RESEARCH ARTICLE

Mercury methylation in *Sphagnum* moss mats and its association with sulfate-reducing bacteria in an acidic Adirondack forest lake wetland

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Abstract

Processes leading to the bioaccumulation of methylmercury (MeHg) in northern wetlands are largely unknown. We have studied various ecological niches within a remote, acidic forested lake ecosystem in the southwestern Adirondacks, NY, to discover that mats comprised of *Sphagnum* moss were a hot spot for mercury (Hg) and MeHg accumulation (190.5 and 18.6 ng g^{-1} dw, respectively). Furthermore, significantly higher potential methylation rates were measured in *Sphagnum* mats as compared with other sites within Sunday Lake's ecosystem. Although MPN estimates showed a low biomass of sulfate-reducing bacteria (SRB), 2.8×10^4 cells mL^{-1} in mat samples, evidence consisting of (1) a twofold stimulation of potential methylation by the addition of sulfate, (2) a significant decrease in Hg methylation in the presence of the sulfate reduction inhibitor molybdate, and (3) presence of *dsrAB*-like genes in mat DNA extracts, suggested that SRB were involved in Hg methylation. Sequencing of *dsrB* genes indicated that novel SRB, incomplete oxidizers including *Desulfobulbus* spp. and *Desulfovibrio* spp., and syntrophs dominated the sulfate-reducing guild in the *Sphagnum* moss mat. *Sphagnum*, a bryophyte dominating boreal peatlands, and its associated microbial communities appear to play an important role in the production and accumulation of MeHg in high-latitude ecosystems.

Introduction

Atmospheric deposition of mercury (Hg) coupled with forest and wetland cover, oligotrophic surface waters, and elevated inputs of acidic deposition has resulted in elevated methylmercury (MeHg) accumulation in aquatic biota in lake ecosystems of the Adirondack mountains, a 'biological Hg hotspot' in the northeastern United States (Driscoll *et al.*, 1995, 2007). Sunday Lake watershed, for instance, experiences 8.1 μg THg m^{-2} $year^{-1}$ and 0.04 μg CH_3Hg^+ m^{-2} $year^{-1}$ wet deposition (Network, 2003; Demers *et al.*, 2007; Selvendiran *et al.*, 2009). In this remote lake, fish tissue contained an average of 1.0 ± 0.47 ng g^{-1} THg (w/w), with 95% of fish samples exceeding the EPA fish tissue MeHg criterion of 0.3 μg g^{-1} and 45% exceeding the FDA advisory level of 1 μg g^{-1} (McLaughlin, 2003). However, the linkage between atmospheric Hg deposition and MeHg accumulation in fish of the Adirondacks is not clear, but possibly involves complex biogeochemical processes. Previous studies in this ecosystem focused on the biogeochemical cycling and aquatic trophic transfer of Hg (Driscoll *et al.*, 1998, 2007; McLaughlin, 2003; Demers *et al.*, 2007), but little attention has been paid to the structure and function of the microbial assemblages that may be involved in Hg transformations.

Freshwater wetlands are important sites for Hg methylation and are a major source of MeHg to associated lakes and streams (St. Louis *et al.*, 1994, 1996; Heyes *et al.*, 2000). These wetlands are characterized by dynamic nutrient cycling, active aerobic and anaerobic microbial processes, and fluctuating hydrology. Unique taxonomic groups were described in the microbial communities of remote northern wetlands, including acidic peatlands, which represent 50% of the global wetland area (Dedysh *et al.*, 1998; Sizova *et al.*, 2003). Hg biotransformations, primarily methylation and

FEMS MICROBIOLOGY ECOLOGY

demethylation, involve intertwined microbial processes including sulfate (Gilmour *et al.*, 1992) and iron reduction (Fleming *et al.*, 2006) and possibly methanogenesis, acetogenesis, and syntrophy (Pak & Bartha, 1998; Barkay & Wagner-Döbler, 2005). An understanding of the community structure and its mechanistic connection with measured relevant biogeochemical processes in wetland ecosystems is critical. Microbial community structure and composition are also important for the characterization of the biodiversity of Adirondack wetlands, and might provide an ecological context to the function of target microbial groups (Gutknecht *et al.*, 2006).

Sulfate-reducing bacteria (SRB) are a phylogenetically diverse group that mediates important metabolic functions by performing sulfate reduction, terminal oxidation of organic carbon, and degradation of contaminants in anaerobic environments (Smith & Klug, 1981; Barton & Tomei, 1995; Coates *et al.*, 1996; Muyzer & Stams, 2008). Experiments have shown that SRB methylate Hg both as pure cultures and as consortia in natural habitats such as salt marsh, estuarine, and freshwater sediments (Compeau & Bartha, 1985; Gilmour *et al.*, 1992; King *et al.*, 2000; Benoit *et al.*, 2001). Within these groups of bacteria, a recent study by Ranchou-Peyruse *et al.* (2009) suggested that only SRB that belong to the *Deltaproteobacteria* methylate Hg. Culture-independent methods have successfully been used to detect SRB using hybridization with 16S rRNA gene oligoprobes (Devereux *et al.*, 1992), PCR with primers specific to dissimilatory (bi)sulfite reductase (*dsrAB*) genes, which catalyzes the reduction of sulfite to sulfide in anaerobic sulfate reduction pathways (Wagner *et al.*, 1998; Stahl *et al.*, 2002), and PCR with SRB group-specific primers to 16S rRNA genes (Daly *et al.*, 2000). Understanding the linkage between ecological functions (e.g. sulfate reduction) and SRB is crucial. Although few studies have linked community structure of SRB to Hg methylation in estuarine sediments or mine tailings (King *et al.*, 2000; Winch *et al.*, 2009), none has examined this process in freshwater wetlands.

