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Linking Microbial Activities and Low-Molecular-Weight Thiols to Hg Methylation in Biofilms and Periphyton from High-Altitude Tropical Lakes in the Bolivian Altiplano

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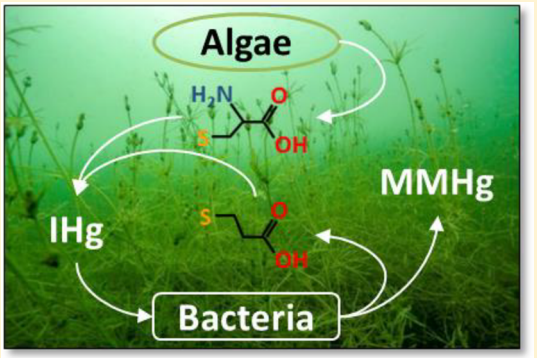
Supporting Information

ABSTRACT: The sources and factors controlling concentrations of monomethylmercury (MMHg) in aquatic ecosystems need to be better understood. Here, we investigated Hg transformations in sediments, periphyton associated with green algae's or aquatic plants, and benthic biofilms from the Lake Titicaca hydrosystem and compared them to the occurrence of active methylating microorganisms and extracellular Hg ligands. Intense Hg methylation was found in benthic biofilms and green algae's periphyton, while it remained low in sediments and aquatic plants' periphyton. Demethylation varied between compartments but remained overall in the same range. Hg methylation was mainly carried out by sulfate reducers, although methanogens also played a role. Its variability between compartments was first explained by the presence or absence of the *hgcAB* genes. Next, both benthic biofilm and green algae's periphyton exhibited a great diversity of extracellular low-molecular-weight (LMW) thiols (13 or 14 compounds) present at a range of a few nmol L^{-1} or μmol L^{-1} but clearly dominated by cysteine and 3-mercaptopropionic acid. Hg methylation was overall positively correlated to the total thiol concentrations, albeit to different extents according to the compartment and conditions. This work is the first examining the interplay between active methylating bacterial communities and extracellular ligands in heterotrophic biofilms and supports the involvement of LMW thiols in Hg methylation in real aquatic systems.



INTRODUCTION

Anthropogenic activities have greatly increased Hg dispersion and concentrations in ecosystems,[1] but globally, human populations remain mainly exposed through the consumption of fish products,[2] preferentially accumulating MMHg from their diet. At the base of the food web, MMHg results from the concurrent methylation and demethylation reactions, both occurring in virtually all environmental compartments. Still, identifying the main loci of MMHg production and factors controlling its final concentration is crucial to predict and reduce exposure risk. It has long been thought that MMHg was mainly produced in sediments[3,4] and then exported to the water column and food webs.[5−7] This paradigm is challenged nowadays, with evidence accumulating that methylation can occur at (i) low to moderate rates in marine or fresh water column,[8,9] likely through bacteria, such as sulfate reducers[10] associated with plankton[11] or particle aggregates[12] and (ii) moderate to (very) high rates in biofilms and periphyton.[13−20] The importance of these compartments needs to be confirmed given the expansion of dead zones and eutrophication.

The methylation of inorganic Hg (IHg) into MMHg is mainly a biotic mechanism relying on the presence of a two-gene

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cluster,[21] widespread among anaerobic bacteria[22] and commonly found in aquatic environments.[23] It is carried out by specific strains belonging mostly to sulfate-reducing bacteria (SRB), iron-reducing bacteria (FeRB), Firmicutes, and methanogens.[17,22,24−26] The methylation rates are usually highest under suboxic to anoxic conditions[27,28] due to an optimal balance between the activities of methylating bacteria and IHg bioavailability. The former depends on many factors such as the availability of electron acceptors[29] and carbon sources.[30] The knowledge of their phylogeny, habitat, and the effect of their metabolism is currently greatly growing with the implementation of molecular probes targeting the *hgcAB* genes in field and model studies.[31−35] On the other hand, our understanding of the IHg bioavailability for bacteria has also improved over the past few years, although mainly with lab studies. It has been demonstrated that the addition of low-molecular-weight (LMW) thiol compounds (RSH) to bacterial cultures can enhance its cellular uptake and subsequent methylation[36] compared with inorganic ligands such as chlorides or sulfides. However, the effect was not the same for every LMW thiol and bacterial strain, suggesting that the uptake might be modulated by differences in $Hg(SR)_2$ properties, bacterial transport systems, and binding sites.[37,38] It has been suggested that IHg can be internalized directly under its complexed form by a thiol transporter or after an exchange of Hg from the complex to some transporter located on the membrane surface of the cell.[39] Whatever the mechanism, in situ data on the identity and concentrations of LMW thiols are still scarce in important environmental compartments regarding methylation to support their involvement in the reaction.

Previous studies highlighted high concentrations of Hg in food webs from Bolivian lakes,[40] but the origin of MMHg has never been clearly ascertained, although preliminary works pointed to sediments and periphyton as important compartments for both MMHg production[41] and accumulation.[42] Moreover, an increase in the MMHg pool was recently observed in the eutrophicated parts of the Altiplano lakes,[40] and we hypothesize that it could be related to the development of periphyton and/or biofilms. In this work, we concomitantly investigated the transformations of Hg species in various compartments from high-altitude tropical lakes located in the Titicaca hydrosystem (Bolivian Altiplano, 3600−3800 m above sea level). A total of five sites representative of the different sub-ecosystem characteristics of these lakes (shallow versus deep and pristine versus eutrophicated or contaminated) were selected to evaluate the role of sediments, periphyton associated with aquatic plants, or green algae and benthic biofilms in Hg transformations. We carried out incubation experiments with stable isotopic tracers combined with a characterization of bacterial communities and extracellular ligands to determine methylation and demethylation rates in each compartment and decipher the underlying mechanism.

