Dissolved organic matter and inorganic mercury loadings increase methylmercury production, *Clostridia* abundance, and recalcitrant organic matter processing in oligotrophic sediments

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Abstract.

Advances in genetics have allowed for greater investigation into the complex microbial communities mediating mercury methylation in the environment. In wetlands in particular, dissolved organic matter (DOM) may influence methylmercury production both through direct chemical interactions with mercury and through substrate effects on the environmental microbiome. We conducted microcosm experiments in two chemically disparate wetland environments (unvegetated and vegetated sediments) to examine the impact of DOM from leachate of local vegetation and inorganic mercury loadings on microbial community membership, metagenomic potential, DOM processing, and methylmercury (MeHg) production. We show that while DOM loadings impacted the microbiome in both environment types, sediment with high organic carbon content was more resistant than oligotrophic sediment to changes in microbiomes. Oligotrophic sediments receiving DOM produced significantly more MeHg than unamended microcosms, coincident with an increase in putative chemoorganotrophic methylators within the class *Clostridia*. Further, metagenomic shifts toward fermentation, and secondarily iron metabolisms, in these microcosms as well as degradation of complex DOM indicated by fluorescence indices also support a possible association between rarely acknowledged microbial metabolisms and MeHg production. Our research provides a basis for future investigation into the role of fermenting organisms in mercury toxicity and generates a new hypothesis that DOM can stimulate mercury methylation in oligotrophic environments either 1) via direct methylation by fermenting bacteria or 2) via enhancing the bioavailability of simple carbon compounds for sulfate- and iron-reducing bacteria through breakdown of complex DOM.

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 freshwater wetlands (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 (reviewed in Hsu-Kim *et al.* 2013) – have been demonstrated under contrasting environmental conditions (Graham *et al.* 2013, Hsu-Kim *et al.* 2013, Ravichandran 2004). 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 (reviewed in Hsu-Kim *et al.* 2013, Podar *et al.* 2015).

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. 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 thought to be 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).

Recent work has increased knowledge on the microbiology of mercury methylation, expanding potential microorganisms mediating methylation beyond sulfate-reducing bacteria (Compeau and Bartha 1985, reviewed in 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 (Gilmour *et al.* 2013, Hsu-Kim *et al.* 2013). Gilmour *et al.* (2013) 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 plant leachate and inorganic mercury loadings on net methylmercury (MeHg) production in a contaminated freshwater estuary at the base of Lake Superior. 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 oligotrophic versus high carbon (C) sediments associated with unvegetated and vegetated 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 and inorganic mercury. Our results provide evidence for the involvement of metabolisms that ferment recalcitrant organic matter in mercury methylation, particularly within oligotrophic environments, an effect that may be imperative to understanding and mitigating human exposure to MeHg with increasing rates of anthropogenic eutrophication.

Methods.

Field site.

The St. Louis River Estuary is home to the largest U.S. port on the Great Lakes and covers roughly 12,000 acres of wetland habitat directly emptying into Lake Superior. Mining in the headwaters, industrial discharge in the port, and atmospheric deposition have left a legacy of mercury contamination in the sediment. We obtained sediment samples from wild rice (*Zizania palustris*, 46° 40.855’ N, 91° 59.048’W) and unvegetated (46° 41.918’ N, 92° 0.123’ W) patches in Allouez Bay, which has high mercury concentrations relative to other areas of the estuary (data not shown). We also obtained wild rice plant matter from nearby Pokegama Bay (46.683448^{o}N, 92.159261^{o}W). Both habitats are clay influenced embayments that drain an alluvial clay plain created by deposition during the retreat of the last glaciation approximately 10,000 years BP.

Experimental design.

A total of 20 anoxic microcosms were constructed in September 2013 to investigate relationships between sediment microbiomes, DOM chemical quality, and mercury methylation. Sediment was obtained in 250-mL amber Nalgene bottles from the top 10 cm of sediment using a block sampling design described in the Supplemental Material. Leachate was extracted using 1 g dried, ground plant matter:20 mL of Nanopure water, filtered through Whatman 0.7 µm GFF filters (Whatman Incorporated, Florham Park, NJ, USA). Microcosms were constructed in 500-mL airtight glass mason jars and stored at room temperature in the dark in Mylar bags with oxygen-absorbing packets between subsampling. Our experiment was designed to promote microbial MeHg production by minimizing abiotic photo-methylation and -demethylation (Morel *et al.* 1998) and sustaining a low redox environment to inhibit demethylation (Compeau and Bartha 1984). A full-factorial design was employed with two environments (vegetated and unvegetated sediment) and two treatments (plant leachate and Nanopure water). Each microcosm received 100 g wet sediment, and 250 mL solution consisting either of DOM at 100 mg/L and $HgCl_2$ at 20 mg/L (50 µg/g wet sediment) in Nanopure water (DOM-amended replicates) or solely of $HgCl_2$ at 20 mg/L in nanopure water (controls). Addition of $HgCl_2$ at high concentration negated initial differences in mercury and provided substrate for the duration of the experiment. $HgCl_2$ concentrations were comparable to microcosm experiments of similar design (Harris-

