Environmental Science & Technology

ARTICLE

pubs.acs.org/est

Methanogens: Principal Methylators of Mercury in Lake Periphyton

Stéphanie Hamelin,[*,†] Marc Amyot,[‡] Tamar Barkay,[§] Yanping Wang,[§] and Dolors Planas[†]

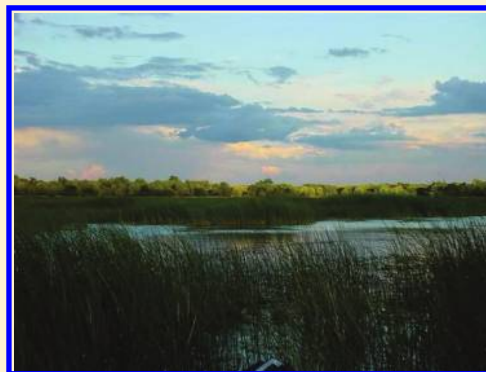
[†]GEOTOP and Groupe de Recherche Interuniversitaire en Limnologie et en Environnement Aquatique (GRIL), Université du Québec à Montréal, C. P. 8888, Succursale Centre Ville, Montréal, Québec, Canada H3C 3P8

[‡]Université de Montréal, Groupe de Recherche Interuniversitaire en Limnologie et en Environnement Aquatique (GRIL), Département des sciences biologiques, C.P. 6128, Succursale Centre-ville, Pavillon Marie-Victorin, Montréal, Québec, Canada, H3C 3J7

[§]Department of Biochemistry and Microbiology, Rutgers University, 76 Lipman Drive, New Brunswick, New Jersey 089015

S *Supporting Information*

ABSTRACT: Mercury methylation and demethylation rates were measured in periphyton biofilms growing on submerged plants from a shallow fluvial lake located along the St. Lawrence River (Quebec, Canada). Incubations were performed in situ within macrophytes beds using low-level spikes of ^{199}HgO and $Me^{200}Hg$ stable isotopes as tracers. To determine which microbial guilds are playing a role in these processes, methylation/demethylation experiments were performed in the absence and presence of different metabolic inhibitors: chloramphenicol (general bacteriostatic inhibitor), molybdate (sodium molybdate, a sulfate reduction inhibitor), BESA (2-bromoethane sulfonic acid, a methanogenesis inhibitor), and DCMU (3-(3,4-dichlorophenyl)-1,1 dimethyl urea, a photosynthesis inhibitor). Active microbes of the periphytic consortium were also characterized using 16S rRNA gene sequencing. Methylation rates in the absence of inhibitors varied from 0.0015 to 0.0180 d^{-1} while demethylation rates ranged from 0.083 to 0.217 d^{-1}, which corresponds to a net methylmercury balance of −0.51 to 1.28 ng gDW periphyton^{-1} d^{-1}. Methylation rates were significantly decreased by half by DCMU and chloramphenicol, totally inhibited by BESA, and were highly stimulated by molybdate. This suggests that methanogens rather than sulfate reducing bacteria were likely the primary methylators in the periphyton of a temperate fluvial lake, a conclusion supported by the detection of 16S rRNA gene sequences that were closely related to those of methanogens. This first clear demonstration of methanogens' role in mercury methylation in environmental periphyton samples expands the known diversity of microbial guilds that contribute to the formation of the neurotoxic substance methylmercury.



INTRODUCTION

Mercury (Hg) methylation is a key process in the understanding of the Hg biogeochemical cycle. Once produced in an aquatic system, methylmercury (MeHg), the neurotoxic form of Hg, is bioaccumulated in organisms and bioamplified through food webs. In freshwater systems, it is generally accepted that Hg methylation takes place in deep and littoral sediments,[1−3] and in anoxic hypolimnions of lakes.[4] However, some studies have also demonstrated the ability of periphyton to methylate Hg.[5−9] For instance, in the Everglades and Brazil, periphyton methylated Hg at higher rates than sediments,[8,10] which can be explained by the higher microbial biomass per gDW in periphyton than in sediments. MeHg concentrations generally account for 3 to 50% of total Hg (THg) measured in wetland periphyton (Hamelin et al., unpublished data). High Hg methylation (MHg) rates and MeHg concentrations in periphyton could lead to high MeHg concentration in fish, since periphyton is a more direct food source than sediment microbes for benthic primary consumers.[11−13] Considering the rapid turnover of the periphytic biofilm organisms,[14,15] the amount of Hg that can be transferred to the top of the food chain could be very high.

Differences between biofilm microbial composition and MeHg concentrations in the ingested food could partly explain the marked differences observed between mercury levels in grazers from different aquatic systems.[16] In aquatic environments, mercury methylation is generally ascribed to the activity of anaerobic bacteria, mainly to sulfate-reducing bacteria (SRB). This conclusion principally rests on the common observation that inorganic Hg methylation rates declined when sediments were treated with molybdate, a specific inhibitor of sulfate reduction.[17−19] Even in periphyton, SRB have been shown to play an important role in mercury methylation.[5,7,8] However, recent studies reported that other microorganisms, namely iron reducing bacteria, may methylate inorganic Hg.[20,21]

In this study, our objectives were the following: (1) to measure Hg methylation and demethylation rates by periphyton in the wetland area of a large shallow fluvial lake, using stable isotope tracers; (2) to identify the main methylators by the addition of metabolic inhibitors; and (3) to characterize the active microbes in the periphytic biofilms using 16S rRNA gene sequencing. In most studies where MHg or demethylation (DHg) rates were

Received: March 25, 2011
Accepted: August 15, 2011
Revised: July 17, 2011
Published: August 29, 2011

 © 2011 American Chemical Society

related to the composition of the microbial community, organisms previously known as Hg methylators, like SRB, were the only ones targeted by molecular probes.[7] Here, the whole community RNA, rather than DNA, was used and thus, mostly microbes that were metabolically active were detected. Results of metabolic inhibitor experiments, together with the molecular characterization of a large sprectrum of periphyton microorganisms, suggests for the first time a role for methanogens in periphyton mercury methylation.

■ MATERIALS AND METHODS

Study Site. The study took place in aquatic meadows of Lake St. Pierre, a widening of the St. Lawrence River (46° 09′ 82″ N; 72° 59′ 10″ W) between Sorel and Trois-Rivières, Québec, Canada. More than half of this fluvial lake (total area of 375 km^2) is covered by macrophyte beds and wetlands. The dominant macrophyte species observed in our sampling site were *Potamogeton perfoliatus* (Perfoliate pondweed), *Elodea canadensis* (Canadian waterweed), *Scirpus fluviatilis* (River bulrush), and *Thypha angustifolia* (Narrowleaf cattail).