EXPERIMENTAL SECTION

Study Sites. Lake Titicaca (3809 m above sea level., Figure SI1) is composed of two nearly separate basins, the great lake (7131 km^2, mean depth of 100 m) and the small lake (1428 km^2, mean depth of 9 m), fed by multiple streams that bring in various pollutants according to their watershed characteristics (urban, industrial, mining, or agricultural activities). The lake banks are extensively colonized by totoras (*Schoenoplectus californicus* sp.), a subspecies of the sedge family. Sediments in the photic zones (<15m) are covered by macrophytes, the most-abundant group being Characeae spp., which colonized a third of the small lake[43] and represent more than 60% of its total biomass and productivity. Lake Uru Uru (3686 m above sea level, 120 to 350 km^2 and 0.25 to 1 m depth) is a man-made reservoir located in the central part of the Bolivian Altiplano, where numerous mining and smelting activities are concentrated. Both lakes are influenced by a tropical climate (rainy season between December and March), and their hydrological regime is dominated by evaporation, resulting in alkaline pH of 8.2−9.7, O_2-depleted waters with salinity ranging from 0.56 to 0.86 PSU for the Titicaca, and more-variable pH (6.7 to 13.3) and higher salinity (1.05 to 3.53 PSU) for Uru-Uru. Sampling stations were selected to be representative of various conditions of the hydrosystem (Table SI1): CH is an oligotrophic deep station (38 m), while HU and BS present intermediate depths (5 and 25 m, respectively) and mesotrophic conditions but BS receives limited mining inputs in addition. BC and UU are both shallow (0.25−2 m) eutrophicated stations receiving mixed pollutions. A pair of campaigns were carried out in 2014, the first by the end of the rainy season (April−May) and the second by the end of the dry one (October−November).

Sampling and Incubations. Scuba divers sampled sediments from shallow sites (HU and BC) by hand-coring, while sediments from deeper sites (CH and BS) were taken with a gravity corer (Uwitec, Austria). Scuba divers also retrieved Characeaes by hand and epibenthic biofilms with syringes, while totora periphyton were collected by hand from a boat by scraping them off of their stems. All incubations were carried out in triplicate; therefore, standard deviation or relative standard deviation values thereafter are based on $n = 3$; in polypropylene jars (Nalgene, ThermoFisher) following a similar protocol. Surface sediments (0−2 cm) from multiple cores were pooled, homogenized, and distributed in jars (∼25 g of wet sediment in each). The various inhibitors (molybdate (Mo), bromoethanesulfonate (BES) and diuron (Di); Sigma-Aldrich) for sulfate reduction, methanogenesis, and photosynthesis, respectively, were first added to the corresponding jars at concentrations between 20 and 50 mM, and then isotopic enriched tracers were added at concentrations close to ambient ones (1 to 10 $ng{\cdot}g^{-1}$ for ^{201}MMHg and 17 to 180 $ng{\cdot}g^{-1}$ for ^{199}IHg). Controls (t_0) were immediately frozen, while t_f were incubated in the dark at an in situ temperature for 24 h and then stopped by freezing. For biofilms and periphyton (∼10 g of wet material each), incubations were carried out over 36 h at in situ temperature under dark or ambient light (i.e., diurnal cycle) conditions, and jars of samples were sacrificed at intermediate time-points for Hg, sulfides, thiols, and microbial analyses. For sulfides and thiols, the dissolved phases were sampled with plastic syringes and filtered through 0.45 μm nylon filters (Minisart, Sartorius) before preservation. Aliquots for sulfides (5 mL) were injected through a rubber septa in N_2-flushed vacutainers containing 0.5 mL of a diamine mixture, while aliquots for total thiol measurements were derivatized according to the protocol described in Liem-Nguyen et al.[44] Briefly, a reduction agent (TCEP, Sigma-Aldrich) was added at 40 μM, samples were hand-shaken and left 10 min to react, and then the derivatization agent (PHMB, Sigma-Aldrich) was added at the same concentration. Samples were shaken again, left to react for another 10 min, and then kept frozen at −20 °C until analyzed. Samples for microbial analyses were taken in cryovials with sterile spatula, immediately frozen in liquid N_2 and kept at −80 °C until analysis.

Laboratory Analyses. *Sulfides and Thiol Analyses.* Hydrogen sulfide (H_2S) was determined using a modification of

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methods described previously.[45,46] Briefly, *N,N-p*-phenylenediamine sulfate and iron chloride were prepared separately in 6 mol L^{-1} HCl and mixed as suggested elsewhere.[47] Subsamples (20 μL) of the sample−diamine mixture were analyzed with an Agilent high-performance liquid chromatography−UV instrument made of a 1260 quaternary pump and a diode array detector (DAD) detector set at 292 nm. A Poroshell 120 EC-C18 column (3 × 75 mm, 2.7 μm, Agilent) was used under isocratic elution conditions: 20% acetonitrile, 18% methanol, and 20% sodium acetate buffer (pH 5.2, 0.05 mM) at 35 °C and 1.1 mL min^{-1}. H_2S calibrations were performed using Radiello solution code 171.

