Periphyton and Flocculent Materials Are Important Ecological Compartments Supporting Abundant and Diverse Mercury Methylator Assemblages in the Florida Everglades

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ABSTRACT Mercury (Hg) methylation in the Florida Everglades is of great environmental concern because of its adverse effects on human and wildlife health through biomagnification in aquatic food webs. Periphyton and flocculent materials (floc) overlaying peat soil are important ecological compartments producing methylmercury (MeHg) in this ecosystem. These compartments retain higher concentrations of MeHg than did soil at study sites across nutrient and/or sulfate gradient(s). To better understand what controls Hg methylation in these compartments, the present study explored the structures and abundances of Hg methylators using genes *hgcAB* as biomarkers. The *hgcA* sequences indicated that these compartments hosted a high diversity of Hg methylators, including *Deltaproteobacteria*, *Chloroflexi*, *Firmicutes*, and *Methanomicrobia*, with community compositions that differed between these habitats. The copy numbers of *hgcAB* quantified by quantitative PCR revealed that floc and soil supported higher numbers of Hg methylators than periphyton in the Everglades ecosystem. The abundance of Hg methylators was strongly positively correlated with concentrations of carbon and nutrients (e.g., phosphorus and nitrogen) according to redundancy analysis. Strong correlations were also observed among numbers of sulfate reducers, methanogens, and the dominant *hgcAB*-carrying groups, suggesting that *hgcAB* would spread primarily through the growth of those assemblages. The abundances of Hg methylators were weakly negatively correlated to MeHg concentrations, suggesting that the size of this population would not solely determine the final concentrations of MeHg in the ecological compartments studied. This study extends the knowledge regarding the distribution of diverse potential mercury methylators in different environmental compartments in a wetland of national concern.

IMPORTANCE Methylmercury is a potent neurotoxin that impacts the health of humans and wildlife. Most mercury in wetlands such as the Florida Everglades enters as inorganic mercury via atmospheric deposition, some of which is transformed to the more toxic methylmercury through the activities of anaerobic microorganisms. We investigated the numbers and phylogenetic diversity of *hgcAB*, genes that are linked to mercury methylation, in the soil, floc, and periphyton in areas of the Everglades with different sulfate and nutrient concentrations. Soil harbored relatively high numbers of cells capable of methylating mercury; however, little detectable methylmercury was present in soil. The greatest concentrations of methylmercury were found in floc and periphyton. The dominant methylators in those compartments included methanogens and *Syntrophobacteriales*. This work provides significant insight into the microbial processes that control methylation and form the basis for accumulation through the food chain in this important environment.

KEYWORDS flocculent, *hgcAB*, methylmercury, peat, periphyton, wetlands

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The Florida Everglades is a large subtropical freshwater wetland covering ~8,000 km² at the southern tip of the U.S. state of Florida. This wetland provides habitats for a diversity of wildlife; however, fish and wildlife are subject to exposure to mercury (Hg) from atmospheric deposition derived from anthropogenic and natural sources (1–3). In natural ecosystems, Hg may be transformed to the more potent neurotoxin methylmercury (CH₃Hg⁺, MeHg) (4, 5). Although the Hg could be methylated abiotically in the presence of suitable methyl donors (6), microorganisms are the prime agents responsible for Hg methylation (7–9). Recent studies using *hgcAB* (essential genes for Hg methylation) (10) as a biomarker have indicated that the potential for Hg methylation is spread much more broadly across more diverse microbial taxa than previously thought and includes the *Deltaproteobacteria*, *Chloroflexi*, *Firmicutes*, and *Euryarchaeota* (11–15). The produced MeHg may flow through food webs and accumulate in top predators through biomagnification (16–19). Due to such adverse effects, MeHg contamination is a major global environmental issue, and great efforts have been made to assess and monitor its concentrations at landscape levels within the Everglades (1, 20).

Among ecological compartments in the Everglades, periphyton and soil have been recognized as important in producing MeHg (18, 21, 22). Periphyton, which are mats comprising diverse microorganisms (e.g., algae, bacteria, and fungi) and particles (mineral and detrital), is present throughout the Everglades, with multiple functions that participate in a range of biogeochemical processes (e.g., oxygen production and nutrient uptake) (23–25). The Everglades soil consists of two layers: flocculent materials (floc) comprised of dead plant debris and periphyton and peat soil formed from decaying emergent macrophytes, such as sawgrass (*Cladium jamaicense* Crantz) or cattail (*Typha* sp.) and residual floc. Interestingly, higher concentrations of MeHg have been reported in periphyton and floc than in soil (18, 20), even though soil is thought to provide the Hg methylating organisms with more favorable conditions, e.g., lower redox potentials and higher nutrient (phosphorus [P] and nitrogen [N]) and carbon (C) concentrations. High MeHg concentrations in periphyton and floc could lead to higher bioaccumulation in invertebrates, small fishes, and amphibians, because these compartments serve as their food sources (26).

In a previous report, we explored the assemblage structures of Hg methylators inhabiting the peat of the Everglades through a culture-independent approach that employed *hgcAB* as a biomarker (11). The previous study concluded that the methylating assemblage was comprised of extremely diverse species of *Bacteria* and *Archaea*, with the syntrophic *Deltaproteobacteria* being the dominant bacterial group. The Hg methylating assemblages in peat soil below the floc differed in composition across study sites that exhibited a range of environmental factors, including gradients of total C (TC), total P (TP), and sulfate concentrations. The previous study focused on peat soils; however, significant concentrations of MeHg are observed in floc and periphyton, suggesting that they also are significant sites for mercury methylation (18, 20). The general environments (available C, N, and P and redox conditions) in floc and periphyton differ from that of peat, such that the distribution of hosts that harbor *hgcAB* might be expected to differ from those in peat. To test the hypothesis that floc and periphyton are hotspots for harboring diverse mercury methylating prokaryotes that differ from those in the soil, this study explored the assemblage structures of Hg methylators in the periphyton and floc of well-characterized regions of the Everglades that represent different nutrient statuses. In parallel, the majority of Hg methylators found in the communities were enumerated by real-time quantitative PCR (qPCR) using newly designed primers targeting *hgcAB* from two of the major Hg methylating clades in order to investigate the correlations between the abundance of Hg methylators, the MeHg concentration in each compartment, and relevant biogeochemical parameters.

RESULTS

Geochemical properties and MeHg concentration. Four sites were selected in the Water Conservation Areas (WCAs) of the Everglades for this study (see Fig. S1 in the

TABLE 1 Summary of geochemical characteristics of the ecological compartments of Everglades wetland

Site	Level (means $\pm$ SD) of ^a :										
	TP (g·kg ⁻¹)			TN (g·kg ⁻¹)			TC (g·kg ⁻¹)			SO ₄ ²⁻ (mg liter ⁻¹)	
	Periphyton	Floc	Soil	Periphyton	Floc	Soil	Periphyton	Floc	Soil	SW ^b	PW ^b
F2 (impacted)	0.89 $\pm$ 0.05	1.31 $\pm$ 0.23	1.20 $\pm$ 0.11	15 $\pm$ 0	26 $\pm$ 3	26 $\pm$ 2	294 $\pm$ 9	424 $\pm$ 93	405 $\pm$ 97	20.8 $\pm$ 1.2	7.1 $\pm$ 4.8
U3 (unimpacted)	0.11 $\pm$ 0.03	0.15 $\pm$ 0.02	0.17 $\pm$ 0.03	10 $\pm$ 1	13 $\pm$ 2	13 $\pm$ 1	216 $\pm$ 11	217 $\pm$ 9	225 $\pm$ 14	21.0 $\pm$ 4.6	5.5 $\pm$ 3.7
DB14	0.15 $\pm$ 0.06	0.38 $\pm$ 0.03	0.05 $\pm$ 0.03	14 $\pm$ 3	39 $\pm$ 4	40 $\pm$ 3	275 $\pm$ 19	443 $\pm$ 16	482 $\pm$ 08	0.2 $\pm$ 0.1	0.1 $\pm$ 0.0
DB15	0.18 $\pm$ 0.12	0.33 $\pm$ 0.04	0.35 $\pm$ 0.03	18 $\pm$ 1	28 $\pm$ 6	38 $\pm$ 1	282 $\pm$ 55	347 $\pm$ 50	433 $\pm$ 12	0.5 $\pm$ 0.5	0.1 $\pm$ 0.0

^aMean values $\pm$ SD were obtained from samples collected in January/February, May, July, and November/December 2015.