Sampling. All sampling devices and tools for Hg measurements were carefully acid-washed and rinsed with nanopure water. Sampling was carried out at the end of summer, when macrophytes densities and water temperatures were high (mid-August 2004). A total of fifteen field replicates (3 per inhibitor treatment and control) of submerged macrophytes and their associated periphyton were sampled using 0.68-L Pac-man boxes (a smaller cylindrical version of the 6-L Downing box,[22] modified by C. Vis, Centre de Service de l'Ontario, Parcs Canada) at 30−45 cm depth. Care was taken during sampling to minimize losses and disturbance of periphyton. Physicochemical characteristics of the water (pH, light, temperature, and dissolved oxygen) were measured in situ with specific probes (Chekmite 4 pH meter, Biospherical QSL-101 quantum meter, WTW Oxi340 oximeter), and water grab samples for DOC, THg_{water}, $MeHg_{water}$ and major ions were also taken once within macrophytes beds at the same depth and the same time that periphyton/macrophytes sampling was conducted. Water chemistry data and details on laboratory analyses for these last samples are presented in the Supporting Information.

Methylation/Demethylation Assays. Filtered lake water (0.20-μm porosity) was spiked with ^{199}HgO and $Me^{200}Hg$ (Oak Ridge National Laboratory) at 3 ng·L^{-1} each, and preincubated for 1 h to allow equilibration with dissolved ligands. Thereafter inhibitors were added before the addition of periphyton−macrophyte complexes (7−10 g DW). Entire macrophytes with their associated biofilm were incubated in situ in clear polycarbonate bottles (total incubation volume of 2 L) within macrophyte beds. Polycarbonate was chosen because this material has optimal light transmittance with minimal Hg and MeHg sorption to container walls.[8,23] All the treatments contained periphyton−macrophytes complexes and ^{199}HgO and $Me^{200}Hg$, and included (1) control (without any inhibitor); (2) BESA (with addition of 5 mM 2-bromoethane sulfonic acid, a methanogenesis inhibitor); (3) DCMU (with addition of 10 μM 3-(3,4-dichlorophenyl)-1,1 dimethyl urea, a photosynthesis inhibitor); (4) molybdate (with addition of 20 mM sodium molybdate, a sulfate reduction inhibitor), and (5) the general bacteriostatic inhibitor chloramphenicol (0.2 mM).[5,24,25] Total incubation time was 48 h, but every 12 h (at dawn and twilight), three replicate bottles were sacrificed by adding 8 mL of 4 N HCl and samples were stored in the dark at 21−23 °C (depending on in situ water temperature). Once in the laboratory, periphyton was separated from macrophytes by mechanical shaking (9 min in a Red Devil paint shaker), a method we had previously tested for removing periphyton efficiently without destroying algal cells. The periphyton suspension was then split in 3 × 100-mL aliquots for each measurement (biomass, THg, MeHg, MeHg stable isotopes, community characterization). MeHg stable isotopes samples were analyzed at Trent University (Dr. Hintelmann's Laboratory), Ontario, Canada. The formation and degradation of MeHg were determined by monitoring the concentrations of the respective isotopes $Me^{199}Hg$ and $Me^{200}Hg$ by gas chromatography−inductively coupled plasma mass spectrometry, following protocols in Hintelmann and Ogrinc.[26] To calculate MHg and DHg rate constants, we used the following equations:[27]

$$\text{NetMeHgproduction} = K_m[Hg^{2+}] - K_d[MeHg^+] \quad (1)$$

where K_m = specific MHg rate constant (in d^{-1}) and K_d = specific DHg rate constant (in d^{-1}). K_m and K_d were calculated from the initial part of the slope of the relation (first 12 h) between isotopic [$MeHg^+$] and time when [$Me^{199}Hg^+$] and [$^{200}Hg^{2+}$] were low enough to simplify eq 1 to

$$K_m = [Me^{199}Hg^+]/(t[^{199}Hg^{2+}]_0) \quad (3)$$

$$[Me^{200}Hg^+] = [Me^{200}Hg^+]_0 e^{-Kdt} \quad (4)$$

where $[Me^{200}Hg^+]_0$ and $[^{199}Hg^{2+}]_0$ are the initial substrate concentrations added to the sample in ng g^{-1}. K_d was obtained by linear regression of ln[$Me^{200}Hg^+$] versus time (t) in days. As the bioavailability of stable isotopes added is probably higher than Hg found in the natural waters, these rates are considered maximum potential rates.

Biomass Determinations. Four 100-mL aliquots of periphyton suspension were filtered on precombusted and preweighed GF/C and kept at −80 °C until analysis. For chlorophyll-α, the extractions were done with hot ethanol.[28] Dry weight was measured by drying filters (45 °C) to constant weight.[29] Identification of algae phototrophes to species level in Lugol's solution was done using an inverted microscope (Leica DMIRB), following Utermöhl's method.[30]

THg and MeHg Analyses. Prior to THg and MeHg analysis, periphyton samples were freeze-dried and weighed. THg concentrations were measured by thermal decomposition using a direct mercury analyzer (DMA 80; Milestone, MLS). From 0.05 to 0.10 g of samples were dried, combusted, and further decomposed on a catalytic column at 750 °C. Mercury vapors were collected on a gold amalgamation trap and subsequently desorbed by heat and then measured by atomic absorption spectrometry at 253.7 nm.[31,32] Samples for MeHg analysis were freeze-dried, weighed, and extracted in KOH/methanol (25%), and extracts were analyzed by cold vapor atomic fluorescence (CVFAS).[33,34] The working detection limit was 0.01 ng g^{-1} for Hg and 0.1 ng g^{-1} for MeHg (three times the standard deviation of 10 procedural blanks). Blanks and certified reference material (TORT-2, SO-2, and IAEA, CNRC) were analyzed every 10 samples to ensure reproducibility and to assess QA/QC. Hg concentrations in reference material varied slightly over time (CVFAS: TORT-2 CV = 1.7%; DMA: TORT-2 CV = 2.2%, SO-2 CV = 1.7%, and IAEA CV = 2.2%) but were not significantly ($p > 0.05$) different from certified values. No substantial Hg contamination was detected in the blanks.

