, FDR-corrected $P = 0.006$), *Peptococcaceae* (*t*-test, FDR-corrected $P = 0.018$), *Dehalobacter restrictus* (*t*-test, FDR-corrected $P = 0.024$), and *Syntrophobotulus glycolicus* (*t*-test, FDR-corrected $P = 0.042$) as well as a possible trend for decreases in *Deltaproteobacteria* (*t*-test, FDR-corrected $P = 0.18$) were also reflected in metagenomic data (Figure 3D).

SIMPER analysis of 16S rRNA genes associated with methylator taxonomy in unvegetated leachate microcosms indicated that two OTUs, in *D. restrictus (*increase) and in *Geobacter* (decrease), significantly contributed to community differences between day 0 and day 28 ($P < 0.05$, Table S1). This was reflective of broader changes in the full community, in which 22.9% of 175 SIMPER-identified OTUs belonged to *Clostridia* (increased from avg. 0.78 OTUs/sample to avg. 17.20 OTUs/sample, Table S3) while 8% belonged to *Deltaproteobacteria* (decreased from avg. 8.5 OTUs/sample to 7.4 OTUs/sample, Table S2).

In total, 7,150 KEGG pathways, 84 COGs, and 79 Pfams were significantly more abundant at day 28 relative to day 0 in unvegetated leachate microcosms (Figure 3A-C). All classfication systems revealed metabolic shifts towards glycosyltranseferases, among other pathways involved in DOM oxidation and in iron and nitrate reduction.

Changes in DOM chemistry.

Details of DOM quantity and quality changes are presented in the Supplemental Material (Figure S3) and regression statistics are presented in Table 2.

DOM fluorescence indices displayed notable changes through time. In the vegetated environment, FI remained stable at a low value in leachate microcosms, indicating plant-derived DOM, and rose in microcosms without leachate indicating greater relative contribution of microbial vs. abiotic processing (Figure 4A and B). In contrast, in the vegetated environment, HIX increased in both leachate and no leachate microcosms indicating processing of more labile vs. recalcitrant DOM (Figure 4C and D). This increase in HIX corresponded with decrease in freshness (Figure 4E and F), further supporting our interpretation. In the unvegetated environment, leachate microcosms (but not microcosms without leachate) increased in FI (Figure

4A and B) denoting progressively microbial DOM sources. There was no change in HIX (Figure 4C and D) suggesting equal processing of labile vs. recalcitrant DOM. Freshness varied non-linearly in leachate microcosms but not those without leachate (Figure 4E and F).

Across environment types, HIX was significantly higher in vegetated microcosms (ANOVA $P < 0.0001$, Tukey HSD, leachate: $P < 0.0001$, no leachate: $P = 0.004$). FI and freshness were higher in unvegetated leachate microcosms than in vegetated DOM-amended microcosms (Tukey HSD, FI: $P = 0.003$, freshness: $P = 0.03$) but did not differ across microcosms without leachate (Tukey HSD, FI: $P = 0.89$, freshness: $P = 0.40$).

Correlation of microbiome, DOM characteristics, and MeHg production.

Given the apparent shift in community structure towards *Clostridia,* and (chemoorganotrophic) *Peptococcaceae* in particular, we examined correlations of this family with the proportion of complex organic matter (HIX) and MeHg production within each environment. We focused on HIX because this index changed consistently and reflected portions of recalcitrant carbon substrate pools utilized by the organisms we identified. Because we only calculated net MeHg production at the conclusion of the incubation, we analyzed these correlations at day 28 and grouped leachate and no leachate replicates within each environment to provide sufficient variation and sample size. *Peptococcaceae* was negatively correlated with HIX and positively correlated with MeHg production in unvegetated microcosms (Pearson's r ($n = 6$), HIX: $P = 0.001$, $r = -0.96$, MeHg: $P = 0.03$, $r = 0.81$). *Peptococcaceae* abundance in vegetated microcosms did not correlate with HIX ($P = 0.20$) or MeHg production ($P = 0.45$).

Finally, despite low statistical power ($n = 3$), we observed marginally significant trends ($P < 0.10$) between key metabolic pathways and HIX (Table 3). In particular, COGs classified

as: Glycosyltransferase, Glycosyltransferases involved in cell wall biogenesis, Glycosyltransferases - probably involved in cell wall biogenesis, and Beta-galactosidase/beta-glucuronidase; and Pfams classified as: Glycosyl transferase family 2, Radical SAM superfamily, and SusD family displayed significant correlations with HIX at the $P < 0.10$ level. Only Pfam PF00593, TonB dependent receptor, correlated with MeHg production ($P < 0.001$, $r = -1.00$, Table 2).

Discussion.

Mercury methylation across environments.