^bSW, surface water; PW, porewater.

supplemental material). Sites F2 (impacted) and U3 (unimpacted) in WCA-2A represent relatively sulfate-rich regions resulting from the discharge of drainage waters from the Everglades Agricultural Area (EAA), while sites DB14 and DB15 are located in the interior of WCA-3A and represent low-sulfate regions due to less impact from agricultural drainage. F2 was included in this study as a representative location that is highly impacted by the phosphorus discharged from EAA (27) and Lake Okeechobee. The sulfate enrichment in WCA-2A sites was confirmed by the significantly higher mean sulfate concentrations in surface water (15 to 29 mg·liter⁻¹, $\bar{X}$ [mean] = 20.9 mg·liter⁻¹, n = 21) and porewater (1.1 to 17.0 mg·liter⁻¹, $\bar{X}$ = 6.2 mg·liter⁻¹, n = 44) than in those in WCA-3A sites (0.1 to 1.3 mg·liter⁻¹, $\bar{X}$ = 0.36 mg·liter⁻¹, n = 18 in surface water and 0.1 to 0.2 mg·liter⁻¹, n = 18 in porewater) (Fig. S2). The WCA-2A sites also showed significantly higher total sulfide (TS; H₂S + HS⁻ + S²⁻) concentrations in porewater (0.9 to 5.0 mg·liter⁻¹, $\bar{X}$ = 3.1 mg·liter⁻¹, n = 20) than did WCA-3A sites (0.02 to 0.16 mg·liter⁻¹, $\bar{X}$ = 0.075 mg·liter⁻¹, n = 18), indicating a higher activity of sulfate reducers at the WCA-2A sites. Likewise, TS concentrations in surface waters were significantly higher in WCA-2A sites (0.02 to 0.10 mg·liter⁻¹, $\bar{X}$ = 0.036 mg·liter⁻¹, n = 21) than in the WCA-3A sites ($\leq$ 0.02 mg·liter⁻¹).

The study sites revealed a significant variation in the concentrations of total C (TC) and total organic C (TOC) among ecological compartments (periphyton, floc, and soil) (mean values are presented in Table 1 and Table S1 and Fig. S3). Soil harbored levels of TC (212 to 495 g·kg⁻¹, $\bar{X}$ = 386 g·kg⁻¹, n = 15) and TOC (132 to 533 g·kg⁻¹, $\bar{X}$ = 344 g·kg⁻¹, n = 15) similar to those in floc for TC (207 to 491 g·kg⁻¹, $\bar{X}$ = 358 g·kg⁻¹, n = 40) and TOC (128 to 484 g·kg⁻¹, $\bar{X}$ = 321 g·kg⁻¹, n = 39) over all sites. The C levels in these two compartments were 30 to 50% higher than those of periphyton, TC (202 to 361 g·kg⁻¹, $\bar{X}$ = 267 g·kg⁻¹, n = 33) and TOC (122 to 336 g·kg⁻¹, $\bar{X}$ = 194 g·kg⁻¹, n = 33), over all sites. Unlike other sites with a large difference between soil/floc and periphyton, site U3 (unimpacted) exhibited similar levels of TC and TOC in periphyton (TC, 202 to 233 g·kg⁻¹, $\bar{X}$ = 216 g·kg⁻¹, n = 12; TOC, 122 to 158 g·kg⁻¹, $\bar{X}$ = 141 g·kg⁻¹, n = 12), soil (TC, 212 to 239 g·kg⁻¹, $\bar{X}$ = 225 g·kg⁻¹, n = 3; TOC, 132 to 199 g·kg⁻¹, $\bar{X}$ = 170 g·kg⁻¹, n = 3), and floc (TC, 207 to 439 g·kg⁻¹, $\bar{X}$ = 234 g·kg⁻¹, n = 13; TOC, 128 to 207 g·kg⁻¹, $\bar{X}$ = 149 g·kg⁻¹, n = 12), which was due to a large portion of periphyton components remaining in floc and soil at this site. The variation of TC and TOC was also significant between sites, as illustrated in Fig. S3. Site U3 (unimpacted) revealed the lowest TC (207 to 439 g·kg⁻¹, $\bar{X}$ = 225 g·kg⁻¹, n = 28) and TOC (122 to 207, $\bar{X}$ = 148 g·kg⁻¹, n = 27) in all compartments, while DB14 exhibited the highest TC (247 to 489, $\bar{X}$ = 377 g·kg⁻¹, n = 27) and TOC (175 to 448, $\bar{X}$ = 336 g·kg⁻¹, n = 27).

Similarly, variation in total N (TN) was observed among ecological compartments, revealing a similar trend with TC and TOC, that is, significantly higher levels (P < 0.05) in soil (12 to 43 g·kg⁻¹, $\bar{X}$ = 29 g·kg⁻¹, n = 15 over all sites) and floc (11 to 45 g·kg⁻¹, $\bar{X}$ = 27 g·kg⁻¹, n = 40) than periphyton (8 to 32 g·kg⁻¹, $\bar{X}$ = 14 g·kg⁻¹, n = 33) (Fig. S3). Site U3 revealed the lowest TN value in all compartments (8 to 15 g·kg⁻¹, $\bar{X}$ = 11 g·kg⁻¹, n = 27) among sites.

FIG 1 MeHg concentrations in periphyton, floc, and soil across sites F2 and U3 within WCA-2A and DB14 and DB15 within WCA-3A. Error bars in each data point (circle symbol) represent ± 1 standard deviation ($n \geq 3$). Box-and-whisker plots were generated from the pooled data obtained from each compartment at individual sites. Boxes show the medians (horizontal lines in the boxes) and the lower and upper quartiles (bottoms and tops of the boxes, respectively). The vertical bars (whiskers) on the box plots represent the maximum and minimum values, excluding outliers. Statistical differences between compartments are indicated by capital letters in the panel representing compartments. The difference between sites for each compartment is represented by a small letter(s). Different letters between compartments or between sites mean a significant difference ($P \leq 0.05$ by the Tukey-Kramer HSD test in ANOVA).

Total P (TP) showed a totally different pattern of variation among sites and ecological compartments from TC/TOC and TN. For all compartments, F2 revealed the highest level of TP (846 to 1,610 $\text{mg} \cdot \text{kg}^{-1}$, $\bar{X} = 1,206 \text{ mg} \cdot \text{kg}^{-1}$, $n = 18$), while U3 had the lowest values (69 to 200 $\text{mg} \cdot \text{kg}^{-1}$, $\bar{X} = 134 \text{ mg} \cdot \text{kg}^{-1}$, $n = 27$) among the sites (Fig. S3).