Variation in SRB taxonomic diversity as related to Hg methylation is expected in freshwater wetlands as compared with marine habitats. Freshwater sediments are characterized by low sulfate reduction rates due to low concentrations of sulfate (Bak & Pfennig, 1991a, b). Studies in forest fen soils (Loy *et al.*, 2004), freshwater wetlands (Castro *et al.*, 2002; Chauhan *et al.*, 2004), and rice rhizosphere (Scheid & Stubner, 2001) have shown the presence of novel phylogenetic groups of SRB as compared with marine ecosystems (Hines *et al.*, 1999; Dhillon *et al.*, 2003; Bahr *et al.*, 2005), likely due to evolution in habitats with low sulfate.

The goals of this study were to relate community structure to Hg methylation activities in a freshwater wetland by (1) examining the potential for microbial Hg methylation in different microbial habitats within a wetland ecosystem, (2) describing the diversity of SRB populations, and (3) assessing the possible role of SRB in methylation.

Materials and methods

Site description and sample collection

The study site, Sunday Lake (44°20′N, 74°18′W), is located in the southwestern Adirondack Mountains, NY. The watershed (996 ha) is densely covered with 70% deciduous and 30% coniferous forest (Driscoll *et al.*, 2003; McLaughlin, 2003), and is remote from direct human disturbance and atmospheric emission sources. This forest–wetland–lake ecosystem has 7.7 ha of lake surface area and 204 ha of wetland drainage. At least one-third of the wetlands, including most of the lake margin, is colonized by ground-growing peat moss *Sphagnum* spp., which by itself can create a moderately acidic bog habitat (Crum & Anderson, 1981).

Samples were collected at two depths (0–15 and 15–30 cm) from six main locations representing distinct habitats within the forest–wetland–lake ecosystem. Locations included two riparian zones on the banks of the major inlet to Sunday Lake, 1 m from the bank (SURN) and 5 m from the bank (SURF), a well-drained upland forest soil (Upland soil), a wetland site dominated by *Carex* spp. sedge (Sedge), a bog dominated by *Sphagnum* spp. approximately 100 m from the lake (BOG), and a single depth sample from the lower portion of a mat floating on the lake surface (MAT), which was dominated by *Sphagnum* spp. mixed with ericaceous shrubs (*Ledum groenlandicum*, *Chamaedaphne calyculata*). The MAT and BOG sites were always water saturated, while the SURN, SURF, and Sedge sites were subject to frequent fluctuations of moisture due to changes in inlet or ground water table. The Upland site was largely unsaturated due to well-drained sandy soils. Sediment cores for Hg analysis were collected in June 2006 from the bottom of Sunday Lake by SCUBA divers using hand-held coring equipment.

Samples were collected from 2004 to 2007. Chemical and molecular biological analyses and Hg methylation assays were conducted with samples taken in July 2005. Samples were collected using clean plastic gloves and placed in sterile air-tight Falcon tubes on ice with clean procedures, transported to the laboratory within 8 h, and stored at −80 °C before analysis. Samples for Hg methylation were collected in acid-cleaned glass jars, which were filled completely with wetland water and sealed. These samples were kept on ice during transport to the laboratory where they were stored at 4 °C until analysis. Microbial mat samples were also collected in June 2005 and July 2007 from a *Spartina* spp. saltmarsh in Cheesequake State Park, NJ, where SRB were expected to be abundant. The samples served as positive controls for community analysis, MPN estimations, and

chemical analysis, respectively. In addition, sediments were taken in July 2007 for chemical analysis from Berry's Creek in the Meadowlands, NJ, a superfund site highly contaminated with Hg (Schaefer *et al.*, 2004).

Analytical methods

Sample pH was measured using an Accumet 915 pH Meter (Fisher Sci.). Aliquots of samples were completely oxidized in a muffle furnace at 550 °C for 4 h, and the remaining weight of the ashed sample was described as the oxidized sample mass. The ashed samples were then sieved through a #270 mesh (53 μm opening with 50 μm delineation between sand and silt) to determine soil texture as the weight percentage ratio of sand and silt with clay. Organic matter content was analyzed using the loss-on-ignition method (Nelson & Sommers, 1996).