Geochemical and microbial characteristics varied across environments, resulting in differential patterns of net MeHg production. First, our work indicated a strongly different capacity of vegetated vs. unvegetated wetland sediments to cycle mercury. Without leachate addition, MeHg production in vegetated sediments was two orders of magnitude higher than in unvegetated sediments (Figure 1). As such, vegetated sediments may be considered potentially important locations for mercury methylation when mercury is present in the environment. Such a dynamic may be due to either higher overall activity of microorganisms or the unique microbiomes contained within these sediments. Within the high-C vegetated environment, leachate did not influence the sediment microbiome or net MeHg production to the same extent as within the more oligotrophic unvegetated environment (Figure 1, Figure S1). Given high ratios of C:N, high OC content, and low NO_3^- concentrations in our vegetated sediment (Table 1), N-limitation may have mitigated net MeHg production in vegetated environments relative to the unvegetated environment (Taylor and Townsend, 2010), which had substantially lower concentrations of all measured C and nutrient concentrations. Both ambient MeHg levels and net MeHg production were dramatically higher in the vegetated environment, supporting other

findings that plant-microbe interactions facilitate MeHg production (Roy et al., 2009; Windham-Myers et al., 2014; Windham‐Myers et al., 2009). Indeed, the vegetated environment displayed higher ambient DNA concentration, enzyme activities, and methylator abundance, underlying a higher *in situ* rate of biological activity.

By contrast, the unvegetated environment experienced a dramatic increase in MeHg (Figure 1) in response to leachate that correlated with changes in the sediment microbiome (Figure 2 and 3, Figure S1). Carbon limitation has been widely demonstrated as a constraint on microbial activity (Bradley et al., 1992; Brooks et al., 2005; Wett and Rauch, 2003); thus, leachate may bolster MeHg production in C-limited ecosystems via impacts on microbial activity. In our system, net MeHg production in the unvegetated environment was possibly also constrained by low *in situ* rates of microbial activity and by low N concentration, and net MeHg production in response to leachate stimulus never increased to vegetated levels. Importantly, leachate enhanced the relative abundance of putative methylators within the microbiome in both environments, indicating that mercury methylation rates may be dually influenced by the sediment microbiome and by organic matter (Aiken et al., 2011; Hsu-Kim et al., 2013).

Microbiome response to leachate addition.

Our results bolster support for recent work demonstrating preferential organic degradation by *Clostridial* fermentation over oxidation by *Deltaproteobacteria* (Reimers et al., 2013) and provide support for the involvement of this clade in MeHg production. Within both environments, leachate altered the sediment microbiome, with structural shifts denoting an increase in *Clostridia* and decrease in *Deltaproteobacteria*. Unvegetated microcosms displayed greater changes in these clades, supporting a greater role for environmental filtering by DOM

within oligotrophic environments (Barberán et al., 2012; Stegen et al., 2012). *Clostridia* are obligate anaerobes with the ability to produce labile carbon compounds via fermentation of recalcitrant organic matter (Reimers et al., 2013; Ueno et al., 2016). Recent work has shown organic carbon degradation via *Clostridial* fermentation to operate at comparable rates to more energetically favorable carbon processing pathways[62]. Moreover, organic acids (*e.g.*, lactate and acetate) produced through these pathways can be subsequently utilized as a carbon source by sulfate- and iron- reducing *Deltaproteobacteria* (Guerrero-Barajas et al., 2011; Reimers et al., 2013; Zhao et al., 2008). Importantly, microbiome changes were mirrored when examining putative methylators independently. Specifically, *Deltaproteobacteria* and *Clostridia*, respectively, were the most abundant methylating organisms at the end of the incubation in all experimental groups except no leachate vegetated microcosms.

In unvegetated sediments, although no methylating pathways were identified, metagenomic analyses indicated an increase in carbon, and secondarily, iron metabolisms, supporting a role for microbial carbon and iron cycling in mercury methylation (Gilmour et al., 2013; Hamelin et al., 2011; Kerin et al., 2006; Podar et al., 2015). Carbon metabolisms were the primary KEGG category increasing in abundance within metagenomes (Figure 3A), and several COG pathways and Pfams indicated a possible metabolic shift favoring glycosyltransferases that convert starches, sugars, and nitroaromatics into a wide range of compounds (Bowles et al., 2005; Ramli et al., 2015) (Figure 3B and C). Further, metagenomic increases in Beta-galactosidase/beta-glucoronidase (lactose to galactose/glucose) (Martini et al., 1987), sugar phosphate isomerase/epimerases (sugar metabolism) (Yeom et al., 2013), and lactoylglutathione lyase (detoxification for methyglyoxal fermentation byproduct) (Inoue and Kimura, 1995) and the SusD family (glycan binding) (Martens et al., 2009) provide additional evidence increases in