Among the three ecological compartments monitored between 2013 and 2015, soils harbored the lowest MeHg concentrations (0.04 to 1.24 $\text{ng} \cdot \text{g}^{-1}$, $\bar{X} = 0.27 \text{ ng} \cdot \text{g}^{-1}$, $n = 39$) over all sites (Fig. 1). Floc and periphyton contained higher MeHg concentrations than soil ($P < 0.05$), averaging 3.02 and 2.31 $\text{ng} \cdot \text{g}^{-1}$, respectively. Each compartment also showed spatial variation in MeHg concentrations among the sites. Soil tended to have higher MeHg concentrations in WCA-3A sites (0.29 to 1.24 $\text{ng} \cdot \text{g}^{-1}$, $\bar{X} = 0.68 \text{ ng} \cdot \text{g}^{-1}$, $n = 3$ in DB14 and 0.20 to 0.48 $\text{ng} \cdot \text{g}^{-1}$, $\bar{X} = 0.32 \text{ ng} \cdot \text{g}^{-1}$, $n = 3$ in DB15) than in WCA-2A sites (0.08 to 0.73 $\text{ng} \cdot \text{g}^{-1}$, $\bar{X} = 0.27 \text{ ng} \cdot \text{g}^{-1}$, $n = 18$ in F2 and 0.04 to 0.61 $\text{ng} \cdot \text{g}^{-1}$, $\bar{X} = 0.19 \text{ ng} \cdot \text{g}^{-1}$, $n = 15$ in U3). This trend was also observed in floc; that is, higher MeHg concentrations were observed in WCA-3A sites (1.88 to 11.6 $\text{ng} \cdot \text{g}^{-1}$, $\bar{X} = 5.07 \text{ ng} \cdot \text{g}^{-1}$, $n = 9$ in DB14 and 1.45 to 7.02 $\text{ng} \cdot \text{g}^{-1}$, $\bar{X} = 4.69 \text{ ng} \cdot \text{g}^{-1}$, $n = 9$ in DB15) than in WCA-2A sites (0.85 to 2.32 $\text{ng} \cdot \text{g}^{-1}$, $\bar{X} = 1.33 \text{ ng} \cdot \text{g}^{-1}$, $n = 12$ in F2 and 0.32 to 7.95 $\text{ng} \cdot \text{g}^{-1}$, $\bar{X} = 2.05 \text{ ng} \cdot \text{g}^{-1}$, $n = 15$ in U3). This trend was not observed in periphyton, with similar levels between WCA-2A (0.81 to 3.17 $\text{ng} \cdot \text{g}^{-1}$, $\bar{X} = 1.77$, $n = 9$ in F2 and 0.46 to 6.75 $\text{ng} \cdot \text{g}^{-1}$, $\bar{X} = 2.57 \text{ ng} \cdot \text{g}^{-1}$, $n = 33$ in U3) and WCA-3A (0.51 to 4.78 $\text{ng} \cdot \text{g}^{-1}$, $\bar{X} = 1.74$, $n = 9$ in DB14 and 1.35 to 4.1 $\text{ng} \cdot \text{g}^{-1}$, $\bar{X} = 2.45$, $n = 9$ in DB15).

Assemblage structures of Hg methylators. We initially attempted to amplify *hgcAB* in periphyton and floc with PCR using primers *hgcA-F/hgcB-R*, which had previously been used to amplify those genes in the Everglades soils (11). However, that primer set did not amplify the appropriate gene fragments from periphyton, even though it successfully amplified DNA from floc. Therefore, we designed forward primers using the *hgcA* sequences formerly obtained from the Everglades soils (11), with the

FIG 2 Phylogeny of Hg methylators detected using *hgcAB* as a gene marker from the periphyton, floc, and peat. (Left) The phylogeny was determined by maximum likelihood constructed using HgcAB protein sequences deduced from 774 *hgcAB* sequences collected in the Everglades samples through our previous (220 sequences) (11) and current (554 sequences) studies. (Right) Pie graphs represent the composition of major taxa in the ecological compartments of the sulfate-enriched site U3 within WCA-2A and the sulfate-depleted site DB15 within WCA-3A.

intention to reduce degeneracy and to increase specificity for the Everglades samples. Among the newly designed candidate primers, primer *hgcA_EvG-F* was chosen as the partner for the reverse primer *hgcB-R*, because this combination produced amplicons with clear single bands in gel electrophoresis from all compartments (i.e., periphyton, floc, and soil) used in this study.

The assemblage structures of Hg methylators were determined for the three compartments from sites U3 and DB15, representing sulfate-rich and sulfate-poor environments, respectively. A total of 411 *hgcAB* sequences obtained from six clone libraries (three compartments from two sites) were translated *in silico* to amino acids (HgcAB protein sequences). The HgcA sequences were grouped into 169 operational taxonomic units (OTUs), defined by a 5% difference in protein sequences for the phylogenetic and diversity analysis. The OTUs were distributed across 10 clades belonging to *Deltaproteobacteria* (clades I, II, and III), *Chloroflexi* (IV), *Firmicutes* (V, VI, VII), and *Euryarchaeota* (VII, IX, X) within the phylogenetic tree that was constructed using all 774 HgcA sequences obtained from the Everglades samples in the previous and current studies (Fig. 2 and Table S2).

The composition of Hg methylator assemblages differed from one another depending on the compartments and sites. Within site U3 of WCA-2A, the soil was dominated by *Deltaproteobacteria* (57% of the total assemblage), followed by *Euryarchaeota* (35%). Floc supported similar proportions (31% to 38%) of *Firmicutes*, *Deltaproteobacteria*, and *Euryarchaeota*. Representatives of the *Deltaproteobacteria* (47%) were the dominant group in the periphyton, followed by *Firmicutes* (31%) and *Euryarchaeota* (19%). All of the *Deltaproteobacteria* detected from U3 were affiliated with the order *Syntrophobacterales* (clade III). U3 supported all three clades of *Euryarchaeota*: VIII (*Methanomicro-*

FIG 3 PCoA plot showing the beta diversity of Hg methylator assemblages between three ecological compartments (i.e., soil, floc, and periphyton). The plot was constructed based on UniFrac weighted distance matrix.

biales and *Methanosarcinales*), IX (*Methanocellales*), and X (the cluster of unidentified sequences), with clade X ($\geq 67\%$ of *Euryarchaeota*) as the majority. The majority of *Firmicutes* in site U3 belonged to clade VII and were related to *Dethiobacter alkaliphilus* AHT 1, a strain that was isolated from soda lake sediments (28). This clade dominated the floc of U3, making up 37% of the total *HgcA* sequences.

Within site DB15, *Deltaproteobacteria* (51%) dominated the soil Hg methylator assemblage, while *Euryarchaeota* (48% to 67%) dominated in floc and periphyton. The majority of *Deltaproteobacteria* in all the compartments of site DB15 were the *Syntrophobacteriales* ($\geq 90\%$ of this class), even though a few sequences belonged to *Geobacter* (clade I) and *Desulfovibrionales* (clade II). Clade X (unidentified methanogens) was a dominant group of *Euryarchaeota* in these compartments. As in U3, *Dethiobacter*-like Hg methylators (clade VI) constituted a major portion of the *Firmicutes* ($\geq 64\%$). *Chloroflexi* (IV) sequences represented a minority ($< 5\%$ of total Hg methylators) in both sites.

The difference of Hg methylator assemblages between compartments within each site was also indicated by their diversity within a compartment (referred to as alpha diversity) and between compartments (referred to as beta diversity). The Chao1 richness estimator (an alpha diversity index) indicated that soil harbored the highest diversity of *hgcAB* OTUs (158 to 162), followed by floc (57 to 103), and periphyton (12–26) (Table S3). This tendency was in good agreement with the Shannon diversity indices, with 3.9 to 4.0 in soil, 3.0 to 3.1 in floc, and 2.1 to 2.8 in periphyton.