The concentrations of 15 thiols (Figure SI2) were determined according to Liem-Nguyen et al.[44] after adapting the method for an ultraperformance liquid chromatography−electrospray ionization−mass spectrometry (UPLC-ESI-MS) instrument made of an Acquity UPLC system (Waters, Milford, MA) including a binary solvent pump and a cooled autosampler connected to a Xevo TQ mass spectrometer equipped with an orthogonal Z-spray-electrospray interface. Thiol separation was achieved using an Acquity UPLC BEH C18 column (2.1 × 50 mm, 1.7 μm, Waters) with the matching Vanguard precolumn. Aliquots of 50 μL were injected in a 0.5 mL·min^{-1} flow rate for the mobile phase consisting of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The elution gradient was as follow: 95% A for 0.5 min, then a linear gradient to 5% A in 3.85 min, and finally held for 0.65 min before re-equilibration to the initial stage. The column temperature was regulated to 45 °C, while the autosampler tray temperature was 5 °C. The electrospray capillary was set at 1.4 kV, desolvation temperature at 550 °C, and cone gas flow rate and desolvation gas flow rate were at 50 and 900 L·h^{-1}, respectively. DLs ranged from 0.1 to 25 nmol L^{-1}.

Hg Species Concentrations and Transformation Rates. Concentrations of Hg species and transformation rate constants were determined according to Rodriguez-Gonzalez et al.[48] Briefly, IHg and MeHg were extracted from 200 mg of freeze-dried samples with 6N HNO_3 (Trace metal grade, Fisher) under a focused microwave (Discover, CEM Corporation) treatment (85 °C for 5 min) and analyzed by species-specific isotope dilution using a complementary pair of isotopes (^{198}IHg and ^{202}MMHg), gas chromatography−inductively coupled plasma−MS (Thermo-Electron Series XII). The concentrations of the added and formed Hg species deriving from the enriched isotopes 199 and 201 were calculated by isotopic pattern deconvolution methodology, and methodological detection limits for Hg species were 0.03 ng·g^{-1}. The extraction and quantification were validated with CRMs IAEA 405 (estuarine sediment, certified for both HgT and MMHg) and 450 (phytoplankton, certified only for HgT). Concentrations were always found within the certified values, and recoveries ranged from 93 to 101%.

Microbial Analyses. DNA and RNA were purified using RNA Pro Soil-Direct kits (MP Biomedicals) coupled to DNA/RNA mini kits (Qiagen) following the manufacturer's instructions. The RNA purity was tested by direct PCR without reverse transcription, and the cDNA synthesis was carried out using the M-MLV reverse transcriptase (Invitrogen) and 50 ng of random hexamers (Roche) with 15−20 ng of RNA. For diversity analysis, the 16S rRNA gene was amplified from DNA and cDNA, with primers 28F and 519R targeting V1−V3 region of the 16S rRNA gene, coupled to MiSeq (Illumina) adaptors as described by Aubé et al.[49] Amplicons were sequenced with MiSeq V3 (Illumina) technology, and data were analyzed with Mothur (1.34.2). Chimeras and singletons were removed using the "chimera.uchime" and "remove.rare" command lines, while normalization was performed with the "sub.sample" line. Operational taxonomic units (OTUs) affiliation was performed using Silva database. The detection of *hgcAB* genes was performed by polymerase chain reaction (PCR) using ORNL-HgcAB-uni-F and ORNL-HgcAB-uni-R primers[31] on DNA extracted from the various samples as the matrix. Briefly, PCR reactions contained 5 ng of matrix DNA, 1 μM of each primer, and 1× AmpliTaq Gold 360 Master Mix (ABI) and were run in a touchdown PCR.[31]

RESULTS AND DISCUSSION

Hg Transformations and Bacterial Diversity in Sediments and Biofilms. *Hg Transformations in Sediments.* Transformation rate constants in sediments are given in Figure 1.

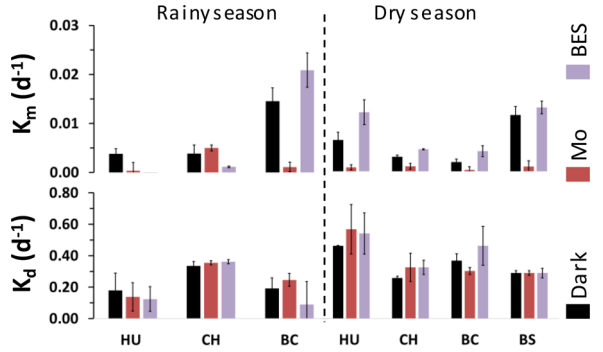

Figure 1. Hg methylation (K_m) and demethylation (K_d) rate constants in sediments at each site for the two seasons under various conditions: dark and with the addition of molybdate (Mo) and bromoethanesulfonate (BES). Error bars represent the standard deviation from triplicate incubations.

Under dark conditions, methylation (K_m) ranged from 2.10^{-3} to $1.5.10^{-2}$ day^{-1} (average relative standard deviation of 25%) and remained in the same range for both seasons at HU and CH (3.10^{-3} − 6.10^{-3} day^{-1}), while at BC, it declined by an order of magnitude from rainy to dry season ($1.5.10^{-2}$ to 2.10^{-3} day^{-1}). Demethylation (K_d) appeared randomly distributed across sites or seasons and only varied within a factor 3 (0.18−0.48 day^{-1}, average relative standard deviation of 18%). These rate constants are consistent with earlier studies on Hg transformations in tropical and temperate freshwater or marine sediments (cf. Table 3 in Alanoca et al.).[41] There was a strong inhibition of methylation when Mo was added (84−92%), except in one case (CH, rainy season) where it increased by 30%. On the other hand, BES enhanced methylation by an average of 63% (13−118%) except for HU and CH during the rainy season, in which it also strongly inhibited it (70−100%). Inhibitors thus demonstrate a predominant role of SRB in methylation with a significant contribution from methanogens during the rainy season likely following larger inputs of OM and nutrients. The increase often observed with BES can be explained by a decreased competition for organic substrates between methanogens and SRB[17,26] or by the use of sulfonates by SRB.[50] Surprisingly, the additions of Mo and BES had little effect on demethylation, while SRB and methanogens have been previously involved in it.[17,51−54] Many microorganisms may, however, degrade MMHg as C1 compounds,[51] and abiotic pathways could also prevail over biotic ones.