Hellal *et al.* 2009, Poulain *et al.* 2006, Ruggiero *et al.* 2011, Zhou *et al.* 2012). 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. All sample processing was conducted in an anaerobic glovebox containing 85% N_2, 5% CO_2, and 10% H_2 gas mix at the USGS in Boulder, CO.

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 (T0) and final (T4) subsamples were frozen at -70°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) and acid digestion (Method 5A-7). 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 unvegetated controls beyond T0 (*n* = 4, one replicate destroyed during experiment).

Dissolved organic matter characteristics.

Water subsamples were collected at 7-day intervals (T0, T1, T2, T3, T4) 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 (reviewed in 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 (Supplemental Material, Caporaso *et al.* 2012). 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 DOM-amended replicates at T0 (before leachate additon) and T4 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 (Supplemental Material, Caporaso *et al.* 2010). 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, 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 T0 with Hotelling's T-square Test and post hoc Student's *t*-tests. MeHg production was calculated by subtracting T0 from T4 MeHg concentrations; values below detection limit were assigned the detection limit as a value for a conservative estimate of change. MeHg production in DOM-amended vs. control microcosms across environment types were compared using ANOVA followed by Tukey HSD post hoc tests. Because of extreme differences in mercury concentrations in vegetated vs. unvegetated sediment (Table 1), paired unequal variance Student's *t*-tests were used to test for within environment (control vs. DOM) differences in net MeHg production. Changes in DOM indices (FI, freshness, HIX) through time (T0-T4) 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 T0 and T4 (DOM vs. controls) using paired unequal variance Student's *t*-tests.

Changes in community membership through time (T0-T4) were assessed with ANOSIM in QIIME. Differences in alpha diversity at T0 were assessed using paired unequal variance Student's *t*-tests. Relative abundances of major clades were assessed between vegetated and unvegetated environments at T0 and changes in clades through time (T0-T4) 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 T0 and T4 in microcosms receiving DOM. Correlations between methylating clades that exhibited significant changes (Kruskal-Wallis, T0-T4), HIX, and MeHg production were assessed at T4 using the Pearson product-momentum correlation coefficient, grouping DOM-amended and control microcosms within each environment in a single analysis to provide sufficient variation.

Increases in the frequency of COGs, Pfams, and KEGG pathways at T4 relative to T0 were evaluated using binomial tests. Targets more abundant at T4 (FDR-corrected $P < 0.01$) were examined for correlations with HIX and MeHg production at T4 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).

Microbial community structure was significantly different between the two environments (ANOSIM, $P = 0.001$, R= 1.00), though alpha diversity did not differ and major phyla were similar (Table 1). The relative abundance of methylators was higher in the vegetated environment (*t*-test, $P = 0.02$), and the structure of potential methylators within microbial communities also differed between environments (ANOSIM, $P = 0.001$, R = 0.99).

Microbiome response to $HgCl_2$ and DOM addition.

Over the course of the incubation, microcosms with vegetated, high-C sediment produced over ten times more MeHg than unvegetated sediment microcosms, regardless of DOM amendment (ANOVA P = 0.002, Tukey HSD, control P = 0.01, DOM P = 0.007, Figure 1). In addition, mercury methylation was significantly enhanced by DOM addition within the unvegetated nutrient-poor environment with roughly two to four times more production in microcosms receiving DOM as compared to controls (*t*-test, P = 0.04, Figure 1). However, DOM addition did not stimulate significantly more MeHg production than control replicates in the vegetated environment (*t*-test, $P > 0.05$, Figure 1).