fermentation processes in response to leachate. Increases in TonB dependent receptors (Moeck and Coulton, 1998), amidohydrolase (Seibert and Raushel, 2005), and NRAMP (Cellier et al., 1995) suggest a secondary importance of iron processing and/or transport of large organic compounds across cellular membranes. Finally, our results provide a possible genetic mechanism connecting iron, sulfur, carbon, and mercury cycling, as the radical SAM superfamily, which facilitates methyl transfers via the use of a [4Fe-S]+ cluster (Booker and Grove, 2010), increased in concert with net MeHg production. In total, the metabolic potential of the sediment microbiome indicates changes in carbon and iron metabolisms within microcosms experiencing higher net MeHg production in response to leachate, supporting past work that suggests a linkage between mercury methylation and these factors (Gilmour et al., 2013; Hamelin et al., 2011; Hsu-Kim et al., 2013; Kerin et al., 2006; Podar et al., 2015).

Lastly, at high taxonomic resolution in both environments, leachate increased the proportion of methylating organisms classified as *Peptococcaceae* within *Clostridia*, despite drastic differences in sediment chemistry (Figure 2D). Specifically, the two OTUs displaying the greatest change are thought to generate energy via organohalide respiration (*D. restrictus*) and fermentative oxidation of organic matter (*S. glycolicus*, also capable of syntrophy) (Han et al., 2011; Stackebrandt, 2014). The relative abundance of *Peptococcaceae* was positively correlated with MeHg production in the unvegetated environment, and other methylating organisms did not increase in abundance, as would be expected if the activity of these organisms was enhanced by leachate.

Associations between microbiology, DOM processing, and net MeHg production.

437 The processing of proportionally more labile (microbe-preferred) organic matter would
438 be expected to result in decreases in DOM freshness and increases in HIX. However, our results
439 suggest substantial contributions of recalcitrant organic matter processing within the unvegetated
440 environment (but not the vegetated environment which followed expectations). In unvegetated
441 microcosms (both leachate and no leachate), HIX did not rise through time indicating relcatritant
442 matter processing (Figure 4C and D). Further, leachate unvegetated microcosms, which
443 experienced pronounced changes in the sediment microbiome and high MeHg production, HIX
444 was significantly lower than in all other experimental groups (ANOVA, $P < 0.0001$, all Tukey
445 HSD $P < 0.0001$). While most microorganisms preferentially degrade labile C sources, the
446 degradation of recalcitrant organic matter can contribute substantially to aquatic carbon cycling
447 (Mcleod et al., 2011). Leachate unvegetated microcosms also exhibited large increases in
448 microbially-derived DOM (FI) through time, demonstrating a noticeable contribution of
449 microbial activity to the DOM pool (Figure 4A).
450 The abundance of methylating *Peptococcaceae* in unvegetated microcosms negatively
451 correlated with HIX, denoting an apparent contribution of these members or co-occuring
452 community members to DOM processing, but the mechanisms behind these shifts remain
453 unclear. Metabolism of recalcitrant organic matter by fermenting organisms may influence
454 mercury methylation via direct and indirect mechanisms. Members of *Clostridia* can generate
455 MeHg themselves, and *Clostridial* degradation of recalcitrant organic matter can also produce
456 bioavailable carbon substrates for sulfate- and iron- reducing organisms that produce MeHg
457 (Reimers et al., 2013).
458 Changes in metagenomes in responses to leachate elucidate metabolic pathways that may
459 be involved in recalcitrant organic matter processing and MeHg production. For example, both

460 COG and Pfam glycosyltransferases were negatively correlated with HIX, suggesting a role for
461 starch, sugar, and nitroaromatic fermentation in response to DOM loading. As well, a negative
462 correlation between HIX, and the radical SAM superfamily provides a possible mechanistic
463 linkage between methyl transfers and recalcitrant organic matter processing. Conversely, Beta-
464 galactosidase/beta-glucuronidase, and the SusD family were positively correlated with HIX,
465 indicating a co-association with labile C processing rather than recalcitrant organic matter. Only
466 one abundant Pfam – a TonB dependent receptor, signaling enzyme that may be involved in iron
467 cycling (Moeck and Coulton, 1998) – and no COGs was correlated with MeHg production.
468 Although our results do not provide a direct linkage between metabolic pathways and mercury
469 methylation, it is notable that no pathways associations involve sulfate-reducing or methanogenic
470 methylators.
471
472 *Conclusions.*
473 Our work shows clearly distinct mercury cycling dynamics between the vegetated and
474 unvegetated sediments of the St. Louis River Estuary environment. While substantially greater
475 MeHg production is observed in vegetated sediments, unvegetated sediments stand to respond
476 more strongly to DOM additions in driving increases in MeHg production. While we observed
477 evidence for changes in the microbiome of both high-C and nutrient-poor sediment, the more
478 oligotrophic environment showed greater responses in the sediment microbiome and in mercury
479 methylation to the addition of DOM, an important insight given increasing risks of
480 anthropogenic eutrophication. Microbiome shifts towards fermentation pathways, increases in
481 chemoorganotrophic *Clostridia*, degradation of recalcitrant organic matter, and increases in
482 MeHg within oligotrophic environments begin to elucidate the microbial ecology of mercury