Algal and Microbial Production. In parallel to MHg/DHg measurements, determination of algal and microbial production was performed by incubating periphyton/macrophytes complexes during 48 h (with subsampling every 12 h) in order to assess the efficiency of inhibitors in controlling periphyton metabolic activity. Algal primary production was measured by oxygen (O_2) production directly in the bottles before and after MHg/DHg incubations (YSI model 59, precision of $\pm$0.01 mg $O_2 \cdot L^{-1}$). Net primary production was calculated by subtracting initial O_2 concentration measured at time zero from the O_2 concentration measured after incubation. Community respiration was measured by the O_2 consumption in dark bottles kept under the same conditions as clear ones. For gross primary production calculation, community respiration was added to the net O_2 production in the clear bottles.[35]

Production by the entire microbial community was estimated from ^{3}H-thymidine incorporation.[36,37] Assumption was made that most of the bacteria and archaea were able to incorporate thymidine. One formaldehyde-sterilized control and three replicate subsamples from MHg/DHg bottles were spiked with 5 nM thymidine (specific activity = 85 μCi nmol^{-1}) and incubated for 30 min in glass vials. Incubations were conducted in a dark insulated box containing water from the sampled station. At the end of the incubation, thymidine incorporation was stopped using formaldehyde (2% v/v final concentration). Cells were collected on 0.2-μm Gelman polycarbonate filters, incubated for 10 min, and rinsed twice with 5 mL of cold 5% trichloroacetic acid. Filters were stored at 4 °C and analyzed later by a liquid scintillation counter (Beckman LS1801, Beckman Instruments).

Microbial Community Characterization. Biofilm RNA was chosen rather than DNA in order to identify which microbes from the biofilm were actively metabolizing and thus could have played a role in MHg/DHg processes. The validity of this approach rests on the fact that active microbes contain more rRNA than inactive ones.[38] The methods for RNA extraction, DNase treatment, and end-point reverse transcription (RT)-PCR were used as described previously.[39] Briefly, total RNA from the periphyton samples was extracted using the DNAeasy Kit (Qiagen). The extracted crude RNA samples were then diluted to 50 μg$\cdot$mL^{-1} for DNase treatment with the TURBO DNA-free kit (Applied Biosystems) according to the manufacturer's instructions. The DNase-treated RNA samples were then diluted to 10 μg$\cdot$mL^{-1} and 1 μL (10 ng) of each RNA sample was used as a template in each RT-PCR reaction, by following the protocol for the Access RT-PCR System kit (Promega life science). For bacterial sequences, PCR amplifications of the 16S rRNA gene were carried out with primers 27f and 519r,[40] whereas for archaean sequences, amplifications were carried out with primers 344f and 907r.[41,42] Control reactions did not include RT. Reactions were incubated at 45 °C for 45 min for reverse transcription to produce the first-strand cDNA, followed by 94 °C for 2 min to denature the RT, and followed by 40 amplification cycles of 94 °C for 30 s, 53 °C for 20 s (for bacteria), or 48 °C for 30 s (for archaea), 68 °C for 1 min, and final extension at 68 °C for 10 min.

Six clone libraries for the three most interesting treatments—control, molybdate, and BESA (33 clones in each library)—were constructed with the PCR products of 16S rRNA genes of bacteria and archaea, individually. The 16S rRNA gene PCR product of each sample was extracted from 1% agarose gel by using the QIAquick Gel Extraction Kit (Qiagen Sciences, MD), and was then cloned into pGEMT-easy vector (pGEMT-easy Vector System, Promega, Madison, WI) according to the manufacturer's instructions. The ligation mixture was transformed into *Escherichia coli* DH10B competent cells (Invitrogen, USA), and transformants were selected on LB (Luria broth) agar plates supplemented with ampicillin (100 μg$\cdot$mL^{-1}) and X-gal (40 μg$\cdot$mL^{-1}). White colonies were picked and inoculated in LB broth containing ampicillin, and plasmids were extracted from these cultures to screen for insert-containing clones. For restriction fragment length polymorphism (RFLP) analysis, the clones were digested with *Eco*RI and *Hae*III and run on 1% agarose gel. At least three clones of each RFLP pattern were chosen; in total 31 clones were sent to Genewiz (South Plainfield, NJ) for sequencing.

The resulting sequence from each clone was used as query in searches performed using BlastN (National Center for Biotechnology Information, Bethesda, MD) and myRDP (Ribosomal Database Project II, East Lansing, MI) to identify the sequences most closely related to the periphyton 16S rRNA genes. When more than one sequence was found as top hits for a single sequence, the highest common taxonomic rank shared by all hits was chosen as the most closely related to the environmental clone. In the case that a common rank did not exist among the hits, we eliminated those that, based on their known characteristics (e.g., extreme halophiles, thermophiles, or acidophiles), were not likely to be found in the studied periphyton. The sequences have been deposited in GenBank under accession numbers HQ848523−HQ848553.

RESULTS

Periphyton Biomass, Total and Methylmercury Concentrations. The algal community was mainly dominated by diatoms (*Coconeis placentula*, *Navicula radiosa*, *Fragilaria capucina*), with few chlorophytes (*Stigeoclonium nanum*, *Protoderma viride*, *Spyrogyra* sp.) and cyanophytes (*Oscillatoria tenuis*, *Coelosphaerium kuetzingianum*).

Periphyton biomass varied from 1.5 to 4.8 mg DW gDW of macrophytes^{-1} and algal biomass represented 678−773 μg Chl-α gDW of macrophytes^{-1}. Mean THg and MeHg concentrations in periphyton were 88 $\pm$ 30 and 3.9 $\pm$ 0.3 ng gDW^{-1} respectively.

Mercury Methylation and Demethylation Rates. In the absence of inhibitors, methylation rates (K_m) varied from 0.0015 to 0.0180 d^{-1} while demethylation rates (K_d) ranged from 0.083 to 0.217 d^{-1}. A mass balance between MHg and DHg was calculated considering in situ [THg] and [MeHg]; we obtained a net variation of MeHg from −0.51 to 1.28 ng gDW periphyton^{-1} d^{-1}. These theoretical variations of MeHg are consistent with the seasonal variations in [MeHg] measured in situ (Hamelin et al., manuscript in preparation).

Effect of Inhibitors on Primary Production and Microbial Production. Net primary production without inhibition was 0.14 $\pm$ 0.01 mg O_2 gDW^{-1} h^{-1}. DCMU totally suppressed periphytic photosynthesis while the other inhibitors did not affect algal metabolism (Figure 1A). Microbial thymidine incorporation in control treatment was 6.15 $\pm$ 0.80 $\times$ 10^{-15} mole thymidine gDW^{-1} h^{-1}. BESA and chloramphenicol significantly decreased microbial heterotrophic production by 50% while molybdate stimulated it by 20% (Figure 1B).