Samples taken from Sunday Lake were freeze-dried before Hg analysis (Labconco Corp., MO) in order to calculate Hg concentrations on a dry weight basis and to prevent Hg losses during processing. Samples (~30 mg) were analyzed sequentially for total mercury (THg) using a DMA-80 Direct Mercury Analyzer (Milestone, CT) utilizing thermal decomposition, catalytic reduction, amalgamation, desorption, and atomic absorption spectroscopy. For MeHg analysis, samples (~20 mg) were first digested (Hintelmann & Nguyen, 2005), and then analyzed via aqueous ethylation with sodium tetraethylborate, purging and trapping, adsorption and desorption, separation by a GC (Clarus 500, Perkin Elmer, CT), reduction by a pyrolytic column, and detection by cold vapor atomic fluorescence spectroscopy (TEKRAN Model 2500, TN) modified from EPA Method 1630 (EPA, 2001). Quality control experiments with reference material and Hg- or MeHg-spiked samples showed an average of 107% recovery rates for the THg measurements and 88–93% for the MeHg analysis.

Reference strains

Six reference SRB strains, each representing one of six groups of SRB (SRB1–6) as defined by Daly *et al.* (2000), referred to as the classic or common SRB groups hereafter, and *Syntrophobacter fumaroxidans* (DSM 10017), were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). The SRB strains included were *Desulfotomaculum nigrificans* (DSM 574, SRB1), *Desulfobulbus propionicus* (DSM 2032, SRB2), *Desulfobacterium autotrophicum* (DSM 3382, SRB3), *Desulfobacter curvatus* (DSM 3379, SRB4), and *Desulfosarcina variabilis* (DSM 2060, SRB5). *Desulfovibrio desulfuricans* G200 (SRB6) was kindly provided by Dr Judy Wall. The strains were cultured by strict anaerobic techniques using media and conditions suggested by the DSMZ. Strain G200 was grown in LS medium (Rapp & Wall, 1987).

Potential rates of Hg methylation

Homogenized samples, 3 mL (in triplicate), were placed into a 13-mL serum vial in a N_2-filled glove bag and vials were sealed with Teflon-lined butyl rubber stoppers. Water from the sampling site was deaerated with N_2 for 20 min and then 3 mL was injected into each vial. After a 24-h preincubation, 5 μL of 0.2–0.5 μCi of $^{203}HgCl_2$ (generously provided by D. Barfuss, Georgia State University, Atlanta) was injected into each vial, which represented ~40–100 ng Hg(II) mL^{-1} sediment. After incubation for 43 h in the dark at ambient temperature, 1.0 mL of 3 N HCl was injected into each vial to stop microbial activity and vials were stored frozen until analysis. Preliminary studies showed that this incubation period was adequate to obtain rates that were above the blank's means plus 3 SDs. The amount of radiotracer added was the smallest that we could use considering the activity of radioisotopes. Procedural blanks were processed exactly the same, except that acid was added before the injection of $^{203}HgCl_2$. In addition, we obtained nearly identical blank results when we compared these procedural blanks with those obtained from samples sterilized using γ radiation (^{90}Co; UMass Lowell reactor). Radiolabeled MeHg was extracted from slurry incubations as described by Hines *et al.* (2006) and $Me^{203}Hg$ was quantified by scintillation counting. Potential Hg methylation rates (% day^{-1}) were calculated from results of triplicate samples per treatment.

To investigate what microbial guilds were involved in Hg methylation, slurries of samples from the MAT, BOG, and SURN sites were amended with compounds known to affect microbial processes. Amendments included a terminal electron acceptor (2.0 mM sulfate), an inhibitor of methanogenesis (2.0 mM bromoethane sulfonic [BES] acid), an inhibitor of sulfate reduction (2.0 mM sodium molybdate), and the combined addition of sulfate, BES, and sodium molybdate at 2.0 mM each (Mix). To enrich SRB, slurries of SURN, BOG, and MAT samples were prepared as described above, amended with K_2SO_4 to a final concentration of 2.0 mM, and then incubated for 14 days at 17 °C. Subsamples removed from slurries at the end of the incubation period were frozen at −80 °C for later molecular analyses. Results of methylation rates were analyzed by one-way and two-way ANOVA. Specific comparisons among different treatments and sampling sites were performed by Tukey's honest significant difference test using SAS (Cary, NC).

MPN estimates of SRB and propionate-oxidizing SRB (PO-SRB)

An immersed, decomposing lower portion of *Sphagnum* moss from the MAT site was collected for MPN analysis in July 2007. The sample was mixed with approximately 3 vol of site water in presterilized anaerobic tubes, fully filled, and sealed immediately. A saltmarsh mat sample was similarly