Community membership changed through time in vegetated and unvegetated environments amended with DOM (ANOSIM T0-T4, veg.: P = 0.001 R = 0.40, unveg.: P = 0.001 R = 0.43, Figure S1A and B), but not in controls (veg.: P = 0.02, R = 0.19, unveg.: $P > 0.05$, Figure S1C and D), indicating no substantial effect from high concentrations of added mercury. At T4, communities in unvegetated microcosms amended with DOM were different than controls (ANOSIM, P = 0.01, R = 0.54), while structure in vegetated sediment microcosms only weakly differed between control and DOM-amended groups (P = 0.04, R = 0.22). When examining only potential methylators, the relative abundance of methylators was significantly greater in the DOM-amended microcosms versus controls for each environment at T4 (Figure 2A, *t*-test, veg.: P = 0.03, unveg.: P = 0.05). However, for both environments, there were no significant changes in community membership within methylating clades through time (ANOSIM, T0-T4, $P > 0.05$). This result was not unexpected given our small sample sizes (methylator OTUs contained less than 1% of sequences).

Variation in community membership in response to DOM addition 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 DOM addition in both environments.

Changes in the methylating community were more evident at finer taxonomic levels. One family of *Clostridia* (*Peptococcaceae*), sharply increased with DOM addition 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 DOM-amended microcosms indicated that two OTUs, in *D. restrictus* (increase) and in *Geobacter* (decrease), significantly contributed to community differences between T0 and T4 (*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 T4 relative to T0 in unvegetated DOM-amended 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 and regression statistics are presented in Table 2. Control microcosms for both environments displayed increasing NPOC concentrations (Figure S3A), while total fluorescence (Figure S3C) and the ratio of fluorescence to NPOC concentration (Figure S3E) displayed non-significant trends or decreased. Within DOM-amended microcosms, vegetated microcosms displayed a decrease in NPOC concentration (Figure S3B), while unvegetated microcosms had stable NPOC content (Figure S3B). Total fluorescence remained stable in both environments (Figure S3D). The ratio of fluorescence-to-NPOC was variable through time and did not statistically change in either environment (Figure S3F).

DOM fluorescence indices displayed notable changes through time. In the vegetated environment, FI remained stable at a low value in DOM-amended microcosms, indicating plant-derived DOM, and rose in controls indicating greater relative contribution of microbial vs. abiotic processing (Figure 4A and B). In contrast, in the vegetated environment, HIX increased in both control and DOM-amended 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, DOM-amended microcosms (but not controls) 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 DOM-amended but not control microcosms (Figure 4E and F).

Across environment types, HIX was significantly higher in vegetated microcosms (ANOVA $P < 0.0001$, Tukey HSD, DOM-amended: $P < 0.0001$, control: $P = 0.004$). FI and freshness were higher in unvegetated DOM-amended microcosms than in vegetated DOM-amended microcosms (Tukey HSD, FI: $P = 0.003$, freshness: $P = 0.03$) but did not differ across control microcosms (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 MeHg production at the conclusion of the incubation, we analyzed these correlations at T4 and grouped DOM-amended and control 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. Within the vegetated high-C environment, DOM and $HgCl_2$ addition did not influence the sediment microbiome or net MeHg production to the same extent as within the 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 an order of magnitude higher in the vegetated environment, supporting other findings that plant-microbe interactions inherently facilitates 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 oligotrophic unvegetated environment experienced a dramatic increase in MeHg (Figure 1) in response to DOM and $HgCl_2$ addition 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, DOM addition may bolster MeHg production in C-limited ecosystem from resultant increases in 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 DOM stimulus never increased to vegetated levels. Importantly, DOM 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 $HgCl_2$ and DOM addition.

Our results bolster support for recent work demonstrating preferential organic degradation by *Clostridial* fermentation over organic matter processing by *Deltaproteobacteria* (Reimers *et al.* 2013) and provide support for the involvement of this clade in MeHg production. Within both environments, DOM amendment 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 (Reimers *et al.* 2013). 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 vegetated controls.

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 DOM addition. 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 DOM addition, 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, DOM addition 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 DOM addition.

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

The processing of proportionally more labile organic matter would be expected to result in decreases in DOM freshness and increases in HIX. However, our results suggest substantial contributions of recalcitrant organic matter processing within the unvegetated environment (but not the vegetated environment which followed expectations). In unvegetated microcosms (controls and DOM-amended), HIX did not rise through time indicating relcatritant matter processing (Figure 4C and D). Further, DOM-amended unvegetated microcosms, which experienced pronounced changes in the sediment microbiome and high MeHg production, HIX was significantly lower than in all other experimental groups (ANOVA, $P < 0.0001$, all Tukey HSD $P < 0.0001$). While most microorganisms preferentially degrade labile C sources, the degradation of recalcitrant organic matter can contribute substantially to aquatic carbon cycling (Mcleod *et al.* 2011). DOM-amended unvegetated microcosms also exhibited large increases in microbially-derived DOM (FI) through time, demonstrating a noticeable contribution of microbial activity to the DOM pool (Figure 4A).