Effect of Inhibitors on Mercury Methylation and Demethylation Rates. DCMU and chloramphenicol significantly decreased K_m by half in periphyton and the addition of BESA severely inhibited it by nearly 100% (Figure 1C). In contrast, molybdate addition enhanced methylation rate up to 45 fold

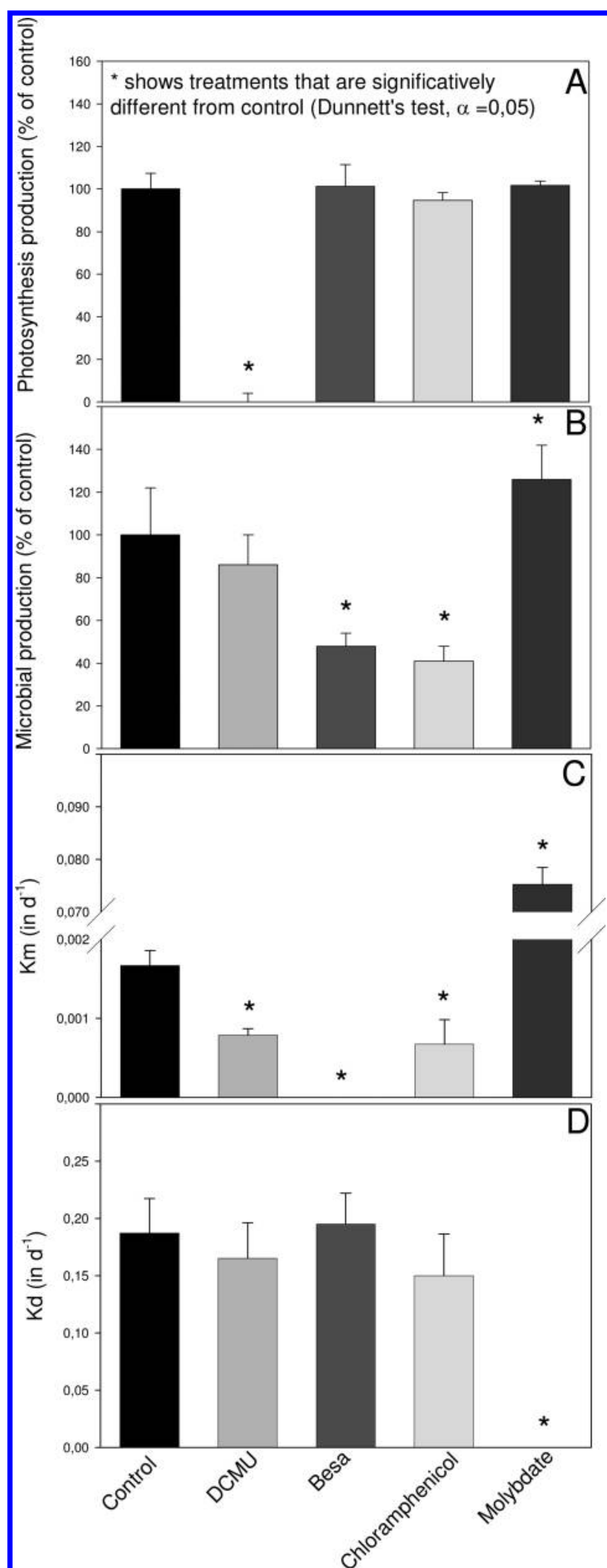


Figure 1. Effect of inhibitor addition (mean ± standard error) on epiphytic community as compared to control treatment during 48 h incubation: (A) photosynthesis production, (B) microbial production, (C) mercury methylation rate constants (K_m d^{-1}), and (D) mercury demethylation rate constants (K_d d^{-1}). * shows treatments that are significantly different from control (Dunnett's test, a = 0.05).

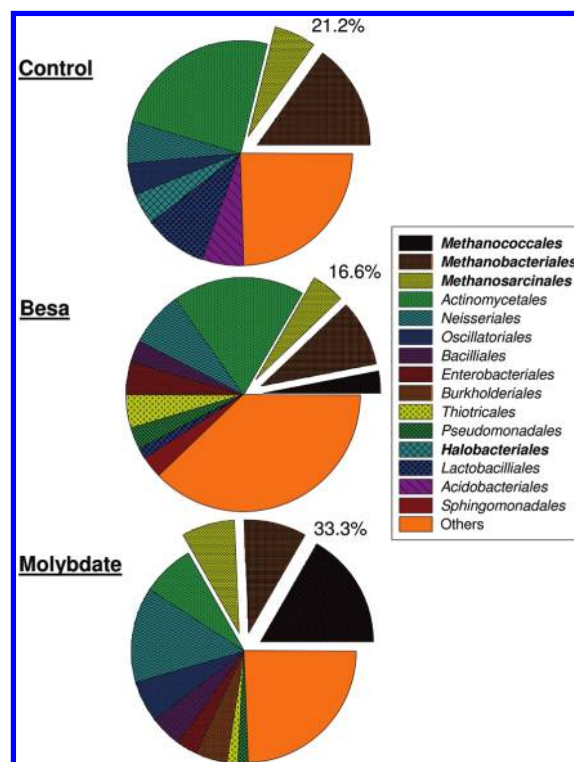


Figure 2. Proportion of each taxa representing active bacteria and archaea in periphyton samples from MHg/DHg incubations with and without metabolic inhibitors. Clone identification is based on DNA sequences and groups established by RFLP patterns. The libraries obtained with primer sets targeting archaeal (344f/907r) and bacterial (27f/519r) were combined for presentation of each treatment results. The decaled portions and the associated percentage correspond to the methanogens. Archaeal orders are denoted by bold italic font and bacterial orders are denoted by italic font only.

relative to unsupplemented controls (Figure 1C). Molybdate, the only addition to affect demethylation rates, completely suppressed it (Figure 1D).

Characterization of the Active Microbial community. Groups of active bacteria and archaea were identified at the end of the MHg/DHg experiments, for the treatments with BESA, molybdate, and for the control. From the 16S rRNA clone libraries, we obtained 9−12 sequences per treatment. Because of the low number of clones that were sequenced, these sequences represent highly dominant active taxa in the periphyton community (Figure 2). Sequences representing taxa known to include Hg methylators such as those of the *Deltaproteobacteria*, were not detected in any of the libraries. On the other hand, sequences most similar to those of methanogens were present in the three archaean libraries. The control included 2 sequences most similar to those of methanogens (Methanococcales and Methanobacteriales), while BESA and molybdate treated peripyton included three such sequences (Methanobacteriales, Methanococcales, and Methanosarcinales).