The weighted UniFrac test revealed a significant difference between assemblages in all pairwise comparisons ($P < 0.001$), with scores between 0.5365 and 0.7348 representing the fraction of branch lengths between two communities (Table S4). Principal coordinate analysis (PCoA) based on the UniFrac distance matrix illustrates the distribution of these communities along axes 1 and 2, explaining 24.6% and 24.2% of the variation, respectively (Fig. 3). On the PCoA plot, two assemblages from soils clustered together while the assemblages from floc and periphyton did not show a clear separation.

Abundance of Hg methylators, methanogens, and sulfate reducers. The numbers of Hg methylators inhabiting the three ecological compartments (i.e., soil, floc, and periphyton) within each study site were estimated by the numbers of *hgcAB* copies that were measured with qPCR using the group-specific primers. The group-specific primers targeted the majority of Hg methylators; that is, the *Syntrophobacteriales* (here referred to as *hgcAB_SYN*) within the aforementioned clade III and three methanogenic OTUs (*hgcAB_MGXa-c*) within clade X (Fig. 2).

FIG 4 Numbers of *hgcAB* copies from *Syntrophobacteriales* and methanogenic OTUs *hgcAB_MGXa-c* in periphyton, floc, and soil sampled in January/February (denoted J), May (M), and November/December (D) 2015. Error bars on each data point (circle symbol) represent ± 1 standard error ($n = 3$). Box-and-whisker plots were constructed from the pooled data within each compartment. Boxes depict the medians (horizontal lines in the boxes) and the lower and upper quartiles (bottoms and tops of the boxes, respectively). The vertical bars (whiskers) show the highest and the lowest values, excluding outliers. Significant differences between compartments in individual sites are denoted on the boxes with letters; different letters mean significant differences ($P < 0.05$ by the Tukey-Kramer HSD test). The significant differences between compartments across all sites are denoted on the right side of the graph.

The abundances of group *hgcAB_SYN* varied significantly with time ($P < 0.05$), especially within periphyton and floc of individual sites (Fig. S4). The more obvious trend in variation was found among compartments. Across all sites, soil (4.1×10^7 to 7.4×10^8 g⁻¹, $\bar{X} = 2.0 \times 10^8$ g⁻¹, $n = 27$ for combined data from all) had a significantly ($P < 0.05$) higher number of *Syntrophobacteriales* than periphyton (2.7×10^4 to 2.4×10^8 g⁻¹, $\bar{X} = 2.3 \times 10^7$ g⁻¹, $n = 21$) (Fig. 4). Floc (1.4×10^5 to 4.0×10^9 g⁻¹, $\bar{X} = 3.3 \times 10^8$ g⁻¹, $n = 42$) also harbored significantly higher numbers of *hgcAB_SYN* than the periphyton across all sites ($P < 0.05$) but did not show a statistical significance within sites U3 and DB15 individually. The results from two-way analysis of variance (ANOVA) indicated that the temporal variation significantly interacted with the variation of *hgcAB_SYN* in the compartments within sites F2_WCA2A and DB15_WCA-3A (Fig. S4).

OTUs *hgcAB_MGXa-c* were observed in all compartments, with a significant variation with time ($P < 0.05$) within compartments in many cases (Fig. S4). Across all sites combined and within individual sites, OTUs tended to be more numerous in soil and floc than in periphyton (Fig. 4). OTU *hgcAB_MGXa* had significantly higher numbers combined across sites ($P < 0.05$) in soils (2.7×10^6 to 1.6×10^8 g⁻¹, $\bar{X} = 2.6 \times 10^7$ g⁻¹, $n = 23$) and floc (8.4×10^4 to 3.3×10^8 g⁻¹, $\bar{X} = 4.6 \times 10^7$ g⁻¹, $n = 38$) than in

periphyton (2.7×10^4 to 2.7×10^7 g⁻¹, $\bar{X} = 3.7 \times 10^6$ g⁻¹, $n = 21$) (Fig. 4). Likewise, OTU *hgcAB*_MGXc revealed significantly higher numbers across all sites in soil (5.1×10^6 to 3.2×10^8 g⁻¹, $\bar{X} = 5.3 \times 10^7$ g⁻¹, $n = 24$) and floc (5.7×10^5 to 6.1×10^8 g⁻¹, $\bar{X} = 1.2 \times 10^8$ g⁻¹, $n = 39$) than in periphyton (3.7×10^5 to 2.7×10^7 g⁻¹, $\bar{X} = 4.2 \times 10^6$ g⁻¹, $n = 21$). Although not supported by statistical significance, OTU *hgcAB*_MGXb also revealed higher numbers combined in soil (8.9×10^5 to 7.4×10^7 g⁻¹, $\bar{X} = 1.1 \times 10^7$ g⁻¹, $n = 18$) and floc (2.9×10^4 to 1.4×10^8 g⁻¹, $\bar{X} = 2.8 \times 10^7$ g⁻¹, $n = 39$) than in periphyton (4.9×10^4 to 1.8×10^7 g⁻¹, $\bar{X} = 1.8 \times 10^6$ g⁻¹, $n = 14$). Two-way ANOVA indicated that there was a significant interaction between time and compartment within site U3 of these methanogenic OTUs in their numbers (gene copies) (Fig. S4).

In addition to the variation among compartments, significant variation in the abundance of Hg methylators occurred between sites. For all the measured groups, site F2 showed significantly ($P < 0.05$) higher numbers of Hg methylators for floc and soil than other sites (Fig. S5). The Hg methylator numbers also varied in periphyton between sites; however, the variation was not significant.

Sulfate-reducing prokaryotes (SRP) and methanogens are the host populations for the syntrophic and methanogenic *hgcAB* genes enumerated above, such that the spread of *hgcAB* genes is likely dependent on the growth of those populations in response to the environmental parameters measured in this study. In order to evaluate how the abundance of those host populations influences the distribution of *hgcAB*_SYN and methanogenic *hgcAB*_OTUs, total SRP and methanogens were enumerated by qPCR using *dsrB* (29) and *mcrA* (30) as gene markers. The estimated numbers of SRP in the same samples used for enumerating Hg methylators ranged from 2.4×10^5 to 6.9×10^9 g dry weight⁻¹ ($\bar{X} = 7.4 \times 10^8$ g dry weight⁻¹) (Fig. S6). As with the syntrophic Hg methylators, the sulfate reducers exhibited higher numbers ($P \leq 0.05$) in floc ($\bar{X} = 2.1 \times 10^9$ g dry weight⁻¹) and soil ($\bar{X} = 7.1 \times 10^8$ g dry weight⁻¹) than in periphyton ($\bar{X} = 4.1 \times 10^6$ g dry weight⁻¹) in all sites. The numbers of methanogens ranged from 2.9×10^5 to 4.2×10^9 g dry weight⁻¹ ($\bar{X} = 7.0 \times 10^8$ g dry weight⁻¹), with significant variation among compartments (Fig. S6). The numbers estimated in floc ($\bar{X} = 1.1 \times 10^9$ g dry weight⁻¹) and soil ($\bar{X} = 6.8 \times 10^8$ g dry weight⁻¹) significantly exceeded ($P \leq 0.05$) those measured in periphyton ($\bar{X} = 1.6 \times 10^6$ g dry weight⁻¹) within all sites compared. Sulfate reducers and methanogens were significantly more numerous in floc and soil of site F2 than in those estimated from other sites (Fig. S7).