483 methylation. Importantly, our results provide evidence for organisms not historically considered
484 in MeHg production and suggest future work into the environmental relevance of these
485 organisms in mercury methylation. *Clostridia* thrive in a variety of anoxic environments from
486 wastewater effluent (Wang et al., 2003) to the human gut (Mahowald et al., 2009), and our work
487 supports the potential for mercury methylation across a broad range of ecological niches
488 (Gilmour et al., 2013; Podar et al., 2015). Taken together, our research provides new insights
489 into microorganisms impacting MeHg production in natural settings in the Great Lakes region
490 and emphasizes the importance of exploring microbial physiology not typically associated with
491 methylating organisms in enhancing mercury toxicity.
492
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503
504 **Conflict of Interest.**
505 The authors declare no conflict of interest.

506

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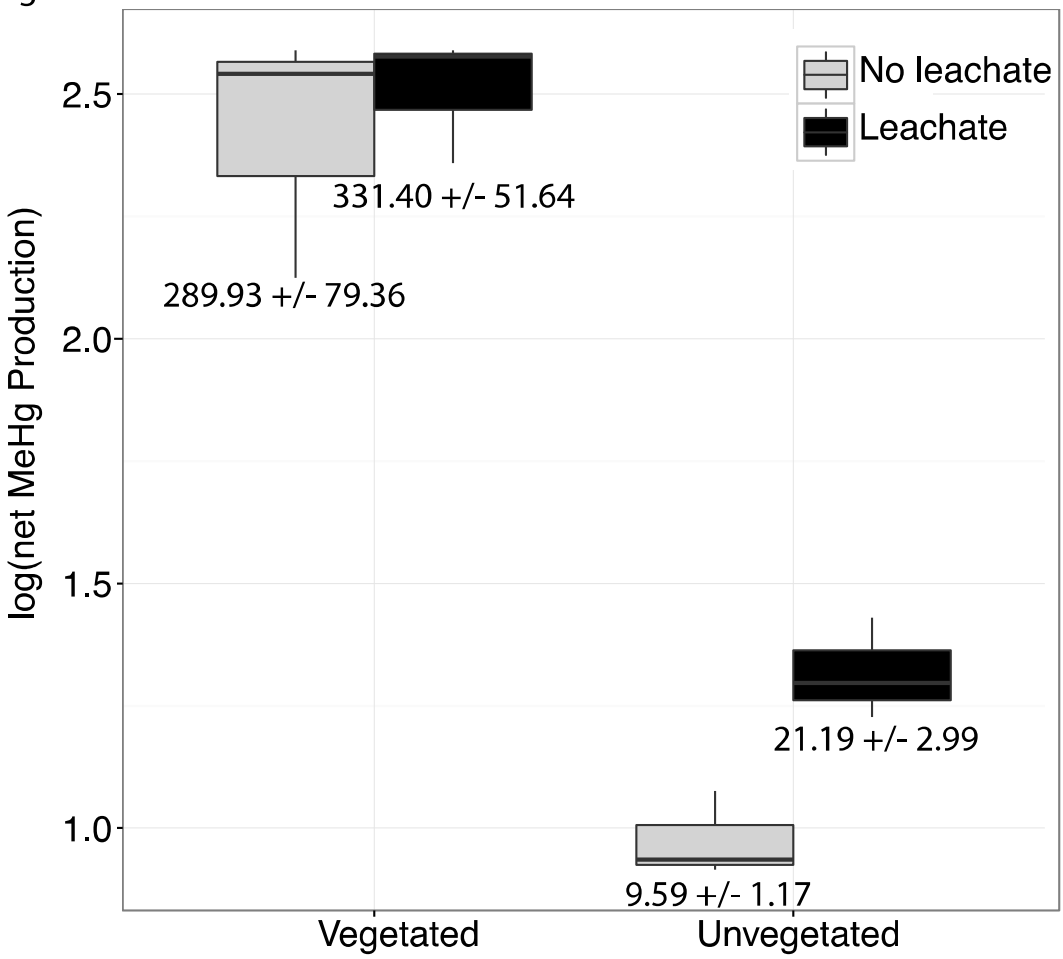
737 **Figures.**