■ DISCUSSION

The most important result reported here is the clear evidence for methylation by methanogens in environmental samples. This conclusion is based on the observation that methylation was totally inhibited by BESA (inhibitor of methanogenesis) and

highly stimulated by molybdate (inhibitor of sulfate-reduction) and is strongly supported by the detection of transcripts of 16S rRNA genes most closely related to those of methanogenes in the active periphyton community. The evidence that methanogens methylate Hg in periphyton closes a circle in methylation research which started in the 1960s by showing that Hg was methylated by extracts of methanogens.[43] Subsequently, many studies with pure cultures and environmental incubations contributed to the broadly accepted paradigm that SRB are the principle Hg methylators.[17,44] Our report brings us back to the conclusion that methanogens in some environments methylate Hg. Together with observations that Fe-reducing bacteria methylate Hg,[20,21] the findings reported here call for a change in our view of methylation from a process attributed to a single microbial guild, SRB, to a process that may be carried out by several guilds of anaerobic microbes. This change would necessitate studies on the microbiological and environmental factors that determine which group methylates under which conditions.

Mercury Methylation and Demethylation Rates in Periphyton. K_m rates in Lake St. Pierre periphyton were slightly lower than those measured by Desrosiers et al. (0.096−1.224 ngMeHg·d^{-1})[5] for epilithon of boreal shield lake, but they were similar to the ones obtained with epiphytes from Florida wetlands (0.2−20%),[6] and higher than values observed with epiphytes from a Wisconsin oligotrophic lake (0.011−0.062%).[45] Microbial community composition and biofilm's structure must be important factors controlling MHg. In Desrosiers et al.,[5] periphyton growing on artificial Teflon substrates were used, whereas in the Everglades[6] macrophytes were chopped in pieces. In this study, epiphytes were collected on natural substrata (macrophytes) and incubations were done without altering the biofilm structure (e.g., without cutting macrophytes into pieces or separating biofilms from their subsrate). In Wisconsin, the periphyton was scraped from the substrate prior to incubation,[45] which may have destroyed the integrity of the mat structure. The multiple layers within periphyton matrix must influence K_m and K_d rates as more layers in the mat may enable a redox gradient, thus creating more niches for different microbial groups to coexist and participate in MHg processes.

The K_ds measured here were similar to the ones reported by Mauro et al. (nd−20%)[6] with dense macrophyte-associated periphyton from highly eutrophied zones of the Everglades. However, our K_ds were up to 35 times higher than the ones measured by Korthals and Winfrey (0.62−1.28%)[45] with periphyton from an oligotrophic lake in Wisconsin. Productivity of the system must be a key factor controlling periphyton net DHg rates and MeHg accumulation.[43]

Effect of Metabolic Inhibitors on Mercury Methylation and Demethylation. Recent experiments using DCMU and chloramphenicol with periphyton have reported a partial decrease of MHg as compared to control samples.[5,8] Here, these inhibitors decreased MHg rates by half, suggesting either direct or indirect contribution of prokaryotes and phototrophs to MHg processes. For instance, algae could directly methylate Hg,[46] or they could indirectly promote Hg methylation by releasing metabolites involved in redox reactions in the biofilm matrix thereby increasing bioavailable Hg concentrations for MHg.[47] They could also excrete more algal organic carbon that would fuel microbial metabolism and thus stimulate Hg methylation. As the inhibition of photosynthesis by DCMU did not affect microbial production, the hypotheses of either the direct effect and/or of controlling Hg bioavailability seem the more plausible.

Partial inhibition by chloramphenicol was also observed in other studies with similar inhibition experiments.[8,48] Chloramphenicol is a broad-spectrum prokaryotic inhibitor of bacterial protein synthesis and is known to inhibit methanogens.[49] However some bacteria, even some strains of SRB methylators, are resistant to this chemical.[50]

By treating incubations with BESA (which inhibits coenzyme M activity), we completely suppressed MHg, and by using molybdate, we enhanced MHg 45 fold. As SRB are strong competitors for the same sources of energy, their inhibition by molybdate would stimulate other microbial guilds, which suggest that methanogens played an important role in mercury methylation of the studied periphyton. Former studies that investigated methanogens and SRB involvement in MHg[51] found a slight inhibition after BESA addition. Compeau and Bartha found also a strong inhibition with molybdate, and they concluded that methanogens played an indirect role and/or that SRB were more efficient in the methylation processes.[17]

In this study, we observed a large increase in mercury methylation following the addition of molybdate. This strongly suggests that SRB are not likely to be significant methylators in our periphyton biofilm even though the large increase in MHg may be partially due to the complete inhibition of DHg by this treatment. Indeed, as seen with the characterization of the active microbial community, none of the identified 16s rRNA gene sequences in the molybdate treatment were related to those of SRB. Most of the studies measuring periphyton MHg rates with molybdate addition found a substantial decrease in MHg rates, from 60 to 95%.[5,8] As discussed before, maybe differences in trophic status of the systems, biofilm tickness, and niche diversity enabled different groups of microbes to methylate. A few studies have reported an absence of inhibition, or a low level of stimulation of MHg, 1.5−1.9 fold, following molybdate addition to lake sediments or bacterial cultures.[21,52] Here, the observed 45 fold increase in MHg is markedly higher than what has previously been reported.

Our results indicate that microbes other than SRB may be important as methylators. In fact, molybdate increased microbial production (Figure 1B), suggesting that molybdate addition changed the dynamics of microbial interactions in the periphyton consortium to favor the activity of the Hg methylators (Figure 1C). Periphytic biofilms are complex communities with species responding differently to the presence of metabolic inhibitors. Competition and also syntrophy between methanogens and SRB is well documented. SRB inhibition by molybdate leads to an accumulation of short-chain fatty acids and hydrogen, as well as acetate, propionate, and butyrate. Some of these substrates can be used by methanogenic archaea in the absence of competition from SRB.[53] As they compete for some of the same nutrients and electron donors,[54] inhibition of one of the two groups may channel the flow of energy toward the other group.

The absence of DHg under molybdate treatment suggests three possible hypotheses: (1) SRB are the major players in DHg under the conditions present in our biofilm, (2) methanogens and other microbes are so efficient to methylate Hg that, even if DHg occurs, it was not detectable because of the fast turnover to MeHg, (3) given that a strong cooperation exists between methanogens and sulfidogens[51] whereby SRB provide metabolic products that are consumed by methanogens, then when SRB are inhibited, maybe methanogens are not able to demethylate. Using a similar approach as in our study, Oremland et al.[55] and Marvin-Dipasquale and Oremland[56] investigated which microbes

and processes were involved in oxidative DHg, the degradation of MeHg to inorganic Hg and CO_2, by sediment incubations and bacterial cultures. Based on experiments with specific inhibitors, they concluded that in anoxic freshwater sediments, both methanogens and sulfidogens contributed to this process. Here, BESA did not have an effect on DHg, suggesting that methanogens were not directly involved in mercury demethylation by periphyton in our samples. Together, this study suggests a clear distinction between the microbial guilds that methylate Hg (methanogens) and degrade MeHg (SRB) in the periphyton in Lake St. Pierre.