Relationship between MeHg and gene abundances with environmental variables. Redundancy analysis (RDA) was performed to evaluate how MeHg concentrations and selected physicochemical parameters may be related to the abundance of *hgcAB* copies measured from the periphyton, floc, and soils in the study sites (F2, U3, DB14, and DB15). Environmental variables in the first two RDA dimensions (axes 1 and 2) explained 82% of the total variance in the abundances of populations with a significant P value (≤ 0.001 in Monte Carlo test) in both canonical axes (Fig. 5). The angles indicated that all Hg methylating populations for which qPCR primer sets have been developed (i.e., *hgcAB*_SYN and OTUs *hgcAB*_MGXa-c) were positively correlated ($< 90^\circ$ between arrows) with each other, along with positive correlations for TC, TOC, TN, TP, and SO_4^{2-} . The numbers of *hgcAB*_SYN and *hgcAB* methanogen populations were also positively correlated with numbers of SRP and methanogens from the same samples (Fig. 5). In contrast, MeHg was weakly or negatively correlated ($\geq 90^\circ$ between arrows) with abundances of the populations and the measured geochemical parameters.

The RDA correlations were in good agreement with the results from Spearman's correlation analyses (Table S5). The Hg methylating populations were significantly positively correlated with each other (correlation coefficient [ρ] = 0.64 to 0.88, $P \leq 0.0001$) and with SRP ($\rho = 0.62$ to 0.94, $P \leq 0.0001$) and methanogens ($\rho = 0.77$ to

FIG 5 Redundancy analysis (RDA) plot representing the relationship between gene copies of *hgcAB*, *dsrB*, and *mcrA*, geochemical parameters, and MeHg concentrations determined in this study. Arrows pointing in the same direction indicate positive correlations, and arrows pointing in opposite directions indicate negative correlations. The arrow length corresponds to the variance explained by the environmental variable. The first two axes explain 82.3% of the total canonical eigenvalues with a significant Monte-Carlo test value ($P < 0.001$). SO₄_PW, sulfate concentration in porewater; SO₄_SW, sulfate concentration in surface water; TCa, total calcium concentration.

0.86, $P \leq 0.0001$). All of the different phylogenetic groups for which qPCR data were collected were positively correlated with TC ($\rho = 0.55$ to 0.76 , $P \leq 0.0034$), TOC ($\rho = 0.60$ to 0.79 , $P \leq 0.001$), TN ($\rho = 0.44$ to 0.61 , $P \leq 0.02$), and TP ($\rho = 0.61$ to 0.83 , $P \leq 0.0006$) but not with SO₄²⁻ in surface water and porewater ($\rho = -0.13$ to 0.28 , $P \geq 0.15$). They were negatively correlated with MeHg ($\rho \leq -0.13$, $P = 0.006$ to 0.529). MeHg was weakly correlated with TC ($\rho = 0.05$, $P = 0.74$) and negatively correlated with TOC ($\rho = -0.004$, $P = 0.98$), TN ($\rho = -0.04$, $P = 0.81$), TP ($\rho = -0.12$, $P = 0.27$), and SO₄²⁻ ($\rho = -0.29$ and -0.28 in surface water and porewater, respectively, $P > 0.05$).

Total calcium concentrations (TCa) showed a strongly negative correlation with the geochemical parameters TC, TOC, TN, and TP ($\rho = -0.69$ to -0.39 , $P = 0.0001$ to 0.08) and Hg methylators ($\rho = -0.96$ to -0.78 , $P < 0.0001$). These negative correlations might be attributable to the fact that an increase in the mass of calcium carbonate proportionally decreased the fraction of organic matter in the compartments.

DISCUSSION

In recent decades, factors controlling the methylation of Hg in the Everglades ecosystem have been the focus of many studies, including characterization of distribution and bioaccumulation (16, 18, 22) and the transformation (e.g., methylation and demethylation) of Hg(II) and MeHg (31–34), including the mechanisms that control Hg methylation (35, 36). Despite many investigations, the little available knowledge on the taxonomic affiliation of Hg methylators limited the insight into the environmental controls on Hg methylation in this wetland ecosystem. In a previous study, we explored the community structure of Hg methylators in the Everglades' soils using *hgcAB* as a genetic marker (11). The present study extended the exploration from soil to periphyton and floc, which are considered important ecological compartments for Hg methylation in the Everglades (1).

Primer *hgcA_EvG-F* was designed as part of this study to increase the specificity and efficiency of PCR amplification for the Hg methylators inhabiting the different environ-

mental compartments in the Everglades. Using this new forward primer and the reverse *hgcB*-R that was developed previously (11), we explored the assemblage structures of Hg methylators at two sites from three ecological compartments: soil, floc, and periphyton. The soil communities at sites U3 and DB15 were dominated by *Syntrophobacteriales* (51% to 57%), followed by *Euryarchaeota* (23% to 35%) and *Firmicutes* (5% to 21%) (Fig. 2).

Hg methylator assemblages in floc materials differed from those in the soil and also differed from each other between sites U3 and DB15. The U3 assemblages were highly enriched with *Syntrophomonadaceae* strains within *Firmicutes* (clade VII in Fig. 2). The family *Syntrophomonadaceae* shares common metabolic characteristics with the order *Syntrophobacteriales* within the *Deltaproteobacteria*. Both groups are capable of establishing syntrophic associations with H₂ consumers such as methanogens (37, 38) or completely oxidize H₂ using the oxidized species of sulfur (e.g., sulfate and thiosulfate) as alternative electron acceptors (28, 39). Even though it is uncertain why the floc was enriched with syntrophic *Firmicutes* in site U3, the high proportion of syntrophs may be related to the high concentration of sulfate to some extent. On the other hand, the floc assemblages in DB15 were dominated by methanogenic archaea rather than the syntrophic *Firmicutes*, probably due to the lower available concentrations of oxidized sulfur.

Periphyton was previously suggested as an ecological compartment that would support Hg methylators other than sulfate reducers. For example, Hamelin and colleagues (40) reported that methanogens were the major contributors to Hg methylation in lake periphyton based on the total inhibition of methylation by treating periphyton with BESA (2-bromoethane sulfonic acid, a methanogenesis inhibitor). Achá and colleagues (41) found that amendment with molybdate (specific inhibitor of sulfate reducers) often did not completely inhibit Hg methylation in periphyton, suggesting the presence of nonsulfate reducers methylating Hg. We determined the whole assemblage structure of Hg methylators in periphyton. The periphyton assemblages were comprised of diverse phylogenetic groups, and their structures differed greatly among the sites (Fig. 2). One of the distinct features between sites was the higher proportion of *Firmicutes* in U3 (31%) than in DB15 (12%). The U3 *Firmicutes* were represented by *Peptococcaceae*, which were not detected in floc materials. This family shares similar characteristics with *Syntrophomonadaceae*, the family within the *Firmicutes* that was found in the floc, with respect to establishing a syntrophic relationship with H₂ consumers (42) or respiration using sulfur compounds as alternative electron acceptors (43, 44). The capability of respiring sulfur compounds might contribute in part to the enriched syntrophic *Firmicutes* in U3 periphyton that would be influenced by high concentrations of sulfate in surface waters. In contrast, periphyton at the low-sulfate site at DB15 was dominated by methanogens over the syntrophic *Firmicutes* and *Deltaproteobacteria*, which lends credence to our assumption that different levels of sulfate concentrations contribute to structuring the Hg methylating assemblages.