collected from Cheesequake State Park, NJ, in August 2007. The numbers of SRB and propionate-oxidizing (PO)-SRB in floating *Sphagnum* MAT and of SRB in the saltmarsh mat were estimated by a modification of the MPN technique (Widdel & Bak, 1992; Brandt *et al.*, 2001). For SRB-MPN, the medium of Widdel & Bak (1992) was modified by adding resazurin (2.0 nM), $FeCl_2 \cdot 4H_2O$ (1.0 mM), which formed black FeS upon commencement of sulfate reduction, and a mixture of sodium salts of acetate, propionate, butyrate, and lactate, 4.0 mM each, as electron donors, and by substituting Na_2S with $Na_2S_2O_4$ (0.2 mM) as the medium reductant to avoid the immediate formation of FeS when $FeCl_2 \cdot 4H_2O$ was added (Brandt *et al.*, 2001). Ambient water pH and temperature in the MAT were 6.0 and 25.5 °C, respectively, at the time of sampling. Preliminary experiments showed little growth of SRB at a pH of < 6.0 and MPN incubations were therefore carried out at pH 6.6. For the saltmarsh sample, the same medium was supplemented with 51.3 mM NaCl. The MPN medium for PO-SRB was adapted from Medium 684 (DSMZ) (Stams *et al.*, 1993), amended with propionate (15.6 mM) and sulfate (19.7 mM) as recommended for the isolation of propionate-oxidizing bacteria (Chen *et al.*, 2005). Five replicate MPN dilution series (1 : 10) were established in Balch tubes (Bellco, NJ) and incubated at 28 °C in the dark. All procedures were performed by Hungate anaerobic techniques in an anaerobic chamber (Coy Laboratory Products, MI) or using a bench top anaerobic manifold. The presence of SRB or PO-SRB was indicated by the formation of black FeS precipitate.

DNA extraction and PCR amplification of 16S rRNA gene, *dsrAB*, and *dsrB* genes

Genomic DNA from pure cultures and MPN series dilution cultures was extracted as described by Wilson (2001). Nucleic acids from natural samples were first recovered by a modification of the bead-beating method of Hurt *et al.* (2001), and RNA and DNA in extracts were separated using Qiagen® RNA/DNA Mini Kit (Qiagen, CA) according to the manufacturer's protocol.

PCR amplification was performed in a GeneAmp PCR System 9700 (Applied Biosystems, CA). Reactions (25 or 50 μL each) included 0.4 μM PCR primers (see Supporting Information, Table S1 for the list of primers), $MgCl_2$ at 1.5 mM (final concentration), except for SRB4 where 1.35 mM was used, 1 × PCR buffer provided by the polymerase manufacturer, 0.2 nM of each deoxynucleoside triphosphate, 0.25 mg of bovine serum albumin per milliliter, 50–250 ng of purified DNA, and 0.025 U of *Taq* polymerase (Denville, NJ). PCR conditions included an initial 5-min hot start at 95 °C, 35 cycles of 94 °C for 1 min, annealing at 45–66 °C (depending on primers used) for 30 s (15 s for PCR of SRB5 and 6), and extension at 72 °C for 1 min, concluding with a final extension at 72 °C for 7 min.

In order to detect the presence of the six groups of classic SRB in the Sunday Lake ecosystem, the nested PCR approach from Daly *et al.* (2000) was used as described by Dar *et al.* (2005) with the exception that new primers for groups SRB5 and 6 were developed (Table S1). Nearly complete product of bacterial 16S rRNA genes was first amplified using primer pair GM3F/GM4R and a touchdown annealing protocol (Muyzer *et al.*, 1995). A second amplification with SRB group-specific primers was performed using 1 : 100 dilution of the PCR products of the first reaction as template. DNA extracts of six pure cultures representing each SRB group served as controls.

The presence of *dsrAB* and *dsrB* genes in environmental samples and in MPN dilution cultures was detected using primers and PCR conditions as suggested by Wagner *et al.* (1998) and Geets *et al.* (2006), respectively.

Clone libraries of *dsrB* genes from native MAT samples

Purified PCR products of *dsrB* genes were ligated into pGEM®-T Easy vectors (Promega, WI) and transformed into *Escherichia coli* DH 10B cells according to the manufacturer's instructions. Randomly selected white colonies were inoculated in Luria–Bertani medium using a Concert™ 96 Plasmid Purification System (Invitrogen, CA). Plasmid DNA was extracted as described by the manufacturer, and inserts were sequenced by GENEWIZ Inc. (North Brunswick, NJ).

Denaturing gradient gel electrophoresis (DGGE) analysis and sequencing of *dsrB* gene fragments

Purified PCR products (250–350 ng) of *dsrB* genes amplified from MAT MPN enrichment cultures were separated by DGGE as described by Muyzer & Smalla (1998) using DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, CA). DGGE gels contained 8% polyacrylamide and a 40–70% denaturant gradient consisting of 7 M urea and 40% (v/v) formamide stock solution according to Geets *et al.* (2006). Electrophoresis was performed in 1 × TAE buffer at 70 V for 13 h at 60 °C.

DGGE gels were stained with 1 × GelStar Nucleic Acid Gel Stain (Cambrex BioScience Rockland, ME) and rinsed quickly with Milli-Q water. Gel images were then acquired under UV light. Gel slices containing DNA bands were excised, the DNA was eluted into Milli-Q water by an overnight incubation at 4 °C, and subjected to another cycle of PCR amplification followed by a second DGGE analysis to check for the purity of the first PCR products. DNA from bands in which the purity was confirmed was amplified,

purified using QIA-quick Gel Extraction Kit (Qiagen), and sequenced by GENEWIZ Inc.