Most of the 16S rRNA gene sequences that were retrieved from control, BESA, and molybdate treatments were most similar to those common in water and sediments from estuaries and freshwaters.[57−60] Some of them are usually found under anaerobic conditions, but they may be found in anaerobic compartments within the aerobic biofilm matrix.[61,62] Sequences most closely related to those of methanogens were common in the three treatments. Moreover, the proportion of methanogens compared to the entire microbial community ranged from 16.6% in BESA to 21.2% in control and to 33.3% in molybdate treatment (Figure 2). Methanogens could be the ones responsible for the high MHg observed in this last treatment. These results provide an excellent starting point for future research into more specifically identifying the species of methanogens responsible for mercury methylation. We cannot exclude the possibility that the other active microbes in our samples, as indicated by similarity of 16S rRNA genes to those of common aerobes (*Neisseiriales*, *Pseudomonadales*, *Oscillatoriales*, *Thiotricales*, *Enterobacteriales*, *Actinomycetales*, *Burkholderiales*, and *Bacilliales*), contributed directly or indirectly to MHg or DHg.

This study reports significant rates of MHg in periphytic biofilms of the largest fluvial lake of the St. Lawrence River. As macrophyte beds are covering more than half of this lake, the contribution of periphyton to the overall MeHg budget of this large river should not be ignored. On a larger scale, recent reports[21,63] and this study may lead to a paradigm shift regarding microbial methylation. Whereas in the past, SRB were seen as the main methylators, it is now clear that in some systems other microorganisms, such as methanogens and iron-reducers, methylate. The biotic complexity of periphytic biofilm and its ever-changing redox conditions probably create a highly competitive environment where populations and their activities change rapidly. More research is needed on the genetic identification of microbes in natural consortiums, synergies and competitions among them, and the in situ conditions determining who are the main Hg methylators in a given environment and under certain conditions.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. More information regarding physicochemical proprieties of the water (Table S1), details about physicochemical analysis (Figure S1), clones identification (Table S2), course of mercury methylation/demethylation during 48 h incubation (Figure S2) and methylation/demethylation rates in all treatments (Figure S3). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: melosira@hotmail.com.

■ ACKNOWLEDGMENT

This study was made possible through funding grant to D.P and M.A. by NSERC−COMERN, the Collaborative Mercury Research Network, a scholarships from UQÀM (faculté des sciences), GRIL and GEOTOP to S.H. In addition, we acknowledge support from the Environmental Remediation Science Program (ERSP), Biological and Environmental Research (BER), U.S. Department of Energy (Grant DE-FG02-05ER63969), and from a European Union Marie Curie Actions − International Incoming Fellowship (FP7-PEOPLE-IIF-2008). We are grateful to Holger Hintelmann's lab for mercury isotopic measurements, to Dominic Bélanger and Riqing Yu for their advice during lab work, and Serge Paquet for his help with statistical analysis. We are also thankful to our field assitants Gabriel Soumis-Dugas, Marianne Métiviers, and Myrianne Joly.

■ REFERENCES

(1) Furutani, A.; Rudd, J. W. M. Measurement of mercury methylation in lake water and sediment samples. *Appl. Environ. Microbiol.* **1980**, *40* (4), 770–776.

(2) Matilainen, T.; Verta, M.; Niemi, M.; Uusi-Rauva, A. Specific rates of net methylmercury production in lake sediments. *Water, Air Soil Pollut.* **1991**, *56* (1), 595–605.

(3) Krabbenhoft, D. P.; Gilmour, C. C.; Benoit, J. M.; Babiarz, C. L.; Andren, A. W.; Hurley, J. P. Methyl mercury dynamics in littoral sediments of a temperate seepage lake. *Can. J. Fish. Aquat. Sci.* **1998**, *55* (4), 835–844.

(4) Eckley, C. S.; Watras, C. J.; Hintelmann, H.; Morrison, K.; Kent, A. D.; Regnell, O. Mercury methylation in the hypolimnetic waters of lakes with and without connection to wetlands in northern Wisconsin. *Can. J. Fish. Aquat. Sci.* **2005**, *62* (2), 400–411.

(5) Desrosiers, M.; Planas, D.; Mucci, A. Mercury Methylation in the Epilithon of Boreal Shield Aquatic Ecosystems. *Environ. Sci. Technol.* **2006**, *40* (5), 1540–1546.

(6) Mauro, J. B. N.; Guimarães, J. R. D.; Hintelmann, H.; Watras, C. J.; Haack, E. A.; Coelho-Souza, S. A. Mercury methylation in macrophytes, periphyton, and water − comparative studies with stable and radio-mercury additions. *Anal. Bioanal. Chem.* **2002**, *374* (6), 983–989.

(7) Achá, D.; Iñiguez, V.; Roulet, M.; Guimarães, J. R. D.; Luna, R.; Alanoca, L.; Sanchez, S. Sulfate-Reducing Bacteria in Floating Macrophyte Rhizospheres from an Amazonian Floodplain Lake in Bolivia and Their Association with Hg Methylation. *Appl. Environ. Microbiol.* **2005**, *71* (11), 7531–7535.

(8) Cleckner, L. B.; Gilmour, C. C.; Hurley, J. P.; Krabbenhoft, D. P. Mercury methylation in periphyton of the Florida Everglades. *Limnol. Oceanogr.* **1999**, *44* (7), 1815–1825.

(9) Huguet, L.; Castelle, S.; Schäfer, J.; Blanc, G.; Maury-Brachet, R.; Reynouard, C.; Jorand, F. Mercury methylation rates of biofilm and plankton microorganisms from a hydroelectric reservoir in French Guiana. *Sci. Total Environ.* **2010**, *408* (6), 1338–1348.

(10) Guimarães, J. R. D.; Meili, M.; Hylander, L. D.; de Castro e Silva, E.; Roulet; Mauro, J. B. N.; de Lemos, R. A. Mercury net methylation in five tropical flood plain regions of brazil: high in the root zone of floating macrophyte mats but low in surface sediments and flooded soils. *Sci. Total Environ.* **2000**, *261* (1−3), 99–107.