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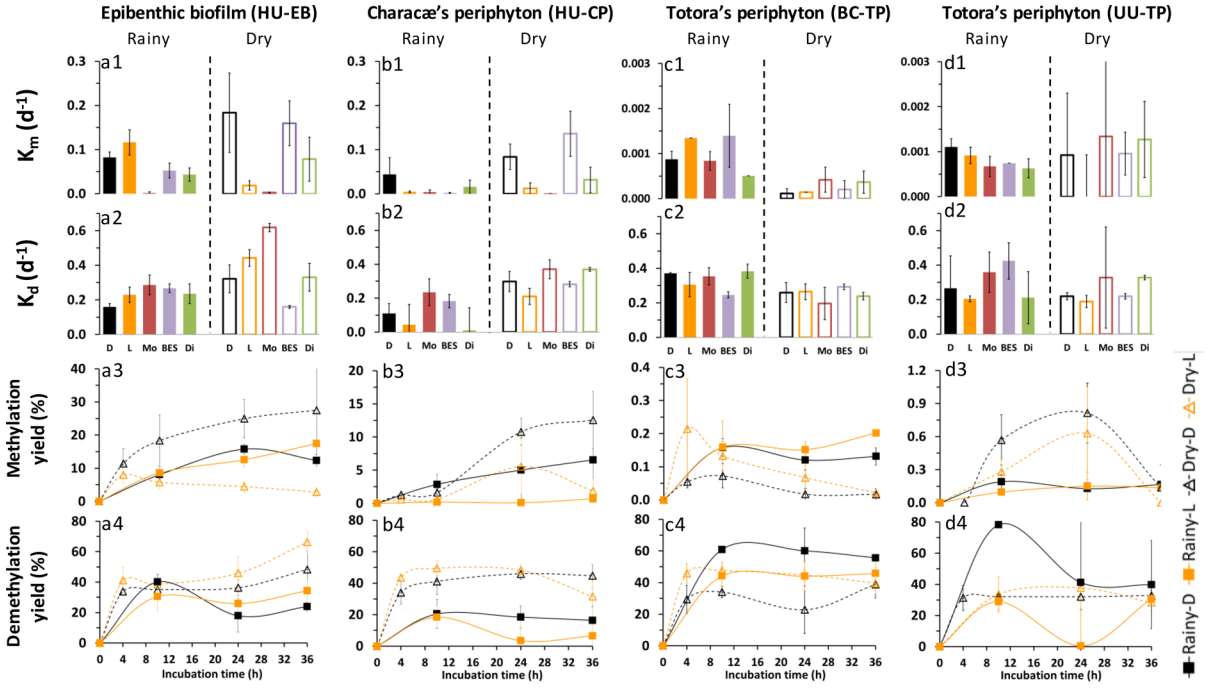

Figure 2. Upper panels: Hg methylation (K_m) and demethylation (K_d) rate constants in epibenthic biofilms from HU, Characeae periphyton from HU and totora periphyton from BC and UU under various conditions (dark (D), ambient light (L), and additions of molybdate (Mo), bromoethanesulfonate (BES), and diuron (Di)). Lower panels: time course of the methylation and demethylation reactions (as percent of added tracers) for both seasons under dark or light conditions without inhibitors. Error bars represent standard deviation from triplicate incubations.

Hg Transformations in Biofilms and Periphyton. Figure 2 presents the transformation rate constants in these compartments (upper panels) and the associated kinetics (lower panels). The highest transformation rates were found during the dry season in the epibenthic biofilm and Characeae periphyton (Figure 2a1,b1). Under dark conditions, K_m values were 0.04 and 0.08 day^{-1} for the periphyton and 0.08−0.18 day^{-1} for the biofilm during the rainy and the dry season, respectively. Under light conditions, rate constants were reduced by 30−90%, except in the biofilm during the rainy season, in which it rose by 40%. The methylation kinetics exhibited various patterns (Figure 2a3,b3), mostly pseudo-first-order reaction in biofilms (BoxLucas fitting, p values of <0.05 except for light conditions during the dry season) with a sharp start followed by a plateau or even a decline, whereas it was delayed in Characeae periphyton and only started after 4 or 10 h with no specific pattern. It suggests that methylation in biofilms is eventually limited by the availability of the added IHg or by the onset of MMHg degradation, while in the periphyton, the bacterial community structure was more critical, as discussed below (see the Microbial Activities section).

Mo and BES always decreased methylation compared with the results in dark conditions with the exception of BES in periphyton during the dry season. The large and constant inhibition by Mo (91−99%) indicates SRB as the main methylators in these compartments. BES only induced a relatively weak to moderate effect in biofilms (13−37% decline), demonstrating that methanogens were not the main methylators but were still involved or indirectly support methylation by SRB.[55] Its effect is in great contrast to that in Characeae periphyton: 95% inhibition during the rainy season but 60% increase during the dry one, indicating a large seasonal shift of their implication as for sediments. Di overall increased methylation compared with results in light conditions (150−300%) except in the biofilm during the rainy season (60% decrease), suggesting that the photosynthetic organisms present (mainly *Chlorophytes* and *Diatoms*) restrained the methylation.