Phylogenetic analyses

Sequences of clone libraries and excised DGGE fragments, and reference sequences obtained from GenBank (http://www.ncbi.nih.gov/) of *dsrB* genes were edited using Contig Express (Vector NTI Advance™ 10; Invitrogen) and Chromas, and aligned by CLUSTALW (Thompson *et al.*, 1994). Phylogenetic trees were constructed by PAUP* (version 4.0 beta 10; Sinaur Associates, MA) and CLUSTALX (Thompson *et al.*, 1997). The robustness of tree topology was tested by bootstrap resampling with 1000 iterations.

Nucleotide sequence accession numbers

GenBank accession numbers of partial *dsrB* gene sequences for clone libraries from native MAT samples and DGGE fragments from the MAT MPN enrichments are HQ148569–HQ148658, and FJ040921–FJ040931, respectively.

Results

Physical and chemical characteristics of the study sites

Samples from the six Sunday Lake sites were divided into those dominated by mineral matter, consisting of $< 20\%$ organic matter, and those dominated by organic matter (Table 1). In the former (SURN, Upland soil, and Sedge), the mineral matter consisted mostly of sand. In the latter, i.e. the MAT and BOG, where *Sphagnum* was the major vegetation, silt and clay dominated. All Sunday Lake samples were acidic, with pH values ranging from 3.00 in the Upland soil to 5.03 in the MAT sample. In comparison, the Cheesequake saltmarsh mat sample was organic rich with mineral matter dominated by clay and silt, and Berry's Creek sediment had low organic content and mineral matter consisting of both sand and silt/clay fractions. Both NJ control sites had near-neutral pH (6.70 in Cheesequake and 7.50 in Berry's Creek).

The highest THg concentration at the Sunday Lake sites occurred in the lake bottom sediment, averaging at 459.9 ng g^{-1} dw (Table 1). With the exception of SURF-top, sample sites that were organic rich had relatively high THg concentrations ranging from 294.1 ng g^{-1} for BOG-top to 190.5 ng g^{-1} for MAT. The mineral-dominated sample sites had lower THg concentrations, ranging from 15.5 to 122.9 ng g^{-1} for the Upland soil and Sedge sites, respectively. Overall, these THg concentrations in Sunday Lake were at least two orders of magnitude lower than the highly contaminated Berry's Creek sample (10 273.2 ng g^{-1}), but also, surprisingly, lower than the THg concentration in the Cheesequake saltmarsh mat (747.7 ng g^{-1}).

The highest values of MeHg in Sunday Lake watershed were found in the BOG-top and floating MAT, ranging from 18.64 to 21.23 ng g^{-1} dw (Table 1). Furthermore, the fraction of THg that was present as MeHg in the BOG-top (7.22%) and MAT (9.78%) samples, both permanently water-saturated sites, was the highest among all samples analyzed. Concentrations of MeHg generally followed the patterns

Table 1. Physical and chemical characteristics of study sites in Sunday Lake, New York (July 21, 2005), Cheesequake saltmarsh (July 23, 2007), and Berry's Creek, NJ (July 26, 2007)

Study site	Temperature (°C)*	pH	Sand (%)	Silt+clay (%)	Organic matter content (%)	THg (ng g^{-1} dw)	MeHg (ng g^{-1} dw)	MeHg fraction in THg (%)
SURN-top†	17.3	4.00	80.4	19.6	18.71	60.4 (1.9)‡	0.49 (0.09)	0.82
SURN-bottom	16.3	4.10	77.5	22.5	20.76	105.8 (8.0)	0.83 (0.08)	0.78
SURF-top	18.5	3.50	39.4	60.6	46.64	86.6 (15.3)	0.63 (0.09)	0.72
SURF-bottom	17.6	3.80	84.8	15.2	7.86	73.9 (9.1)	0.24 (0.11)	0.32
Upland soil	20.5	3.00	87.2	12.8	16.24	15.5 (1.1)	< 0.50§	< 0.03
BOG-top	25.5	4.30	22.0	78.0	74.00	294.1 (0.8)	21.23 (2.52)	7.22
BOG-bottom	23.6	4.05	2.9	97.1	88.37	254.9 (5.1)	4.16 (0.56)	1.63
Sedge	18.8	3.50	83.5	16.5	17.56	122.9 (17.3)	3.85 (0.40)	3.14
MAT	25.8	5.03	0	100.0	91.23	190.5 (14.4)	18.64 (1.55)	9.78
Sunday Lake sediment	15.3	ND	ND	ND	ND	459.9 (0.7)	2.58 (0.47)	0.56
Cheesequake	28.5	6.70	18.1	81.9	40.67	747.7 (55.6)	7.82 (0.86)	1.05
Berry's Creek	29.5	7.50	42.3	57.7	8.9	10 273.2 (363.0)	32.35 (5.79)	0.32

*Values were water temperature for BOG-top, BOG-bottom, and MAT, and soil temperature for other sites.

†Top – bulk sample taken from the upper 15 cm of the soil profile; bottom – bulk samples taken from the bottom 15 cm of the soil profile. Surface bulk samples were collected for Upland soil, Sedge, MAT, and Lake sediment.

‡Values in parentheses represent the SDs.

§Below detection limit.