(11) Hecky, R. E.; Hesslein, R. H. Contributions of benthic algae to lake food webs as revealed by stable isotope analysis. *J. North Am. Benthol. Soc.* **1995**, *14* (4), 631–653.

(12) Cremona, F.; Hamelin, S.; Planas, D.; Lucotte, M. Sources of organic matter and methylmercury in littoral macroinvertebrates: A stable isotope approach. *Biogeochemistry* **2009**, *94* (1), 81–94.

(13) McIntyre, P. B.; Michel, E.; Olsgard, M. Top-down and bottom-up controls on periphyton biomass and productivity in Lake Tanganyika. *Limnol. Oceanogr.* **2006**, *51* (3), 1514–1523.

(14) Robinson, G. G. C.; Gurney, S. E.; Goldsborough, L. G. The primary productivity of benthic and planktonic algae in a prairie wetlant under controlled water-level regimes. *Wetlands* **1997**, *17* (2), 182–194.

(15) Lamberti, G. A. The role of periphyton in benthic food webs. In *Algal Ecology: Freshwater Benthic Ecosystems*; Stevenson, R. J., Bothwell, M. L., Lowe, R. L., Eds.; Academic Press: San Diego, CA, 1996; pp 533−573.

(16) Dominique, Y.; Maury-Brachet, R.; Muresan, B.; Vigouroux, R.; Richard, S.; Cossa, D.; Mariotti, A.; Boudou, A. Biofilm and mercury availability as key factors for mercury accumulation in fish (*Curimata cyprinoides*) from a disturbed Amazonian freshwater system. *Environ. Toxicol. Chem.* **2007**, *26* (1), 45–52.

(17) Compeau, G. C.; Bartha, R. Sulfate-reducing bacteria: Principal methylators of mercury in anoxic estuarine sediment. *Appl. Environ. Microbiol.* **1985**, *50* (2), 498–502.

(18) Kerry, A.; Welbourn, P. M.; Prucha, B.; Mierle, G. Mercury methylation by sulphate-reducing bacteria from sediments of an acid stressed lake. *Water, Air Soil Pollut.* **1991**, *56* (1), 565–575.

(19) Gilmour, C. C.; Henry, E. A.; Mitchell, R. Sulfate stimulation of mercury methylation in freshwater sediments. *Environ. Sci. Technol.* **1992**, *26* (11), 2281–2287.

(20) Kerin, E. J.; Gilmour, C. C.; Roden, E.; Suzuki, M. T.; Coates, J. D.; Mason, R. P. Mercury Methylation by Dissimilatory Iron-Reducing Bacteria. *Appl. Environ. Microbiol.* **2006**, *72* (12), 7919–7921.

(21) Fleming, E. J.; Mack, E. E.; Green, P. G.; Nelson, D. C. Mercury Methylation from Unexpected Sources: Molybdate-Inhibited Freshwater Sediments and an Iron-Reducing Bacterium. *Appl. Environ. Microbiol.* **2006**, *72* (1), 457–464.

(22) Downing, J. A. A regression technique for the estimation of epiphytic invertebrate populations. *Freshwater Biol.* **1986**, *16*, 161–173.

(23) Gorski, P. R.; Armstrong, D. E.; Hurley, J. P.; Shafer, M. M. Speciation of Aqueous Methylmercury Influences Uptake by a Freshwater Alga (Selenastrum Capricornutum). *Environ. Toxicol. Chem.* **2006**, *25* (2), 534–540.

(24) Balch, W. E.; Wolfe, R. S. Specific and biological distribution of coenzyme M (2-mercaptoethane sulfonic acid). *J. Bacteriol.* **1979**, *137*, 264–273.

(25) Oremland, R. S.; Capone, D. G. Use of specific inhibitors in biochemistry and microbial ecology. *Adv. Microb. Ecol.* **1988**, *10*, 285–383.

(26) Hintelmann, H.; Ogrinc, N., Determination of stable mercury isotopes by ICP/MS and their application in environmental studies. In *Biogeochemistry of Environmentally Important Trace Elements*; Cai, Y., Braids, C.O., Eds.; American Chemical Society: Washington, DC, 2003; pp 321−338.

(27) Hintelmann, H.; Keppel-Jones, K.; Evans, R. D. Constants of Mercury Methylation and Demethylation rates in sediments and Comparison of Tracer and Ambiant Mercury Availability. *Environ. Toxicol. Chem.* **2000**, *19* (9), 2204–2211.

(28) Nusch, E. A. Comparison of different methods for chlorophyll and phaeopigment determination. *Archiv Für Hydrobiologie Beiheft Ergebnisse der Limnologie* **1980**, *14*, 14–36.

(29) APHA. *Standard Methods for the Examination of Water and Wastewater*; American Public Health Association, American Water Works Association and Water Pollution Control Federation: Washington, DC, 1989; p 1087.

(30) Lund, J. W. G.; Kipling, C.; Le Cren, E. D. The Inverted Microscope Method of Estimating Algal numbers and the Statistical Basis of Estimation by Counting. *Hydrobiologia* **1958**, *11* (2), 143–170.

(31) Boylan, H. B.; Kingston, S. One-Step mercury analysis. *Am. Lab.* **1988**, *30*, 25–26.

(32) Cizdziel, J. V.; Hinners, T. A.; Heithmar, E. M. Determination of total mercury in fish tissues using combustion atomic absorption spectrometry with gold amalgamation. *Water, Air Soil Pollut.* **2000**, *135*, 355–370.

(33) Bloom, N.; Fitzgerald, W. F. Determination of volatile mercury species at the picogram level by low-temperature gas chromatography with cold-vapour atomic fluorescence detection. *Anal. Chim. Acta* **1988**, *208*, 151–161.

(34) Bloom, N. S. Determination of picogram levels of methylmercury by aqueous phase ethylation, followed by cryogenic gas chromatography with cold vapour atomic fluorescence detection. *Can. J. Fish. Aquat. Sci.* **1989**, *46* (7), 1131–1140.

(35) Vis, C.; Hudon, C.; Carignan, R. Influence of the vertical structure of macrophyte stands on epiphyte community metabolism. *Can. J. Fish. Aquat. Sci.* **2006**, *63* (5), 1014–1026.

(36) Wicks, R. J.; Robarts, R. D. The extraction and purification of DNA labeled with [methyl-3H]thymidine in aquatic bacterial production studies. *J. Plankton Res.* **1987**, *9* (6), 1159–1166.

(37) Stanley, E. H.; Johnson, M. D.; Ward, A. K. Evaluating the influence of macrophytes on algal and bacterial production in multiple habitats of a freshwater wetland. *Limnol. Oceanogr.* **2003**, *48* (3), 1101–1111.