Demethylation rate constants exhibited a wide range across conditions and seasons (0.01 to 0.62 day^{-1}; Figure 2a2,b2) but still less than the methylation ones. For biofilms, K_d averaged 0.24 ± 0.05 and 0.37 ± 0.17 day^{-1} during the rainy and the dry season, respectively, while for Characeae periphyton, it was 0.12 ± 0.09 and 0.31 ± 0.07 day^{-1}. Similarly to methylation, demethylation was thus higher during the dry season, while differences between dark and light conditions were limited. The effect of BES and Di were relatively limited, whereas Mo always increased it (24−115%), indicating that demethylation is not coupled to sulfate reduction in this ecosystem. Demethylation kinetics were variable, following pseudo-first-order reactions for all biofilms except during rainy dark conditions and for Characeae periphyton only during the dry season (BoxLucas fitting, p values of <0.05). It points to the MMHg concentration as a limiting factor for the reaction only in some cases and also highlights the typical variability of such experiments.

Very little or no methylation was found in periphyton associated with totoras located in BC or UU (Figure 2c1,d1), with maximum K_m values of about 1.10^{-3} day^{-1} (DL = 4.10^{-4} day^{-1}). However, demethylation was comparable with results from the other compartments investigated (K_d = 0.19−0.42 day^{-1} and DL = 0.07 day^{-1}; Figure 2c2,d2). Again, the demethylation kinetics were variable, following pseudo-first-order reactions for all periphyton from BC but only during the dry season for periphyton from UU. Totora periphyton contain relatively high concentrations of ambient Hg, averaging 12 and 55 $ng{\cdot}g^{-1}$ dry weight in BC and UU, respectively (Table SI1), of which MMHg makes a significant proportion (57 and 12%, respectively). These periphyton therefore efficiently accumulate MMHg produced elsewhere and likely passes it up to the food web(s) given their large contribution to fish diets in this ecosystem.[42,56,57]

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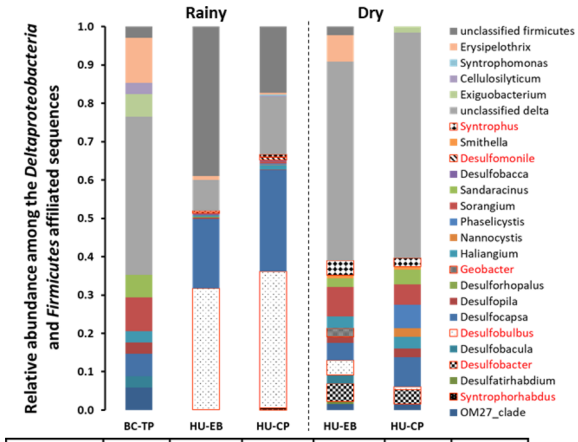
To summarize, methylation rate constants span 3 orders of magnitude, increasing from 1.10^{-3} in totora periphyton to 1.10^{-2} in sediments and 1.10^{-1} day^{-1} in Characeae periphyton and epibenthic biofilms. Both K_m and K_d values (and their responses to inhibitors) are comparable with previous studies on Hg transformations in productive sites from temperate cold[18] or (sub)tropical ecosystems.[13,41,58] Even though the homogenization of samples for incubations induces bias in rate assessments,[20] our data overall demonstrate that methylation can take place in many compartments of this ecosystem even if there is a large variability among them. Net methylation rates ranged from 0.2 to 8.8 and −0.5 to 12.1 ng g^{-1} of dry weight day^{-1} in sediments and biofilms, respectively, demonstrating that these compartments are mostly net contributors of MMHg to the ecosystem. However, they were either slightly positive or clearly negative for periphyton associated with Characeae and totoras (−0.3 to 0.4 and −0.7 to −3.8 ng g^{-1} of dry weight day^{-1}, respectively), the latter therefore being mostly a sink for MMHg. Further investigations are required to estimate rates more precisely, the contribution of each compartment to the bioavailable MMHg pool and potential shifts according to the ecosystem state, such as Hg pollution, eutrophication, and warming.[40]

Bacterial Diversity in Biofilms and Periphyton. In biofilms and Characeae periphyton, bacteria related to Proteobacteria, Cyanobacteria, and Bacteroidetes dominated the active microbial communities (Figure SI3). Totora periphyton from BC was clearly dominated by Chloroplasts followed by Bacteroidetes, Verrucomicrobia, and Proteobacteria, whereas Cyanobacteria were almost absent. The species richness was higher during the rainy season (S ranging from 110 to 144 versus 77−79 during dry season), and their distribution was more even (*J* from 0.51 to 0.78 versus 0.33, respectively). Deltaproteobacteria and Firmicutes were under-represented in totora periphyton, accounting for only about 0.6% of the total community (Figure 3), and a



Firmicutes	0.1±0.2	9.4±2.4	3.7±0.5	0.2±0.03	0.1±0.1	(%)
Deltaproteo	0.5±0.3	14.7±1.1	17.6±1.1	2.4±0.4	2.6±0.2	(%)

Figure 3. Taxonomical affiliation of DNA sequences related to Firmicutes and Deltaproteobacteria (sample code: station-sample). Red-highlighted genera are those for which mercury methylating strains have been described. Relative abundances of the Firmicutes and Deltaproteobacteria within the community (average percentages ± standard deviation) are given in the table below the figure.

closer look at the DNA sequence affiliation reveals the complete absence of sequences related to known methylating bacteria in these groups. Furthermore, the *hgcAB* genes were not detected in totora periphytons but were successfully amplified in the Characeae periphyton and epibenthic biofilm (Figure SI4). This clearly demonstrates that the absence of methylation in the former resulted from a lack of methylating microorganisms.