ND, not determined.

observed for THg, with higher concentrations in samples with high organic content. The MeHg concentrations in the BOG-top and MAT samples were comparable to the concentration measured in Berry's Creek sediment ($32.35\,ng\,g^{-1}$ dw). However, the fraction of THg as MeHg in Berry's Creek sediment was the lowest in the entire data set (0.32%). The remaining Sunday Lake samples that were collected proximal to the lake (BOG-bottom, Sedge, and lake sediment) had a moderate range of MeHg ($2.58–4.16\,ng\,g^{-1}$), while samples more distant from the lake (SURN, SURF, and Upland soil) had $< 1\,ng\,g^{-1}$ MeHg concentrations. These samples also contained a much lower fraction of THg as MeHg; all were $< 2\%$. Together, the analyses showed that THg and MeHg concentrations increased with increasing organic matter content and proximity to Sunday Lake. In particular, samples dominated by *Sphagnum* (i.e. BOG and MAT) seemed to be 'hotspots' for both THg and MeHg accumulation.

Potential Hg methylation rates

A site comparison by two-way ANOVA (Type 3 tests) indicated that Hg methylation rates for SURN, BOG, and MAT generally displayed significant variation, with the highest values for the floating MAT and the lowest in SURN ($P < 0.0001$; Fig. 1). For the amendments of sulfate and BES, significantly higher Hg methylation rates were found in MAT in comparison with BOG ($P < 0.0001$ and 0.009, respectively, Tukey's test) and SURN ($P < 0.0001$ and 0.0001, respectively, Tukey's test). The addition of molybdate and Mix to the slurry incubations, however, did not cause significant differences among the three sites ($P > 0.7$, Tukey's test).

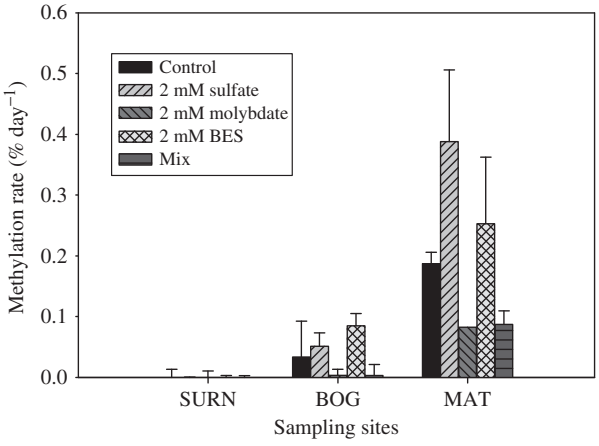

Fig. 1. Potential methylation rates of Sunday Lake samples and the impact of various treatments on these rates. The 'Mix' treatment was amended with a combination of sulfate, molybdate, and BES at 2 mM each.

ANOVA analysis (Type 3 tests) (two-way for sites and treatments, and one-way for treatments per site) of the effect of treatments on potential methylation showed that rates differed significantly, mainly due to the pronounced influence of the sulfate treatment (Fig. 1; $P = 0.0002$). For the MAT sample, potential Hg methylation rates were the highest in the sulfate-supplemented incubation, with activities significantly higher than the unamended control (2.1-fold increase; $P < 0.005$, Tukey's test), the molybdate treatment (4.6-fold; $P < 0.0001$), and the Mix treatment (4.4-fold; $P < 0.0001$). No significant difference in potential methylation rates was found between the sulfate and BES-amended samples (1.5-fold; $P = 0.949$). In comparison with the control, amendment of molybdate in MAT samples apparently inhibited potential methylation rates by 44% reduction ($P = 0.013$, Tukey's test). However, the addition of BES did not significantly change potential methylation rates in the MAT samples, although a moderate rate increase could be observed (1.4-fold; $P = 0.967$). Similarly, the additions of Mix (0.5-fold; $P = 0.437$) in MAT samples did not significantly affect potential methylation rates compared with rates of the control. For the BOG and SURN samples, different amendments did not significantly impact potential methylation rates ($P > 0.05$, Tukey's test).

Detections of dissimilatory (bi)sulfite reductase (*dsrAB*) genes

As Hg methylation is generally attributed to SRB activity (Barkay & Wagner-Döbler, 2005), we examined whether there was a genetic potential for sulfate reduction, as indicated by the presence of *dsrAB* genes, in the communities of various environments within the Sunday Lake ecosystem (Fig. 2). These determinations were performed with native samples and those that were incubated for 14 days with 2 mM sulfate. PCR products corresponding to the expected 1926 bp *dsrAB* product were detected in samples