Notably, Hg methylator assemblages were composed to only a minor degree of *Geobacter* (clade I, iron reducer) and *Desulfovibrionales* (clade II, sulfate reducer), with none exceeding 4% of the total community in any compartment, even though these two taxa had been well-documented as important Hg methylators in other aquatic soils and sediments (7, 8). Recently, Christensen and colleagues (14) described a pair of universal primers, ORNL-HgcAB-uni-F and ORNL-HgcAB-uni-R, that amplified *hgcAB* from diverse strains (cultured) within *Desulfovibrionales* and recommended that their primers would be appropriate for the Everglades soil samples. Based on their recommendation, we determined the assemblage structure of Hg methylators using their primer set and our newly designed primer for DNA isolated from the peat and floc in site DB15, and we found that no more than one OTU (<2% of total sequences detected) of *Desulfovibrionales* was detected in soil and floc with the ORNL primers (see Fig. S8 in the supplemental material). This result confirmed that *Desulfovibrionales* do not represent a dominant group of Hg methylators at this sulfate-limited Everglades site.

The low proportion of *Desulfovibrionales* in Hg methylator assemblages does not

necessarily suggest low sulfate reduction rates by Hg methylators. The soils of WCA-2A showed relatively high TS concentrations, indicating on-going sulfate reduction (Fig. S2). The soils of the Everglades were reported to be dominated by *dsrB* transcripts originating from the strains of *Syntrophobacteriales* (11). Strains of *Syntrophobacteriales* may be metabolically versatile (39, 45) and are capable of reducing sulfate in the presence of sufficiently available sulfate as well as forming syntrophic relationships with methanogens. Therefore, sulfate reduction is very likely to contribute to the spread of *hgcAB* through the growth of *Syntrophobacteriales* rather than *Desulfovibrionales* in the relatively sulfate-rich sites of F2 and U3 in WCA-2A. TS concentrations in the low-sulfate sites of WCA-3A were low, indicating very low sulfate reduction in those sites.

Although qPCR is a powerful method to estimate copies of *hgcAB*, it is difficult to design universal qPCR primers for enumerating the diversity of *hgcA* sequences because there are no well-conserved regions between the cap helix motif in *hgcA* and the 4Fe-4S motifs in *hgcB*. qPCR primers for some specific groups bearing *hgcAB* have been published (e.g., *Deltaproteobacteria*, *Firmicutes*, and *Euryarchaeota*) by Christensen and colleagues (14). However, these primers were designed based on only a few known strains that did not represent a significant proportion, if present at all, in the Everglades samples. We designed group-specific qPCR primers targeting the majority of Hg methylators inhabiting the Everglades: the syntrophic *Deltaproteobacteria* (i.e., *hgcAB_SYN*) and three methanogen OTUs (i.e., *hgcAB_MGXA-c*). Although the present study did not include *Firmicutes* and *Chloroflexi* as target groups for qPCR because of the difficulty in designing primers for them, it should be noted that those taxa occupied an important niche of Hg methylation assemblages in some compartments (e.g., *Firmicutes* with $\geq 31\%$ in the communities of floc and periphyton of site U3). Further study is needed to enumerate those groups for a more comprehensive understanding of their roles in Hg cycling.

The qPCR using the group-specific primers indicated that Hg methylators inhabited all the compartments tested in considerably high numbers. The numbers of *hgcAB_SYN* almost reached the total numbers of sulfate reducers in each compartment (Fig. 4 and Fig. S6), with a significantly positive correlation between *hgcAB* copies from *hgcAB_SYN* and *dsrB* copies in the RDA result (Fig. 5) and Spearman's correlation analysis ($\rho = 0.80$, $P \leq 0.0001$). Likewise, the OTUs of methanogenic Hg methylators revealed the number within the same order of magnitude as the total number of methanogens (Fig. S6), with positive correlations between their *hgcAB* copies and *mcrA* copies in RDA biplot (Fig. 5) and Spearman's analysis ($\rho = 0.77$ to 0.96 , $P \leq 0.0001$). These results suggested that *hgcAB* is widely spread across the sulfate reducers and methanogens in the Everglades ecosystem.

Significantly positive correlations were also found between the copies of all the genes and the levels of carbon (TC and TOC) and macronutrients (TN and TP) (Fig. 5), suggesting that the concentrations of substrates and nutrients affect the numbers of *hgcAB* copies at these sites. At this time, it is not known whether the carbon and nutrient concentrations are directly related to Hg methylation; however, it is clear that these parameters support the growth of sulfate reducers and methanogens. Hg methylation occurs via a reductive acetyl-coenzyme A pathway linked to a putative corrinoid iron-sulfur protein (HgcA) and a [4Fe-4S] ferredoxin (HgcB), without any step potentially yielding energy or precursors of macromolecules (10). Hence, Hg methylation is unlikely to directly contribute to the abundance of *hgcAB*; instead, *hgcAB* would be spread by the distribution and proliferation of microbes carrying these genes in the environment.

For the last few decades, sulfate has been considered a key factor in Hg methylation (8) because SRP have been thought to be primary Hg methylators in many environments (7). Since sulfate serves as the respiration substrate for SRP, its concentration is likely to contribute to the spread of *hgcAB* genes to some extent, especially in the sulfate-rich WCA-2A. RDA results indicate that sulfate concentrations in surface water and porewater has a positive correlation with *hgcAB* copy numbers in *hgcAB_SYN* ($\rho = 0.284$ and 0.25 , respectively) without significant statistical support ($P \geq 0.152$) (Table S5). The positive correlation is primarily due to the high numbers of *hgcAB* copies in the

sulfate-rich site F2. In the RDA that does not include site F2, the *hgcAB*_SYN numbers were strongly negatively correlated with sulfate concentrations, even though inclusion of the relatively high-sulfate site U3 was included in the analysis (Fig. S9). These results suggest that the impact of the sulfate on the *hgcAB* gene distribution would be site (or geographic location) specific and largely reliant on other environmental factors, such as carbon substrates and nutrients.

The copy numbers of *hgcAB* may not be correlated directly with Hg methylation activity; nevertheless, copies of the controlling genes are among the broadly recognized components that control Hg methylation activity. This study evaluated whether this parameter correlated with MeHg concentration. The abundances of Hg methylators were highly variable among compartments and geographical locations at the four sites, although some trends were observed. Among the three compartments, the lowest abundance occurred in periphyton; the highest abundance among sites was at F2. These trends were not in agreement with the MeHg concentration, which tended to be higher in periphyton than in soil and to be lower at F2 than sites DB14 and DB15 within WCA-3A (Fig. 5). These contradictory observations may be explained by the following possibilities: (i) *hgcAB* might not be expressed in proportion to the copy number and/or (ii) the final concentrations of MeHg would be determined by a combination of complex abiotic factors (e.g., MeHg transportation and availability of Hg^{2+}) and biological processes in the Hg cycle. Along with Hg methylation, MeHg demethylation is likely to be a crucial determinant of net concentrations of MeHg, which had been well documented in the three compartments of the Everglades (31, 34). Two pathways have been proposed for microbial MeHg demethylation: the reductive pathway that first cleaves MeHg to Hg(II) and CH_4 by an organomercurial-lyase enzyme encoded by *merB* and then to Hg(0) by mercuric reductase encoded by *merA* of Hg-resistant bacteria (46–50), as well as by the oxidative pathway that completely oxidizes MeHg to CO_2 and Hg(II) by SRP and methanogens (51–54). More recently, a methanotroph was also reported to degrade MeHg via the potential activity of methanol dehydrogenase (55). To gain insight into the detailed mechanisms that determine the fate of MeHg, further efforts should be made to assess the expression of *hgcAB* with the identification of MeHg demethylators and their activities in wetland ecosystems such as the Everglades.