On the contrary, Deltaproteobacteria and Firmicutes were abundant in biofilms and Characeae periphyton during the rainy season (3.7−17.6%, Figure 3) but less so during the dry one (0.1−2.6%). Interestingly, distribution of genera were more similar within seasons than compartments. Among them, OTUs related to the *Desulfobulbus* genus clearly dominate during the rainy season (32−36%), while the relative abundances of *Desulfobacter*, *Desulfobulbus*, *Geobacter*, and *Synthrophus* were similar during the dry one (up to 5% each). The abundances of both Deltaproteobacteria and Firmicutes and genera containing known methylators were higher during the rainy season, while the methylation was lower. Therefore, the extent of Hg methylation in these compartments cannot be predicted by the relative abundances of these genera beyond an "on−off" response.

MMHg Production Explained by Intertwined Microbial Activities and Extracellular Ligands. Given the significant levels of Hg methylation observed during the rainy season (first campaign) in biofilms and Characeae periphyton, we specifically investigated the activities of potential methylators and LMW thiol dissolved concentrations during the dry season (second campaign) to disentangle their respective role in MMHg production.

Microbial Activities. Figure SI5 presents the relative abundances of active genera among Deltaproteobacteria and Firmicutes during biofilm and periphyton incubations. In both compartments, genera belonging to *Desulfobacterales* and *Myxococcales* dominated if unclassified are omitted. *Desulfobacter* and *Desulfobulbus* clearly prevailed in Characeae periphyton, while the distribution was more equilibrated between *Desulfobacter, Desulfobulbus, Desulfomicrobium*, and *Syntrophus* in the biofilm. The communities' structures were stable along incubations, and no major changes of genera occurred within 36 h, even though their relative abundances varied slightly. Remarkably, no genera containing known methylators were detected to be active at t_0 in Characeae periphyton, with *Desulfobacter* and *Desulfobulbus* developing only after 4 h, explaining the lag in methylation observed in this compartment, as mentioned before. Regarding inhibitors, it is noteworthy that Mo only slightly modified the communities' structures but did not suppress SRB activities in biofilms or in periphyton, while the methylation was inhibited by more than 90% in both cases. SRB either switched to another respiration mode or to fermentation upon Mo addition, which is confirmed by the absence of H_2S production (Table 1). However, the effect of BES and Di are contrasted, either enhancing or decreasing the relative abundance of the genera containing methylators in biofilms and periphyton but without a clear pattern with respect to methylation.

The time courses of the combined relative abundances of cDNA affiliated within genera known to host methylators are shown in Figure SI6a. Unfortunately, errors associated with these measurements are large and trends between compartments or dark and light conditions were not significant (Mann−Whitney test, *p* values of >0.05). The methylation level of each compartment, either in the high, intermediate, or low ranges, is thus essentially set by the abundance of active genera, but the latter does not explain the variation of methylation observed within the compartments (Figure SI6b: R^2 = 0.26 and 0.10 in biofilm and periphyton, respectively, none of them significant, *p* value of >0.05).

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Table 1. Concentrations of Extracellular Dissolved Sulfides (μmol L^{-1}) and Low-Molecular-Weight Thiols (nmol L^{-1}) Measured in Incubation Experiments of Epibentihic Biofilm and Characeae Periphyton[a]