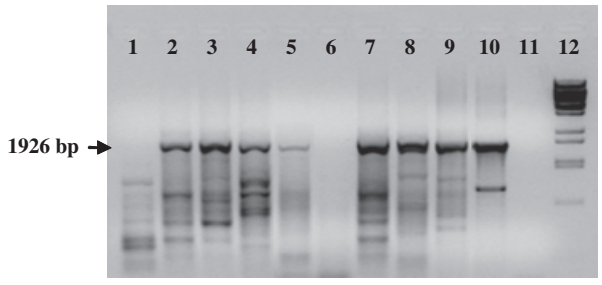

Fig. 2. Dissimilatory sulfite reductase (*dsrAB*) genes in the native and sulfate-enriched samples of Sunday Lake wetland. Lanes marked as: 1, SURN-top; 2, BOG-top; 3, BOG-bottom; 4, Sedge; 5, MAT; 6, SURN-top-SO_4^{2-}; 7, BOG-top-SO_4^{2-}; 8, MAT-SO_4^{2-}; 9, Cheesequake (saltmarsh mat); 10, *Desulfovibrio desulfuricans* G200 (positive control); 11, blank; 12, size marker – λ DNA-BstE II digest.

collected from water-saturated sites in Sunday Lake. These included the top and bottom samples of the BOG, the Sedge, and the MAT (bottom only). No products were detected in samples collected at drier sites such as SURN top (lane 1 in Fig. 2) and bottom (not shown), SURF, and Upland soil (data not shown). Soil incubations with sulfate noticeably enhanced PCR signals in the BOG-top and MAT samples, but not in the SURN-top sample. Thus, the presence of *dsrAB* in microbial biomass was related to potential methylation rates (Fig. 1) with MAT and BOG samples positive for both. On the other hand, the absence of significant methylation activities in SURN (Fig. 1) may be related to low abundance of SRB.

Nested PCR detection of 16S rRNA genes of SRB

Initial surveys using 16S rRNA gene detection suggested that SRB, if present, represented a minor component in the SURN community (Yu *et al.*, unpublished data). We therefore used the nested PCR approach of Daly *et al.* (2000) and Dar *et al.* (2005) to enhance the sensitivity of SRB detection in samples collected at all six Sunday Lake sites. A DNA extract from a saltmarsh mat, where sulfate reduction was possibly the dominant anaerobic electron accepting process, served as a positive environmental control. It was confirmed that the primer sets for SRB1–4 from Daly *et al.* (2000) and new primer sets for SRB5–6 from this study could successfully distinguish the six SRB groups in the Sunday Lake samples by using six reference strains representing each SRB group (Fig. S1).

The nested PCR approach using SRB-specific 16S rRNA gene primers detected SRB1, 2, 5 (Fig. S1), and 6, but failed to detect SRB3 and 4, in all Sunday Lake samples (Table 2). SRB5, consisting of complete oxidizers (Daly *et al.*, 2000), were present in all sampling sites. The most diverse SRB communities were observed in the BOG and MAT samples, niches that are water saturated year round, and the only samples where group 2, related to *Desulfobulbus* spp., were present. The lowest diversity of SRB was found in Upland soil samples, which remained largely aerated year round, and where only SRB5 were detected. Sites that were sampled at two depths (e.g. SURN-top, SURN-bottom) did not differ in SRB distribution.

Enrichment with sulfate had little effect on SRB distribution in the MAT and BOG samples. In SURN, sulfate amendment resulted in loss of organisms representing the three SRB groups that were present in the unamended sample (Table 2). As this sample did not methylate Hg under any of the test conditions (Fig. 1), the significance of this change to MeHg production is probably negligible. These results clearly show that SRB were present in the microbial communities of Sunday Lake, and that amendment with sulfate did not cause a noticeable shift in SRB community structure for the MAT and BOG samples where a potential for Hg methylation was noted (Fig. 1). Because results suggested that the MAT sample was a 'hot spot' for the accumulation of both THg and MeHg, for potential methylation rates, and for diversity of SRB, further work was focused on the characterization of the MAT SRB community.

Phylogenetic analysis of clone libraries of *dsrB* genes from native MAT samples

Of 90 clones of *dsrB* genes that were sequenced from the native MAT samples, only 19 sequences were 78–87% similar to cultured lineages, while the remaining 71 sequences were 74–95% similar to uncultured ones. Phylogenetic analysis (Fig. 3) showed that, with two exceptions, MAT *dsrB* sequences formed large clusters that had no clear similarity to *dsrB* of cultured SRB. 41.1% of these clustered with an uncultured SRB clone W23 (DQ855257) from metalliferous peats in western New York (Martinez *et al.*, 2007). The other 37.8% were weakly affiliated with uncultured *dsrB* from an agricultural grassland soil (AM901622) (Miletto *et al.*, 2010). Clones similar to *dsrB* of known SRB (the two exceptions) included (1) 11 and six clones that clustered with *dsrB* of *Desulfobulbus* spp. (SRB2) and *Syntrophobacter* spp., respectively, and (2) two sequences, which clustered with *dsrB* from *Desulfovibrio aminophilus* (SRB6) and *Desulfobacca acetoxidans*. Sequences similar to Archaeal *dsrB* genes were not found.