The abundance of methylating *Peptococcaceae* in unvegetated microcosms negatively correlated with HIX, denoting an apparent contribution of these members or co-occuring community members to DOM processing, but the mechanisms behind these shifts remain unclear. Metabolism of recalcitrant organic matter by fermenting organisms may influence mercury methylation via direct and indirect mechanisms. Members of *Clostridia* can generate MeHg themselves, and *Clostridial* degradation of recalcitrant organic matter can also produce bioavailable carbon substrates for sulfate- and iron- reducing organisms that produce MeHg (Reimers *et al.* 2013).

Changes in metagenomes in responses to DOM and inorganic mercury addition elucidate metabolic pathways that may be involved in recalcitrant organic matter processing and MeHg production. For example, both COG and Pfam glycosyltransferases were negatively correlated with HIX, suggesting a role for starch, sugar, and nitroaromatic fermentation in response to

DOM loading. As well, a negative correlation between HIX, and the radical SAM superfamily provides a possible mechanistic linkage between methyl transfers and recalcitrant organic matter processing. Conversely, Beta-galactosidase/beta-glucuronidase, and the SusD family were positively correlated with HIX, indicating a co-association with labile C processing rather than recalcitrant organic matter. Only one abundant Pfam – a TonB dependent receptor, signaling enzyme that may be involved in iron cycling (Moeck and Coulton 1998) – and no COGs was correlated with MeHg production. Although our results do not provide a direct linkage between metabolic pathways and mercury methylation, it is notable that no pathways associations involve sulfate-reducing or methanogenic methylators.

Conclusions.

Microbiome shifts towards fermentation pathways, increases in chemoorganotrophic *Clostridia*, degradation of recalcitrant organic matter, and increases in MeHg within oligotrophic environments begin to elucidate the microbial ecology of mercury methylation. While we observed evidence for changes in the microbiome of both high-C and nutrient-poor sediment, the more oligotrophic environment showed greater responses in the sediment microbiome and in mercury methylation to the addition of DOM, an important insight given increasing risks of anthropogenic eutrophication. Importantly, our results provide evidence for organisms not historically considered in MeHg production and suggest future work into the environmental relevance of these organisms in mercury methylation. *Clostridia* thrive in a variety of anoxic environments from wastewater effluent (Wang *et al.* 2003) to the human gut (Mahowald *et al.* 2009), and our work supports the potential for mercury methylation across a broad range of ecological niches (Gilmour *et al.* 2013, Podar *et al.* 2015). Taken together, our research provides new insights into microorganisms impacting MeHg production in natural settings and emphasizes the importance of exploring microbial physiology not typically associated with methylating organisms in enhancing mercury toxicity.

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Figures.