			strict biotic origin							biotic and abiotic origins							
	condition duration		H2S	CYS	HCYS	CYSGLY	NACCYS	GSH	CYST	2MPA	3MPA	MAC	ETH	SUC	PEN	NACPEN	GLYC
	t0	0		1140 ± 130	48 ± 8	17 ± 15	14 ± 5		17 ± 9		76 ± 14		414 ± 264	5 ± 2	56 ± 4	11 ± 1	22 ± 25
		4	6.1 ± 0.2	632 ± 197	133 ± 30	30 ± 16	13 ± 5		10 ± 5	7 ± 4	92 ± 10	30 ± 40	307 ± 78	3 ± 2	29 ± 12	10 ± 1	37 ± 18
		10	46 ± 2	561 ± 47	350 ± 32	36 ± 17	14 ± 8		21 ± 9	15 ± 13	188 ± 36	59 ± 54	361 ± 471	30 ± 14	33 ± 6	12 ± 2	39 ± 22
	dark	24	298 ± 10	598 ± 96	48 ± 26	89 ± 23	8 ± 2		33 ± 8	6 ± 6	1295 ± 95	25 ± 16	259 ± 382	9 ± 3	13 ± 11	14 ± 1	15 ± 2
		36	57 ± 2	595 ± 69	40 ± 23	44 ± 18	14 ± 6		50 ± 23	10 ± 8	1729 ± 153	388 ± 257	380 ± 402	27 ± 10	10 ± 9	16 ± 1	37 ± 9
		4	12.5 ± 0.4	2649 ± 307	200 ± 21		29 ± 6		43 ± 5	16 ± 6	103 ± 11	36 ± 21			106 ± 28	47 ± 7	12 ± 8
eplbenthic blofilm	light	10	1 ± 0.02	220 ± 108	221 ± 43	48 ± 19	19 ± 4		9 ± 3	20 ± 1	58 ± 12		38 ± 14	10 ± 5	121 ± 30	25 ± 3	4 ± 3
		24	19 ± 1	478 ± 235	276 ± 68	376 ± 58	35 ± 8		30 ± 8	26 ± 4	121 ± 13	47 ± 37	21 ± 21	9 ± 2	258 ± 50	47 ± 5	10 ± 3
		36	98 ± 3	215 ± 64	54 ± 6		43 ± 18		25 ± 9	27 ± 5	194 ± 34	51 ± 40	28 ± 28	14 ± 10	308 ± 83	22 ± 1	38 ± 4
	BES dark	36	385 ± 13	668 ± 129	17 ± 1	14 ± 14	7 ± 3		102 ± 34	5 ± 3	804 ± 64	127 ± 66	812 ± 355	15 ± 1	50 ± 26	17 ± 1	40 ± 20
	Mo dark	36		7 ± 4			0 ± 0		28 ± 3	8 ± 4	14 ± 2		53 ± 67		10 ± 6	4 ± 1	22 ± 8
	Di light	36	24 ± 1		37 ± 33	15 ± 15	40 ± 11			30 ± 10	163 ± 16	42 ± 27	12 ± 16	6 ± 3	360 ± 49	15 ± 1	29 ± 1
	t0	0	2.7 ± 0.1	563 ± 73	5 ± 2	65 ± 21	62 ± 10		47 ± 18	5 ± 3	284 ± 27		60 ± 11	4 ± 2	124 ± 20	12 ± 2	
		4	0.40 ± 0.01	657 ± 267	25 ± 7	27 ± 16	68 ± 17	7 ± 1	14 ± 8		571 ± 88	30 ± 38	45 ± 22	9 ± 8	127 ± 19	21 ± 3	5 ± 4
		10	0.37 ± 0.01	617 ± 231	71 ± 15	196 ± 36	52 ± 10		16 ± 8	5 ± 4	421 ± 89		73 ± 60	4 ± 2	95 ± 50	16 ± 3	4 ± 4
	dark	24	0.45 ± 0.02	928 ± 257	10 ± 36	34 ± 17	105 ± 20	25 ± 6	14 ± 8	8 ± 7	641= ± 103	79 ± 72	29 ± 14	4 ± 22	258 ± 61	57 ± 13	13 ± 2
		36	0.95 ± 0.03	1430 ± 493	108 ± 33	10 ± 14	175 ± 45		49 ± 53	25 ± 16	883 ± 189	105 ± 26	153 ± 28	186 ± 53	250 ± 77	128 ± 27	38 ± 14
		4	0.68 ± 0.02	83 ± 29	8 ± 3	28 ± 16	81 ± 7	9 ± 1	5 ± 2		268 ± 42		53 ± 38	3 ± 2	152 ± 14	16 ± 1	
Characae periphyton	light	10	0.12 ± 0.004	184 ± 58	23 ± 4	43 ± 18	63 ± 8	15 ± 15	18 ± 13		127 + 10		18 ± 19	3 ± 2	138 ± 19	8 ± 1	5 ± 4
		24	0.17 ± 0.01	303 ± 63	52 ± 19	93 ± 24	120 ± 6	61 ± 25	6 ± 3	6 ± 2	442 ± 30	97 ± 37	26 ± 26	27 ± 6	220 ± 24	32 ± 4	
		36	0.31 ± 0.01	112 ± 31	31 ± 10	76 ± 22	103 ± 24		12 ± 8	4 ± 5	227 ± 42	121 ± 59	36 ± 26	12 ± 2	198 ± 24	21 ± 4	5 ± 4
	BES dark	36	0.56 ± 0.02	776 ± 281	91 ± 27	77 ± 22	95 ± 18	11 ± 6	15 ± 13	5 ± 2	579 ± 125	74 ± 31	115 ± 105	7 ± 7	193 ± 49	43	9 ± 6
	Mo dark	36		623 ± 274	22 ± 17		78 ± 51		6 ± 3		623 + 87	84 ± 56	46 ± 16	8 ± 4	153 + 45	43 ± 8	17 ± 12
	Di light	36	136 ± 5	584 ± 94	30 ± 10	9 ± 14	175 ± 19		32 ± 9	54 ± 2	740 ± 105	114 ± 49	131 ± 63	53 ± 21	465 ± 30	91 ± 12	28 ± 3

[a]Cells were left blank when values are under DLs.

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Extracellular Dissolved Sulfides and Low-Molecular-Weight Thiol Concentrations. H_2S was overall 2 orders of magnitude lower in periphyton (0−3 μmol L^{-1}, except Di 136) compared to biofilm (0−298 μmol L^{-1}, up to 385 with BES), indicating a much-higher sulfate reduction activity in this latter (Table 1). Over the 15 LMW thiols investigated, 13 were detected in virtually every sample, and some general patterns are worth observing: CYS and 3-MPA were clearly the dominant ones in both compartments (Figure SI7a), with average concentrations of 636 and 443 nmol L^{-1}, respectively, accounting for 37% and 26% of the total thiol concentrations. They were followed by HCYS, CYSGLY, NACCYS, MAC, ETH, and PEN (average concentrations of 87, 66, 61, 89, 151, and 155 nmol L^{-1}, respectively) and then GSH, CYST, 2-MPA, SUC, NACPEN, and GLYC (average concentrations of 21, 26, 15, 22, 30, and 20 nmol L^{-1}, respectively). The LMW thiol distributions followed similar trends between biofilm and periphyton (Figure SI7b) with the same dominant and intermediate compounds occurring in most cases (Mann−Whitney test, p values of >0.05 except for NACYS, GSH, and PEN). An appreciable difference was the complete absence of GSH in the biofilm incubations, while it was occasionally detected in the periphyton at low levels. It can be explained by the fact that the biofilm consortium is dominated by (anaerobic) bacteria that do not use GSH as an antioxidant or for metal detoxification,[59] contrary to algae, in which it is present intracellularly at millimole concentrations.[60] Moreover, the time-course evolution of thiol concentrations are strikingly similar (Figure SI8), continuously increasing under dark conditions while clearly decreasing during light periods with the exception of the biofilm at 4 h. Dark conditions were thus more favorable for thiol production and/or accumulation compared to light ones, similar to methylation.