Figure 1

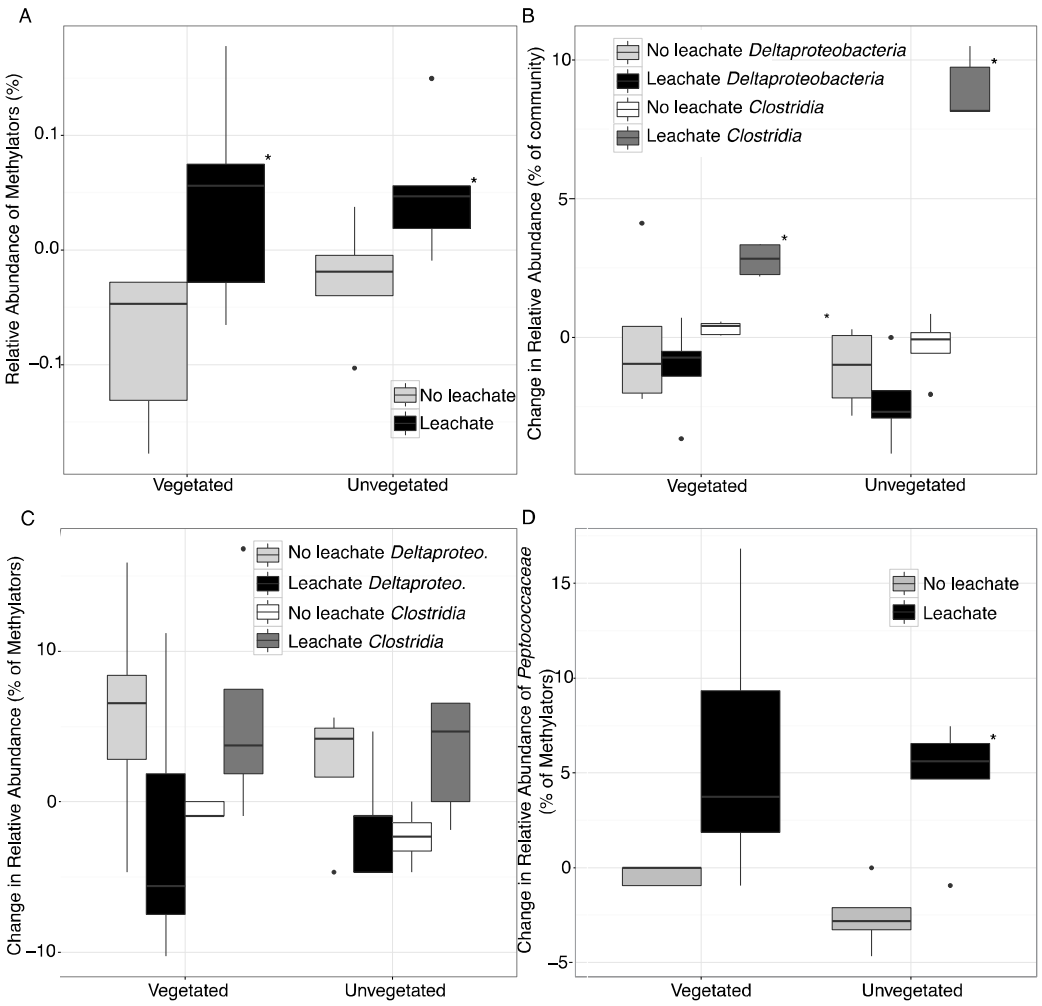


738

739 **Figure 1.** Boxplots are shown for net MeHg production (calculated as concentration at 28 days
740 less the initial concentration), with upper and lower hinges representing the values at the 75th and
741 25th percentiles and whiskers representing 1.5 times value at the 75th and 25th percentiles,
742 respectively. Leachate increased net MeHg production in unvegetated sediment but did not have
743 a large impact within vegetated sediments. Regardless of leachate addition, vegetated sediment
744 experienced an order of magnitude higher rates of net mercury methylation. Mean increase in
745 MeHg production in ng per g dry +/- standard errors are listed below each box.