In conclusion, building on our previous exploration of Hg methylators in soils (11), the present study extended our knowledge of the assemblages of Hg methylators in the flocculent materials and periphyton that are important compartments producing MeHg in the Everglades ecosystem (20, 22). This study indicates that the abundance and assemblage structures of Hg methylators are largely dependent on environmental variations, such as the concentrations of sulfate, available carbon substrates, and nutrients, occurring within environmental compartments. Our findings provide an opportunity to clarify the microbial processes that control Hg methylation by identifying the dominant Hg methylators from individual compartments and suggest the presence of MeHg demethylators.

MATERIALS AND METHODS

Sampling and processing. Triplicates of periphyton, floc (loosely consolidated detrital particles), and peat soil (top 5 cm from surface, exclusive of floc) were sampled during each sampling event conducted during December 2013, September 2014, and January/February, May, July, and November/December 2015 across sites F2 (26°20'32.5" N, 80°22'35.5" W) and U3 (26°16'59.4" N, 80°24'26.1" W) within WCA-2A and sites DB14 (25°58'54.0" N, 80°36'39.9" W) and DB15 (26°2'55.8" N, 80°43'34.4" W) within WCA-3A (see Fig. S1 in the supplemental material). Periphyton (either benthic, floating, or epiphytic on submerged plant stems) was collected with gloved hands. Floc and soil were retrieved by acrylic cores and separated from each other in the field according to particle density. On one occasion, floc was collected by sweep net. Individual samples were mixed in a polyethylene ziplock bag. Three subsamples (50 to 100 g) were soon (≤ 1.5 h) frozen by dry ice for transport to the laboratory in Gainesville, FL, where they were stored at -80°C until isolation of DNA. The remaining samples were placed on ice before being refrigerated at 4°C at the end of the day for analyzing physicochemical parameters in the laboratory by DB Environmental, Inc., Rockledge, FL. Surface and pore waters were collected from the same sites and stored as described previously (11, 56, 57). See the supplemental material for a detailed description of field collection procedures and precautions taken to ensure sample integrity.

DNA isolation. Periphyton, floc, and soil samples were homogenized with a mortar and pestle under liquid nitrogen before isolation of DNA. DNA was extracted from 0.2 g (wet weight) of samples using a Power Soil DNA isolation kit (MoBio Laboratories, Carlsbad, CA). The quality of DNA was assessed by 1.5% ethidium bromide-stained agarose gel electrophoresis. The isolated DNA was stored at -80°C until use.

Primer design for *hgcAB*. *hgcA* and *hgcB* are essential for MeHg production and have been identified from a diversity of known strains within *Bacteria* and *Archaea* (10). *hgcB* (approximately 216 to 372 bp in size) is generally, but not always, immediately downstream from *hgcA* (approximately 924 to 966 bp). Universal forward primer *hgcA*_EvG-F (5'-GGN AAR GGV ACY TTY GGV AC-3') was designed based on the conserved region in the alignment of *hgcA* sequences that had been obtained from the NCBI (<https://www.ncbi.nlm.nih.gov>) and JGI IMG/ER (<https://img.jgi.doe.gov>) public databases and the Everglades soils in our previous study (11). This new primer targeted a region of amino acid sequence, GKGTFGT, that was well conserved in *HgcA* sequences deduced from the Everglades *hgcA* sequences. The targeted region is located at positions 286 to 305 in *hgcA* of *Desulfovibrio desulfuricans* ND132 (58) (Fig. S10). This primer matches well with the sequences from the Everglades soils and previously known Hg methylators belonging to *Deltaproteobacteria* (other than *Desulfovibrionales*), *Firmicutes*, *Chloroflexi*, and *Euryarchaeota*, with ≤ 2 mismatching bases (Table S6 and Fig. S11). The *hgcAB* sequences from *Desulfovibrionales* had ≥ 3 mismatching bases, suggesting that the application of this primer could lead to an underestimation of Hg methylators for an environment harboring a large proportion of *Desulfovibrionales*. The expected size of PCR amplicons with primers *hgcA* EvG-F/*hgcB*-R will be $\sim 900 \pm 50$ bp depending on species.

For qPCR enumeration of *hgcAB* gene copies, group-specific primers were designed based on the *hgcA* sequences originated from the Everglades wetland. While this work was being conducted, a total of 774 *hgcA* sequences were collected from soil, floc, and periphyton in WCA-2A and WCA-3A in our previous (220 sequences) (11) and current study (554 sequences), which were grouped into 355 operational taxonomic units (OTUs), with 5% cutoff dissimilarity in the amino acid sequences in mothur v.1.32.1 (59). The group-specific sequence regions were identified on the alignment of sequences representing each OTU. Initially, 3 to 8 forward primers were designed based on the sequences in conserved regions for each target group. The sequences of primers were evaluated for secondary structures and dimer-forming potential using the Oligo-Analyzer program from Integrated DNA Technologies. These primers were also evaluated with respect to formation of an unexpected amplicon(s) from the Everglades samples in PCR using *hgcB*-F as a reverse primer (11). Through the program and PCR evaluation, we finally selected four forward primers that would have the lowest potential in forming hairpin structures and dimers and would produce a single PCR amplicon with the expected size: one for *Syntrophobacteriales*, *hgcA*_SYN-F (5'-CCV TGG YTB CCR GGB MGD GCV TTY TC-3'), and three for methanogenic *Archaea*, *hgcA*_MGXa-F (5'-CCT GGC TGC CAA CGC ATA AC-3'), *hgcA*_MGXb-F (5'-GGC GGT GGT AAC CTC GGT A-3'), and *hgcA*_MGXc-F (5'-CCA TCG CCA TGC CGT TCT C-3'). Primer *hgcA*_SYN-F covered 81 OTUs (including 171 sequences) out of a total 150 OTUs (312 sequences) affiliated with *Syntrophobacteriales*, with fewer than 2 mismatching bases in primer binding area. Primers *hgcA*_MGXa-F, *hgcA*_MGXb-F, and *hgcA*_MGXc-F targeted three OTUs that were the majority of methanogenic OTUs, including more than 20 sequences in each (total of 64 sequences from methanogenic Hg methylators [269 sequences]). Primer *hgcA*-SYN-F was well matched with the *hgcA* sequences from well-known Hg methylators within the *Syntrophobacteriales* but did not match with strains of other taxa, including the *Deltaproteobacteria* (Table S6). The group-specific primers for methanogens did not match with sequences of strains of known archaeal Hg methylators (Table S6), suggesting a high specificity for the Everglades members. Targeting regions of the group-specific primers are depicted in Fig. S10. In a pair with *hgcB*-R, all these forward primers produced a single fragment of the expected size on agarose gel electrophoresis from all compartments (i.e., soil, floc, and periphyton). The specificity of the primers was verified by sequences amplified by the group-specific primers. Five or ten clones were randomly selected from clone libraries constructed using the amplicons produced by the primers. All sequences from the selected clones were matched to the target groups. The sequence-confirmed clones were used for preparation of standard DNA for qPCR. Additional information on primers used in this study is summarized in Table S7.

PCR, cloning, and sequence analysis of *hgcAB*. The *hgcAB* fragment was amplified using primers *hgcA* EvG-F and *hgcB*-R (5'-CAD GCN CCR CAY TCV ATR CA-3') (11) in DNAs from the periphyton, floc, and soil samples taken from sites U3 and DB15. The reaction mixture contained 10 μl of iQ supermix (Bio-Rad Laboratories), 2 μl of primers (concentration of each primer, 10 $\text{pmol}\cdot\mu\text{l}^{-1}$), and 2 μl of one-tenth-diluted DNA in a 20- μl final volume. The PCR was run under the following thermal cycling conditions: an initial denaturation step at 94°C for 10 min and 6 cycles of touchdown steps at 94°C for 30 s and 60°C for 30 s, decreasing 1°C per cycle, and 72°C for 1 min, followed by 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 5 min. Primers ORNL-HgcAB-uni-F and ORNL-HgcAB-uni-F, which had been previously published as universal primers for *hgcAB* (14), were also applied for amplifying *hgcAB* from floc and soil samples of DB15 in order to compare the diversity of sequences amplified by different primer sequences. The PCR for these primers was run under the same reaction conditions, except for the thermal cycling conditions as described by Christensen and colleagues (14).