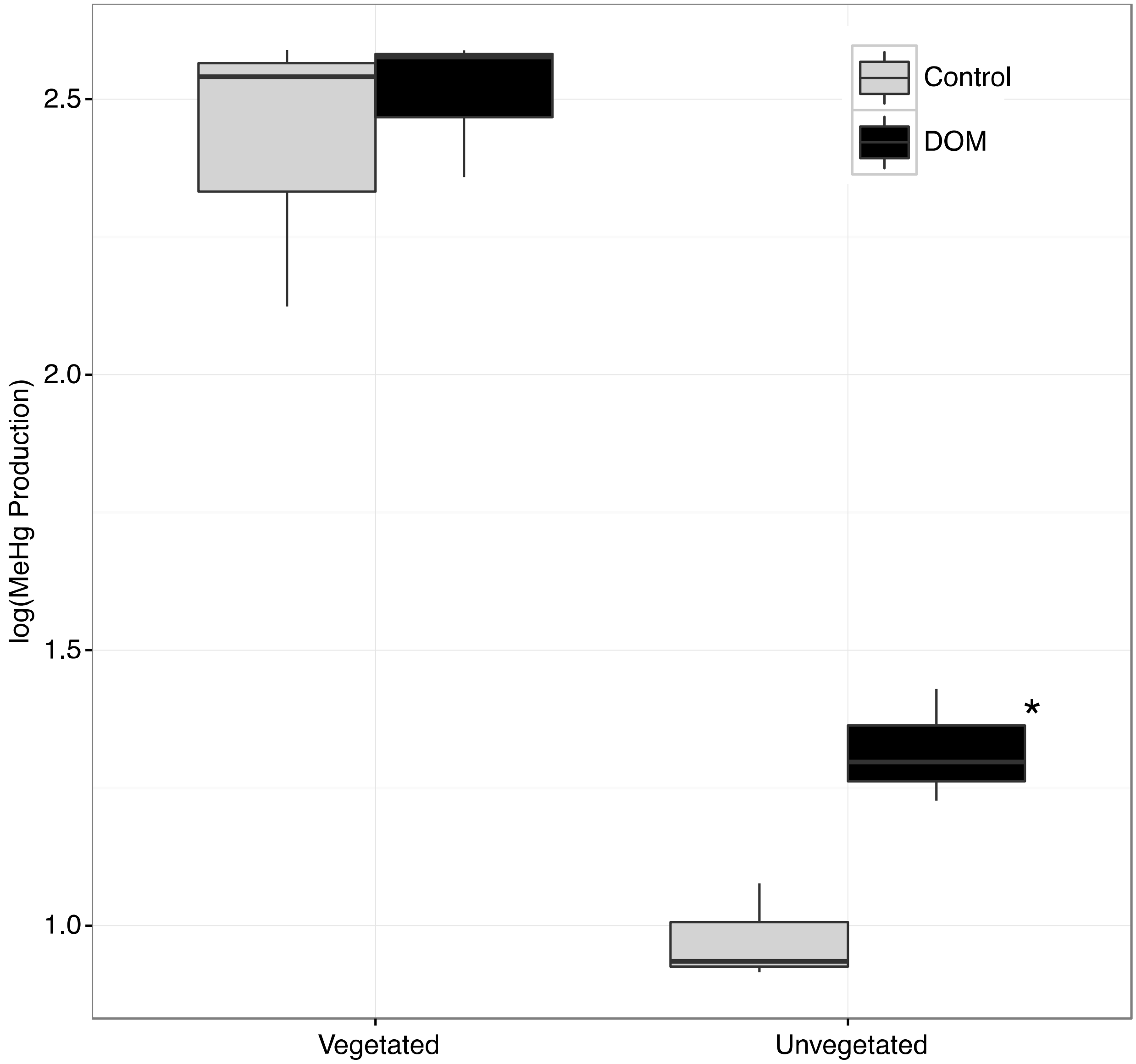

Figure 1. Boxplots are shown for net MeHg production, with upper and lower hinges representing the values at the 75th and 25th percentiles and whiskers respresenting 1.5 times value at the 75th and 25th percentiles, respectively. DOM did not significantly increase net MeHg production in, vegetated sediment, but induced net MeHg production in unvegetated sediment. Regardless of DOM amendment, vegetated sediment experienced an order of magnitude higher rates of net mercury methylation.