The individual thiol concentrations found here are in the same order of magnitude than those reported recently in the dissolved fraction of biofilms,[19] pore waters,[44,61,62] and even a polluted estuary[63] but higher than in open and coastal oceans[64−66] or lake water columns.[67] However, the diversity we observed in these productive biological compartments is much higher than in previous studies, in which two to eight compounds have been detected, highlighting the importance of using a more-comprehensive and sensitive method. It is especially worth mentioning that 3-MPA is here found as a dominant compound because it was previously reported as a central intermediate in the metabolism of dimethylsulfoniopropionate, methionine, and homocysteine in anoxic sediments[68,69] but was never reported in biofilms until now to the best of our knowledge. To a lesser extent, the presence of CYST and NACPEN is also noteworthy because they have not been reported either. However, the complete absence of SULF contrasts with previous studies in which it has been detected in lake and wetland pore waters[44] along with other common thiols.

Hg Methylation vs LMW Thiols. Thermodynamic speciation modeling of our data set was unfortunately not possible because dissolved HgT and OC were not measured due to limited sample availability. Still, based on a previous modeling exercise[70] and realistic values for dissolved IHg (in the nanomolar range) and DOC (30 mg·L^{-1}), we can assume that, given the sulfide concentrations, the dissolved IHg would be dominated by (nanoparticulate) HgS_s and dissolved HgS species, while IHg complexed by DOM and LMW thiols would be a few orders of magnitude lower. However, even the best models currently available are likely not able to capture the complexity of such a system; the inhibition of HgS_s nanoparticles growth and aggregation in the presence of DOM and LMW thiols[71] is especially difficult to account for. With this in mind, it is thus interesting to observe that the concentrations of total thiols were, on average, similar in biofilm and periphyton (1.75 and 1.70 μmol L^{-1}, respectively). However, they were higher under dark conditions compared with results in light conditions and also under BES and Di compared to Mo, closely matching methylation trends. Previous studies found higher methylation rates in periphyton under light conditions and hypothesized that algae either directly methylate Hg or release metabolites (labile carbon, LMW thiols, or both), thereby enhancing bacterial activities and/or IHg bioavailability.[16,17,20] We found opposite results that can be primarily explained by the lower concentrations of LMW thiols in light conditions compared with the results in dark ones, although the production of oxygen could also have inhibited sulfate reduction and other anaerobic activities even if the microbial data are not conclusive on that point. The strong suppression of thiols production in the biofilm incubated with Mo is also especially noteworthy, suggesting it could actually be the cause of the Mo inhibitory effect given that SRB were not repressed.

Hg methylation was overall positively correlated to total LMW thiols concentration, even if data are highly scattered (Figure 4, R^2 = 0.4 for the full data set, p value of <0.005), but

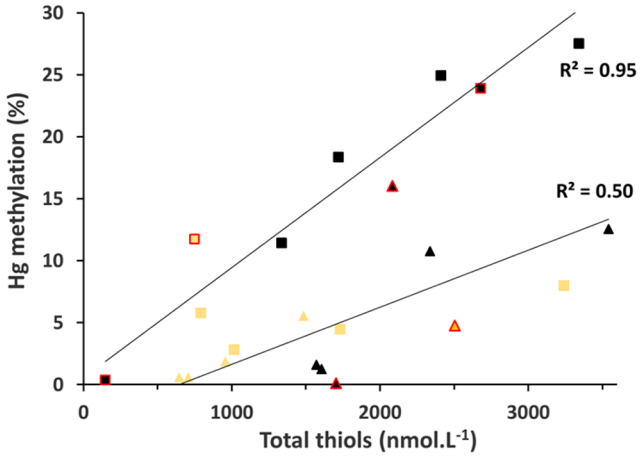

Figure 4. Relationships between Hg methylation yields and total thiol concentrations in the incubations of epibenthic biofilm (squares) and Characeae periphyton (triangles) under dark (black-filled symbols) or light (yellow-filled symbols) conditions. Red borders indicate samples with inhibitors.

each set of incubation needs to be considered individually because the conditions and kinetics are not the same. There is indeed a strong correlation in biofilm incubations under dark conditions (R^2 = 0.95), while it is less so under light ones (R^2 = 0.55, not shown), where the MMHg formed was already degraded after the first time point (4 h, Figure 2a3). These results would therefore support the role of thiols in promoting methylation through maintaining Hg in solution[72] and facilitating its uptake by methylating organisms, as demonstrated in previous lab studies.[37−39] Among the LMW thiols found here, IHg should be mainly complexed by cysteine and 3-MPA given their higher concentrations and stability constants.[62] The effect of cysteine has been extensively studied in model experiments, but 3-MPA would deserve more investigations if its environmental relevance is confirmed. However, for the Characeae periphyton, LMW thiols explained only half the variation in

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methylation (R^2 = 0.5 for the full data set, ranging from 0.45 to 0.99 for dark and light conditions, respectively; not shown). The kinetics do not reveal a large demethylation of the MMHg formed except for light conditions at 36 h, suggesting that thiols are involved in Hg methylation but that other parameters are equally important. Here, the absence of active methylating organisms at the beginning of the incubations was a major controlling factor that lowered the methylation range compared to the biofilm and obscured the potential trends between thiols and methylation.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.8b01885.

Figures showing a map of the study sites, structure of the low-molecular-weight thiols, bacterial community structures based on 16S rRNA gene affiliation, PCR amplification of *hgcAB* genes, the affiliation of cDNA reads related to Deltaproteobacteria and Firmicutes, time-course of the relative abundances, Hg methylation, distribution of extracellular H_2S and low-molecular-weight thiol concentrations, and the time-course evolution of total thiol concentrations. A table showing ancillary parameters and ambient Hg species concentrations. (PDF)

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The authors declare no competing financial interest.

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