746

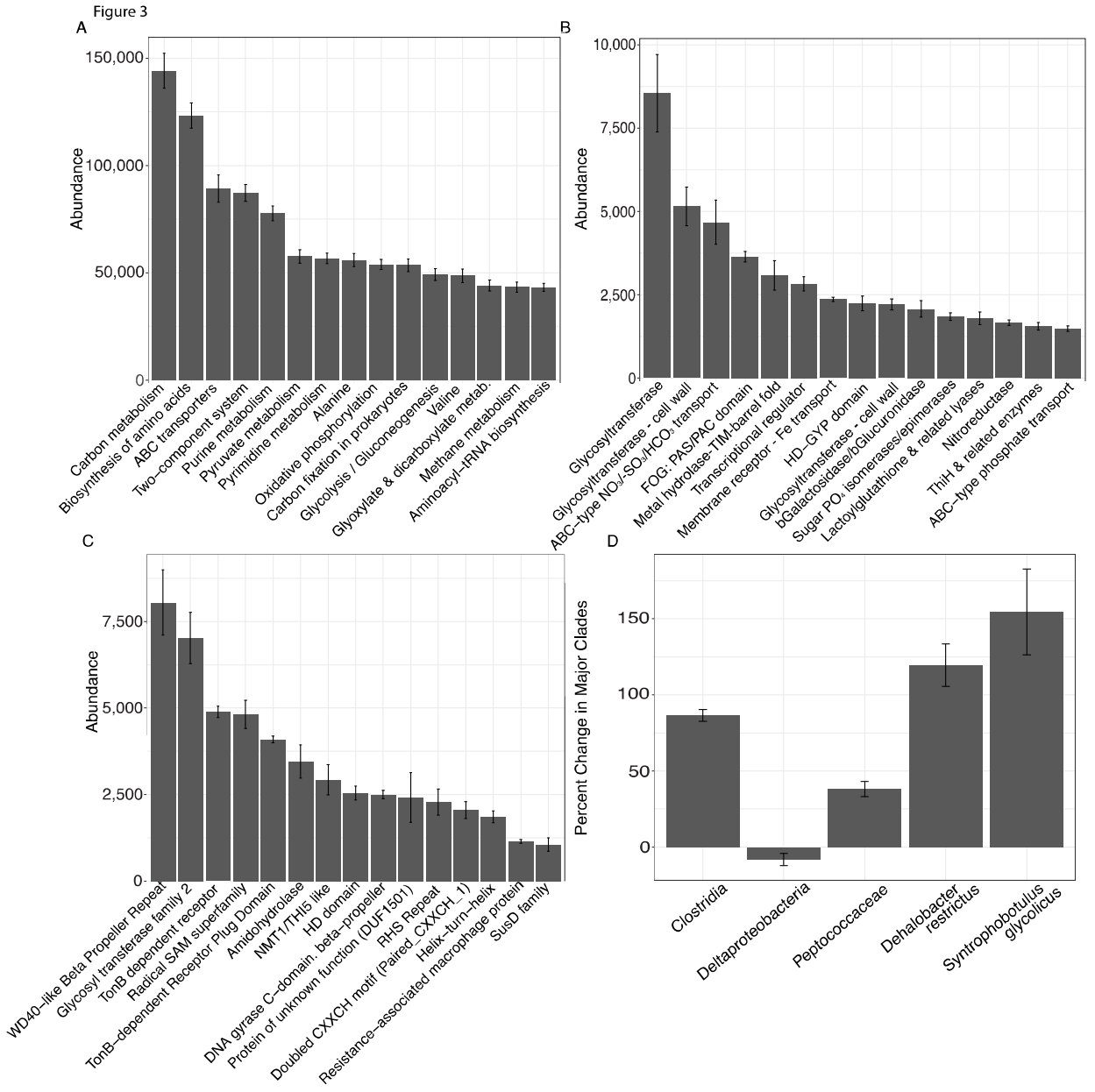
Figure 2



747

748 **Figure 2.** Boxplots are shown for selected changes in abundance of methylator abundance (A)
749 and taxonomy (B-D) in response to leachate addition, with upper and lower hinges representing
750 the values at the 75th and 25th percentiles and whiskers representing 1.5 times value at the 75th
751 and 25th percentiles, respectively. Outliers are plotted as points. Shading for each bar denote
752 taxonomy and leachate vs. no leachate. Significant relationships ($P < 0.05$) are denoted with an
753 asterisk. (A) The relative abundance of methylating organisms increased in both sediment types
754 in response to leachate addition. (B) The addition of leachate decreased the proportion of

Deltaproteobacteria and increased the proportion of *Clostridia* in both vegetated and unvegetated sediment, with greater effects in unvegetated sediment. (C) Within potential methylators, *Deltaproteobacteria* decreased and *Clostridia* increased in response to leachate, (D) driven by changes within the family *Peptococcaceae*. Abundance data are present in Table S3.



761

762 **Figure 3.** Results from analysis of metagenomic shotgun sequences from unvegetated