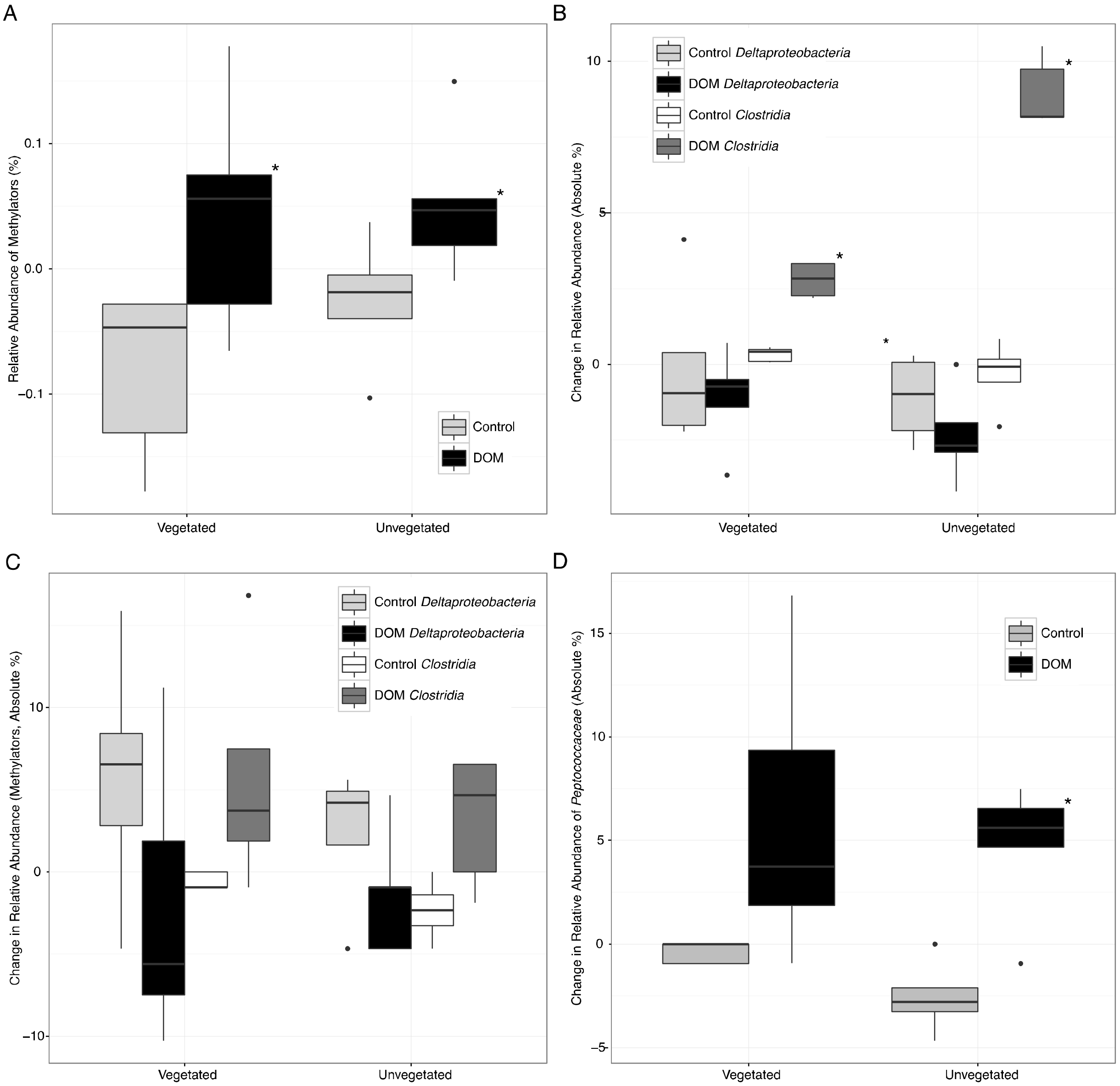

Figure 2. Boxplots are shown for selected changes in abundance of methylator abundance (A) and taxonomy (B-D) in response to DOM addition, with upper and lower hinges representing the values at the 75th and 25th percentiles and whiskers respresenting 1.5 times value at the 75th and 25th percentiles, respectively. Outliers are plotted as points. Shading for each bar denote taxonomy and DOM-amended vs. control. Significant relationships ($P < 0.05$) are denoted with an asterisk. (A) The relative abundance of methylating organisms increased in both sediment types in response to DOM addition. (B) The addition of DOM 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 DOM addition, (D) driven by changes within the family *Peptococcaceae*. Abundance data are present in Table S3.