The PCR products were cloned and transformed into an *Escherichia coli* TOP10 TOPO TA cloning kit for sequencing (Invitrogen, Carlsbad, CA). The transformants were randomly selected on Luria-Bertani (LB) agar plates containing kanamycin (50 $\mu\text{g}\cdot\text{ml}^{-1}$) and sent to the University of Florida Sequencing Core Laboratory (<http://www.biotech.ufl.edu/>) for sequencing of inserts. The quality of sequences was examined in BioEdit (v.7.1.3) (60). All the *hgcA* sequences determined in this and a previous study (a total of

774 sequences) were translated *in silico* into the corresponding amino acid sequences (HgcA sequence) in BioEdit (v.7.1.3), aligned with ClustalX 2.0 (61), and reedited using the BioEdit program. For the phylogenetic analysis, the HgcA sequences were grouped into OTUs, with 5% cutoff dissimilarity in the amino acid sequences in mothur v.1.32.1 (59). The phylogeny of OTUs was determined by their affiliation patterns on a maximum-likelihood (ML) tree constructed using the representative OTUs and reference sequences from NCBI and JGI IMG/ER in MEGA, version 5.2.1 (62), with bootstrap analysis (1,000 resamplings). The mothur program was also used to estimate the diversity of OTUs and the coverage of OTUs sampled within each clone library and to perform PCoA based on UniFrac weighted distance matrix (63).

qPCR. Quantitative real-time PCR (qPCR) was performed for enumerating copies of *hgcAB*, *mcrA*, and *dsrB* from three compartments in all the study sites. For the enumeration of *hgcAB* copies, one of the aforementioned forward primers (i.e., *hgcA_SYN*-F, *hgcA_MGXa*-F, *hgcA_MGXb*-F, and *hgcA_MGXc*-F) was used in combination with *hgcB*-R as a partner reverse primer under the following thermal cycling conditions: an initial denaturation step at 94°C for 10 min and 6 cycles of touchdown steps at 94°C for 30 s and 63°C for 30 s, decreasing 1°C per cycle, and 72°C for 30 s, followed by 30 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s. The addition of a touchdown step resulted in reducing the amplification of untargeted regions from the sample DNAs. Previously published primers and thermocycler conditions were employed for PCR amplification of *mcrA* (64) and *dsrB* (65).

The qPCR was run in a StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA, USA) with a 96-well PCR plate in which each well contains 10 μ l of iQ SYBR green supermix (Bio-Rad Laboratories, Hercules, CA), 2 μ l of a forward/reverse primer mixture (10 pmol μ l⁻¹ each), and 2 μ l of one-tenth-diluted sample DNA or plasmid DNA standard carrying the gene fragment of interest in a 20- μ l final volume of reaction mixture. The sample DNAs were tested for potential inhibitor(s) that may have carried over during DNA purification by spiking 10-fold dilutions of sample DNA in reaction mixtures containing $\geq 10^5$ copies of standard DNA. No significant inhibitory effects were observed. All sample DNAs and standard DNAs were assayed in triplicate. All the PCR runs were followed by an image capture step (15 s at 83°C) after a final extension step of each cycle. The final cycle of PCR amplification followed a melt curve analysis of increasing temperature from 60°C to 95°C in 0.5°C increments every 10 s to check the quality of amplicons (Fig. S12). The products were also examined on 1.5% (wt/vol) agarose ethidium bromide-stained gel. Every qPCR run included serially diluted standard plasmid DNA (over 6 orders of magnitude) carrying the gene fragment of interest in order to make a standard curve. Standard DNA plasmids were made by cloning the target gene fragment amplified from some of the environmental soils as described previously (11, 56). Plasmid DNA was linearized using the endonuclease PstI or SpeI (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions, followed by purification with the QIAquick PCR purification kit (Qiagen, Valencia, CA). The prepared standards were stored in aliquots at -80°C, and a separate standard was used for each qPCR run.

The standard curve was constructed by plotting the relative fluorescence units at a threshold cycle value against the logarithm of the copy number of the standard plasmid DNA. The copy number in each reaction was calculated by the following formula: molecules (gene copies) of DNA = (mass [in grams] $\times$ Avogadro's number)/(average molecular mass of base template length), where the average mass of a base pair was assumed to be 650 Da. The PCR efficiency (E) was calculated from the slope of the standard curve by using the formula $E = 10^{-1/\text{slope}} - 1$. E values from the standard curves are summarized in Table S8.

Analytical methods. The National Environmental Laboratory Accreditation Conference (NELAC) (66) and the NELAC Institute (67) standards and method guidelines were followed for all laboratory analyses, which includes the maintenance of quality assurance/quality control acceptance criteria (see the unnumbered table in the supplemental material). Sulfate concentration was measured with a Dionex ICS-1000 ion chromatograph according to SM4110 B (68) after filtering of the sample through a 0.45- μ m polyether sulfone filter. Total sulfide (TS), which is equal to the sum total of all dissolved sulfide species (i.e., $\text{H}_2\text{S} + \text{HS}^- + \text{S}^{2-}$), was analyzed in the laboratory by ion selective electrode according to a modification of SM4500S-G (69) after preservation with zinc acetate and NaOH in the field. TP concentrations in soil, periphyton, and floc were determined using a wet digestion procedure (70), followed by measurement of soluble reactive P in the digestate by EPA method 365.2 (71). TN, TC, and TOC (after fumigating the sample with HCl to drive off the inorganic carbon as CO_2) concentrations in the same solid matrices were analyzed by combustions finely ground samples (passed through a no. 60 standard sieve) in a carbon-nitrogen elemental analyzer (Costech ECS 4010). Total calcium (TCa) in each compartment was determined by analysis of the digestate (EPA 3050B) by flame atomic absorption spectrometry (72).

Lastly, MeHg analysis on solid matrices was based on NELAC-approved modification of EPA 1630 (73) following digestion of KOH-methanol for soil/floc or HNO_3 for periphyton. Samples were analyzed on a cold vapor atomic fluorescence spectrophotometer (CVAFS).

Statistical analyses. The significant differences of gene copy numbers and physicochemical data between compartments or study sites were computed using ANOVA with JMP (v.10) software (SAS Institute Inc., Cary, NC, USA), followed by the *post hoc* Tukey-Kramer honestly significant difference (HSD) test for ANOVA. Normal distribution of the data were confirmed by the Shapiro-Wilk test in JMP. The two-way ANOVA computation was performed to evaluate an interaction between two independent variables (i.e., time and compartments) with *hgcAB* copy number. Nonparametric Spearman's test was performed to measure rank correlation between two variables with JMP. *P* values of <0.05 were considered significant. An RDA was implemented in Canoco (v.4.5 for Windows) software (74) for determining the relationships between variations in gene copy numbers and geochemical parameters.

The statistical significances of axis and individual parameters were evaluated using a Monte Carlo permutation full model with 999 unrestricted permutations.

Data availability. The GenBank accession numbers for the sequences determined in this study are MK166213 to MK166766.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.00156-19>.

SUPPLEMENTAL FILE 1, PDF file, 4.3 MB.

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