(38) Kemp, P. F.; Lee, S.; Laroche, J. Estimating the Growth Rate of Slowly Growing Marine Bacteria from RNA Content. *Appl. Environ. Microbiol.* **1993**, *59* (8), 2594–2601.

(39) Wang, Y.; Freedman, Z.; Lu-Irving, P.; Kaletsky, R.; Barkay, T. An initial characterization of the mercury resistance (mer) system of the thermophilic bacterium *Thermus thermophilus* HB27. *FEMS Microbiol. Ecol.* **2009**, *67* (1), 118–129.

(40) Lane, D. J.; Pace, B.; Olsen, G. J.; Stahl, D. A.; Sogin, M. L.; Pace, N. R. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82* (20), 6955–6959.

(41) Raskin, L.; Stromley, J. M.; Rittmann, B. E.; Stahl, D. A. Group-Specific 16S rRNA Hybridization Probes To Describe Natural Communities of Methanogens. *Appl. Environ. Microbiol.* **1994**, *60* (4), 1232–1240.

(42) Lane, D. J., 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*; Stackebrandt, E., Goodfellow, M., Eds.; John Wiley & Sons: Chichester, UK, 1991; pp 115−175.

(43) Wood, J. M.; Kennedy, F. S.; Rosen, C. G. Synthesis of methylmercury compounds by extracts of a methanogenic bacterium. *Nature* **1968**, *220*, 173–174.

(44) King, J. K.; Kostka, J. E.; Frischer, M. E.; Saunders, F. M.; Jahnke, R. A. A quantitative relationship that demonstrates mercury methylation rates in marine sediments are based on the community composition and activity of sulfate-reducing bacteria. *Environ. Sci. Technol.* **2001**, *35* (12), 2491–2496.

(45) Korthals, E. T.; Winfrey, M. R. Seasonal and spatial variations in mercury methylation and demethylation in an oligotrophic lake. *Appl. Environ. Microbiol.* **1987**, *53* (10), 2397–2404.

(46) Pongratz, R.; Heumann, K. G. Production of methylated mercury and lead by polar macroalgae - A significant natural source for atmospheric heavy metals in clean room compartments. *Chemosphere* **1998**, *36* (9), 1935–1946.

(47) Price, N. M.; Morel, F. M. M.; Stumm, W. Role of extracellular enzymatic reactions in natural waters. In *Aquatic Chemical Kinetics*; Wiley: New York, 1990; pp 235−257.

(48) Pak, K.-R.; Bartha, R. Mercury Methylation by Interspecies Hydrogen and Acetate Transfer between Sulfidogens and Methanogens. *Appl. Environ. Microbiol.* **1998**, *64* (6), 1987–1990.

(49) Kates, M.; Kushner, D. J.; Matheson, A. T. *The Biochemistry of Archaea (Archaebacteria)*; Elsevier: Amsterdam, 1993.

(50) Henry, E. A. The role of sulfate-reducing bacteria in environmental mercury methylation. Ph.D. Dissertation, Harvard Unuiversity, 1992.

(51) Pak, K.-R.; Bartha, R. Mercury methylation and demethylation in anoxic lake sediments and by strictly anaerobic bacteria. *Appl. Environ. Microbiol.* **1998**, *64* (3), 1013–1017.

(52) Matilainen, T. Involvement of bacteria in methylmercury formation in anaerobic lake waters. *Water, Air Soil Pollut.* **1995**, *80* (1−4), 757–764.

(53) Sørensen, J.; Christensen, D.; Jørgensen, B. B. Volatile fatty acids and hydrogen as substrates for sulphate-reducing bacteria in anaerobic marine sediment. *Appl. Environ. Microbiol.* **1981**, *42*, 5–11.

(54) Oremland, R. S.; Taylor, B. F. Sulfate reduction and methanogenesis in marine sediments. *Geochim. Cosmochim. Acta* **1978**, *42* (2), 209–214.

(55) Oremland, R. S.; Culbertson, C. W.; Winfrey, M. R. Methylmercury decomposition in sediments and bacterial culture: Involvement of methanogens and sulfate reducers in oxidative demethylation. *Appl. Environ. Microbiol.* **1991**, *57* (1), 130–137.

(56) Marvin-Dipasquale, M. C.; Oremland, R. S. Bacterial Methylmercury Degradation in Florida Everglades Peat Sediment. *Environ. Sci. Technol.* **1998**, *32* (17), 2556–2563.

(57) Yoshida, A.; Seo, Y.; Suzuki, S.; Nishino, T.; Kobayashi, T.; Hamada-Sato, N.; Kogure, K.; Imada, C. Actinomycetal community structures in seawater and freshwater examined by DGGE analysis of 16S rRNA gene fragments. *Mar. Biotechnol.* **2008**, *10* (5), 554–63.

(58) Min, K. R.; Rickard, A. H. Coaggregation by the Freshwater Bacterium *Sphingomonas natatoria* Alters Dual-Species Biofilm Formation. *Appl. Environ. Microbiol.* **2009**, *75* (12), 3987–3997.

(59) Berrada, Z. L.; Telford, S. R. Diversity of *Francisella* Species in Environmental Samples from Martha's Vineyard, Massachusetts. *Microb. Ecol.* **2010**, *59* (2), 277–283.

(60) Li, H.; Xing, P.; Chen, M.; Bian, Y.; Wu, Q. L. Short-term bacterial community composition dynamics in response to accumulation and breakdown of *Microcystis* blooms. *Water Res.* **2011**, *45* (4), 1702–1710.

(61) Teske, A.; Wawer, C.; Muyzer, G.; Ramsing, N. B. Distribution of Sulfate-Reducing Bacteria in a Stratified Fjord (Mariager Fjord, Denmark) as Evaluated by Most-Probable-Number Counts and Denaturing Gradient Gel Electrophoresis of PCR-Amplified Ribosomal DNA Fragments. *Appl. Environ. Microbiol.* **1996**, *62* (4), 1405–1415.

(62) Ito, T.; Okabe, S.; Satoh, H.; Watanabe, Y. Successional development of sulfate-reducing bacterial populations and their activities in a waste-water biofilm growing under microaerophilic conditions. *Appl. Environ. Microbiol.* **2002**, *68* (3), 1392–1402.

(63) Warner, K. A.; Roden, E. E.; Bonzongo, J.-C. Microbial Mercury Transformation in Anoxic Freshwater Sediments under Iron-Reducing and Other Electron-Accepting Conditions. *Environ. Sci. Technol.* **2003**, *37* (10), 2159–2165.