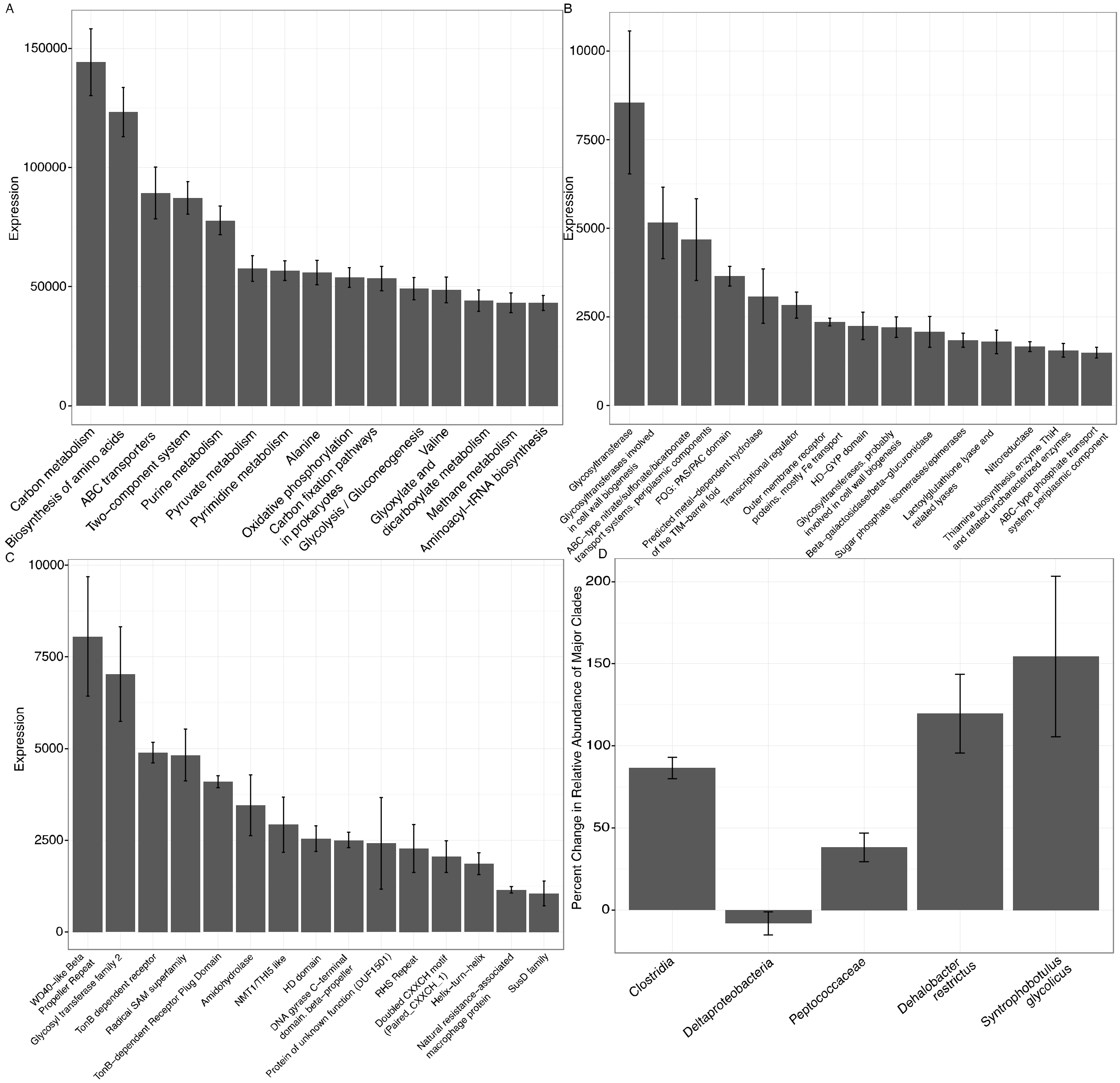

Figure 3. Results from analysis of metagenomic shotgun sequences from unvegetated microcosms are denoted in Figure 3. Panels A, B, and C show the abundance of the top 15 KEGG, COG, and Pfam targets that increased at T4 vs. T0, respectively. Panel D shows percent change in selected taxonomic groups at T4 vs. T0.