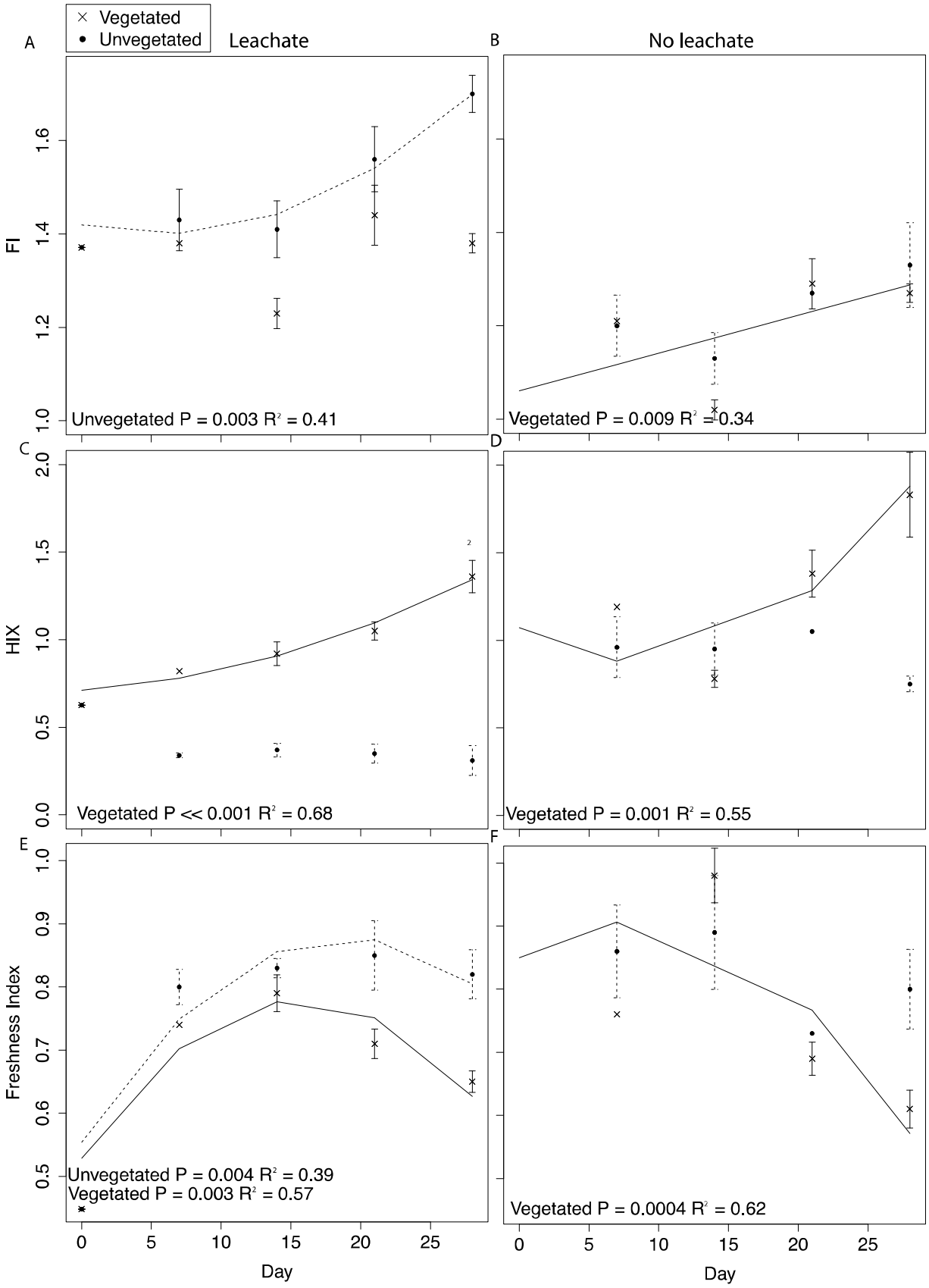
763 microcosms are denoted in Figure 3. Panels A, B, and C show the abundance of the top 15

764 KEGG, COG, and Pfam targets that increased at day 28 vs. day 0, respectively. Panel D shows

765 percent change in selected taxonomic groups at day 28 vs. day 0. Error bars denote standard

766 error.

767



769 **Figure 4.** DOM fluorescence indices were assessed through time with linear and quadratic
770 regressions in each environment and microcosm type. Averages for each environment and
771 microcosm type are plotted at days 0, 7, 14, 21, and 28, with error bars representing the standard
772 error. Plots in the first column are leachate microcosms, while plots in the second column are no
773 leachate microcosms. Unvegetated microcosms are depicted as closed circles with dashed lines
774 showing significant regressions; vegetated microcosms are x's with solid lines showing
775 significant regressions. (A) and (B) denote FI, (C) and (D) denote HIX, and (E) and (F) denote
776 freshness.

777
778

779 **Tables.**

780

781 **Table 1.** Mean chemical and biological characteristics of vegetated (*n* = 5) and unvegetated (n =

782 5) environments are presented Table 1. Asterisks represent significant differences from post hoc

783 *t*-tests, and standard deviations are presented in parentheses.