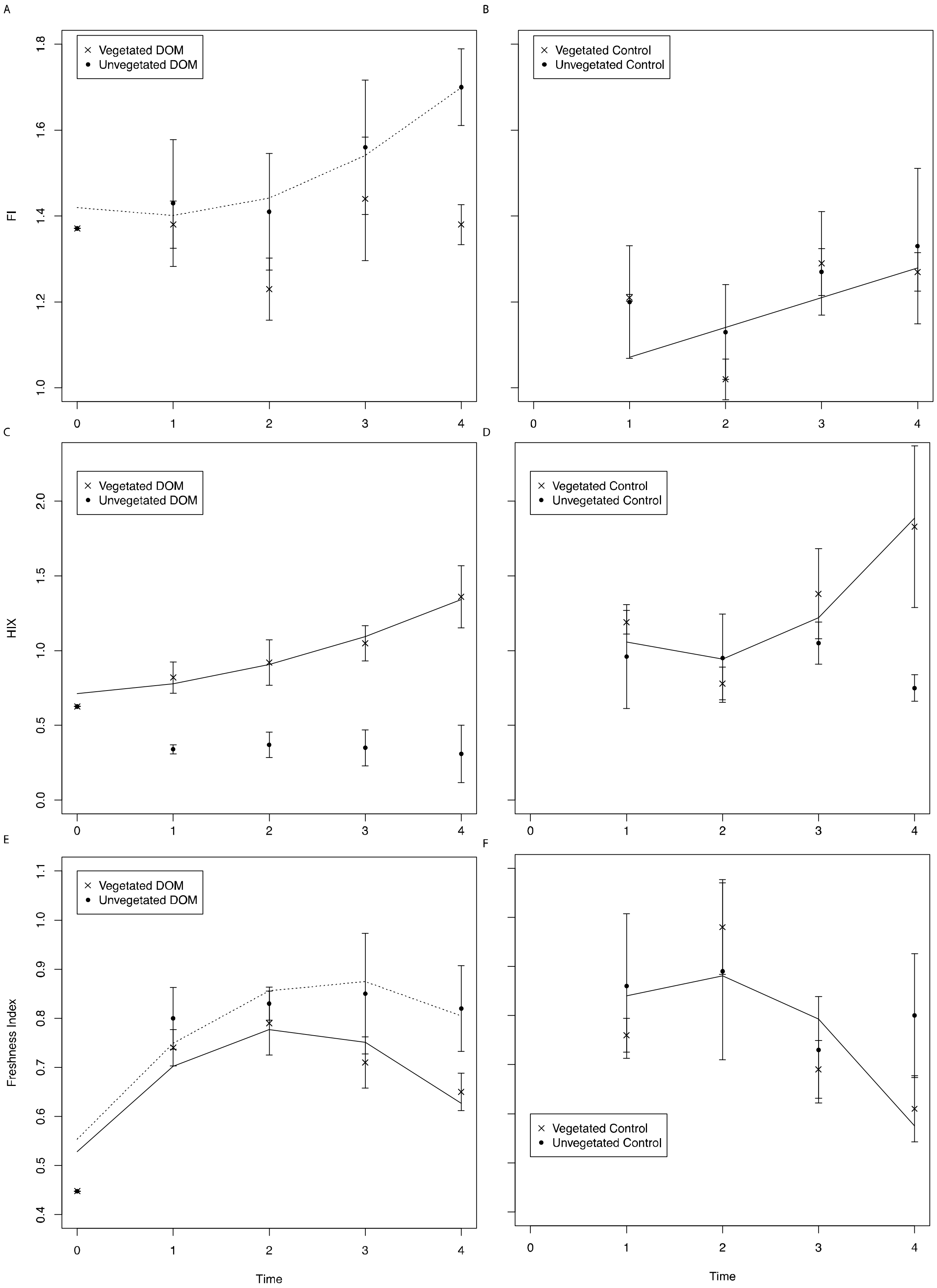
A
B
C
D
E
F
Vegetated DOM
Unvegetated DOM
Vegetated Control
Unvegetated Control
FI
HIX
Freshness Index
Time
1.0
1.2
1.4
1.6
1.8
0.0
0.5
1.0
1.5
2.0
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
0
1
2
3
4

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Figure 4. DOM fluorescence indices were assessed through time with linear and quadratic regressions in each environment and microcosm type. Averages for each environment and microcosm type are plotted at T0-T4, with error bars representing the standard deviation. Plots in the first column at DOM-amended microcosms, while plots in the second column are control microcosms. Unvegetated microcsms are depicted as closed circles with dashed lines showing significant regressions; vegetated microcosms are x's with solid lines showing significant regressions. (A) and (B) denote FI, (C) and (D) denote HIX, and (E) and (F) denote freshness.

Tables.

Table 1. Mean chemical and biological characteristics of vegetated and unvegetated environments are presented Table 1. Asterisks represent significant differences from post hoc *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*	116.73 (6.65)	120.6 (11.33)

*$P < 0.10$ **$P < 0.05$ ***$P < 0.01$

Table 2. R^2 values from regression analysis of changes in DOM properties through time are listed in Table 2. Control microcosms were analyzed from T1-T4 and DOM-amended microcosms were analyzed from T0-T4, with characteristics of the applied DOM represented at T0.

	NPOC (mg/L)	Total Fluorescence	Fluor:NPOC	FI	HIX	Freshness
Vegetated, Control (T1-T4)	0.39**	0.21*	n.s.	0.22**	0.51***	0.52***
Vegetated, DOM-amended (T0-T4)	0.32***	n.s.	n.s.	n.s.	0.68****	0.57***
Unvegetated, Control (T1-T4)	0.64****	n.s.	0.29**	n.s.	n.s.	n.s.
Unvegetated, DOM-amended (T0-T4)	n.s.	n.s.	n.s.	0.41***	n.s.	0.39***

*$P < 0.10$ **$P < 0.05$ ***$P < 0.01$ ****$P < 0.001$

Table 3. The Pearson product-momentum correlation coefficient was used to assess relationships of selected COG and Pfam targets with HIX and net MeHg production at T4 ($n = 3$). 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

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