	Vegetated Environment	Unvegetated Environment
pH*	5.6 (0.09)	5.8 (0.40)
NH4 (mg/L)***	1.49 (0.32)	0.36 (0.14)
TPOC (mg/L)***	1.13 (0.06)	0.09 (0.05)
TDN (mg/L)**	0.06 (0.02)	0.04 (0.01)
percent C***	13.16 (2.20)	1.82 (3.39)
percent N***	0.8 (0.06)	0.1 (0.23)
C:N*	16.43 (1.59)	20.06 (5.36)
DNA concentratin (ng/L)***	28.13 (5.06)	9.31 (3.16)
NAG (nmol/h/g)***	308.94 (81.30)	9.05 (9.29)
BG (nmol/h/g)***	371.22 (81.25)	17.71 (19.29)
PHOS (nmol/h/g)***	393.45 (55.06)	20.69 (17.33)
SMHG (ng/g)**	2.67 (2.18)	0.24 (0.12)
STHG (ng/g)	306.56 (551.07)	3.16 (3.99)
SM/THG	0.02 (0.009)	0.32 (0.45)
Proteobacteria***	0.3 (0.04)	0.43 (0.02)
Chloroflexi***	0.17 (0.01)	0.06 (0.009)
Bacteroidetes	0.11 (0.02)	0.13 (0.03)
Acidobacteria*	0.07 (0.009)	0.08 (0.02)
Nitrospirae***	0.05 (0.009)	0.02 (0.009)
Actinobacteria***	0.03 (0.007)	0.07 (0.01)
Alpha Diversity**	183.8 (6.64)	193.7 (11.33)

*$P \leq 0.10$ **$P \leq 0.05$ ***$P \leq 0.01$

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788 **Table 2.** R^2 values from regression analysis of changes in DOM properties through time are listed in Table 2. No leachate microcosms
789 were analyzed from across days 7, 14, 21, and 28; and leachate microcosms were analyzed across days 0, 7, 14, 21, and 28 (n = 4-5 at
790 each sampling point, no samples were taken in no leachate microcosms at day zero), with characteristics of the applied leachate
791 represented at day 0.

	NPOC (mg/L)	Total Fluoresence	Fluor:NPOC	FI	HIX	Freshness
Vegetated, No leachate (across days 7, 14, 21, 28)	0.39**	0.21*	n.s.	0.22**	0.51***	0.52***
Vegetated, Leachate (across days 0, 7, 14, 21, 28)	0.32***	n.s.	n.s.	n.s.	0.68****	0.57***
Unvegetated, No leachate across days 7, 14, 21, 28)	0.64****	n.s.	0.29**	n.s.	n.s.	n.s.
Unvegetated, Leachate (across days 0, 7, 14, 21, 28)	n.s.	n.s.	n.s.	0.41***	n.s.	0.39***

792 *$P \leq 0.10$ **$P \leq 0.05$ ***$P \leq 0.01$ ****$P \leq 0.001$

793

794 **Table 3.** The Pearson product-momentum correlation coefficient was used to assess relationships of selected COG and Pfam targets
795 with HIX and net MeHg production at day 28 (n = 3). Relationships are presented in Table 3.

	HIX	MeHg
COG		
Glycosyltransferase	0.98*	0.79
Glycosyltransferases involved in cell wall biogenesis	0.96*	0.76
ABC-type nitrate/sulfonate/bicarbonate transport systems, periplasmic components	-0.85	0.55
FOG: PAS/PAC domain	-0.88	0.60
Predicted metal-dependent hydrolase of the TIM-barrel fold	0.88	0.60
Transcriptional regulator	0.999**	0.88
Outer membrane receptor proteins, mostly Fe transport	-0.79	0.45
HD-GYP domain	0.99**	0.84
Glycosyltransferases, probably involved in cell wall biogenesis	0.96*	0.74
Beta-galactosidase/beta-glucuronidase	0.98*	-0.80
Sugar phosphate isomerases/epimerases	-0.73	0.36
Lactoylglutathione lyase and related lyases	-0.93	0.67
Nitroreductase	0.996**	0.86
Thiamine biosynthesis enzyme, ThiH and related uncharacterized enzymes	0.98*	0.80
ABC-type phosphate transport system, periplasmic component	-0.66	0.27
Pfam		
WD40-like Beta Propeller Repeat	0.99**	0.85
Glycosyl transferase family 2	0.97*	0.76
TonB dependent receptor	0.90	0.9999***
Radical SAM superfamily	0.95*	0.75
TonB-dependent Receptor Plug Domain	0.51	-0.83
Amidohydrolase	-0.87	0.57
NMT1/THI5 like	-0.87	0.58
HD domain	0.9997**	0.91
DNA gyrase C-terminal domain, beta-propeller	-0.94	0.70
Protein of unknown function (DUF1501)	-0.46	0.04
RHS Repeat	-0.80	0.46
Doubled CXXCH motif (Paired_CXXCH_1)	0.97*	0.78
Helix-turn-helix	-0.90	0.63
Natural resistance-associated macrophage protein	-0.83	0.51
SusD family	0.99*	-0.82

* $P < 0.10$ ** $P < 0.05$