Table 2. Distributions of SRB groups and effects of sulfate enrichment (2 mM) on this distribution in samples collected at various ecological niches within the Sunday Lake wetland during July 2005 sampling*

Niche	SRB1	SRB2	SRB3	SRB4	SRB5	SRB6
SURN-top†	+	−	−	−	+	+
SURN-bottom	+	−	−	−	+	+
SURF-top	+	−	−	−	+	+
SURF-bottom	+	−	−	−	+	+
Upland soil	−	−	−	−	+	−
BOG-top	+	+	−	−	+	+
Sedge	+	−	−	−	+	+
MAT	+	+	−	−	+	+
SURN+SO_4^{2-}‡	−	−	−	−	−	−
BOG+SO_4^{2-}	+/−	+	−	−	+	+
MAT+SO_4^{2-}	+	+	−	−	+	+

*+ and − indicate the presence and absence, respectively, of PCR products specific for the indicated group.

†Samples were collected from the 'top' 15 cm or the 'bottom' 15 cm of the soil.

‡Sample to which sulfate was added followed by a 3-week incubation before methylation assays and DNA extraction. Both SURN and BOG were from the top layer.

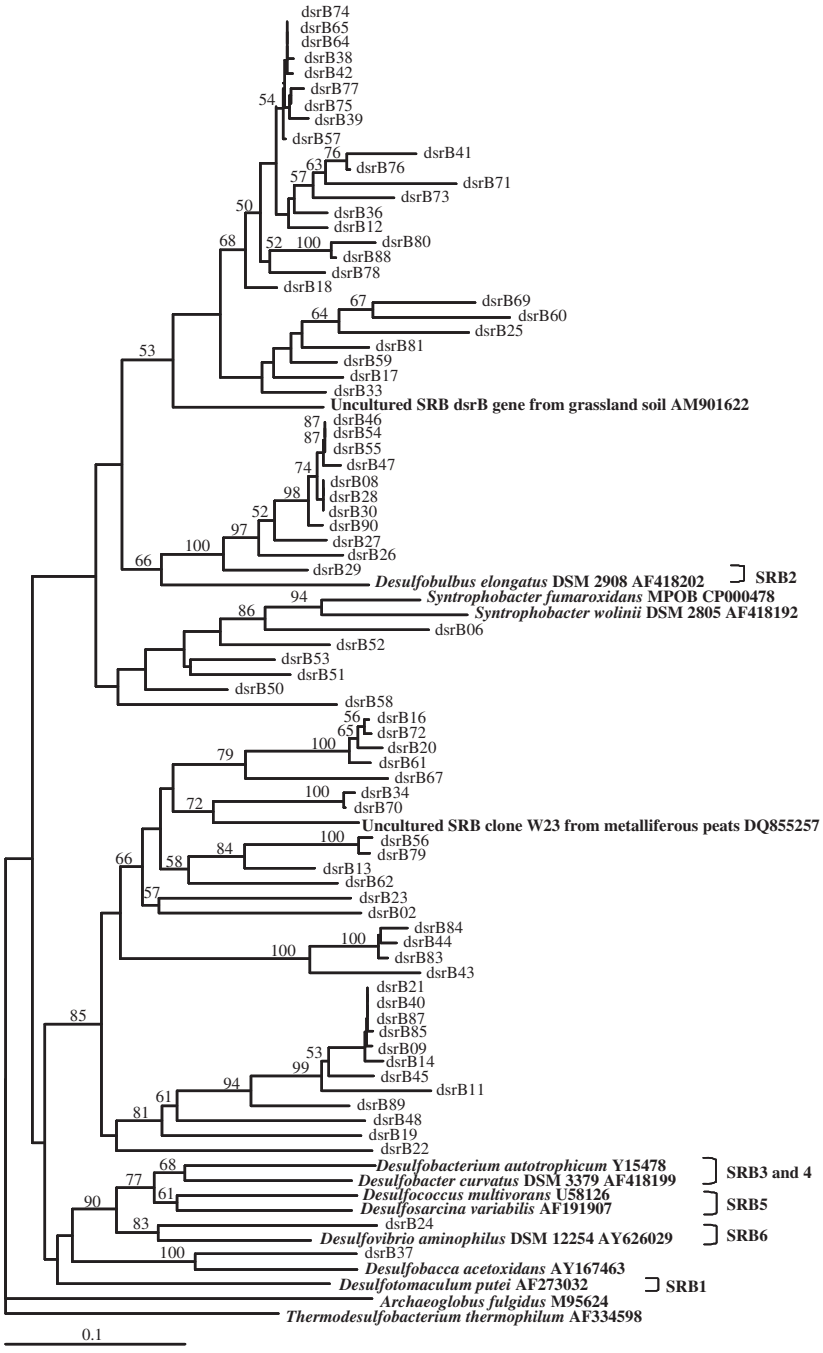

Fig. 3. Phylogenetic analysis of *dsrB* gene sequences in the native MAT (Sunday Lake) sample. The neighbor-joining method was used and bootstrap values were shown at branch points. The scale bar indicated 10% sequence difference.

MPN estimates and molecular characterization of *dsrB* gene of SRB and PO-SRB in MAT enrichments

To further evaluate the abundance of SRB in the MAT sample, we estimated their number using the MPN method. Because syntrophs that switch their metabolism to sulfate reduction are known to oxidize propionate (Harmsen *et al.*, 1998; de Bok *et al.*, 2004; Chen *et al.*, 2005), another set of MPN tubes was set up with propionate as a sole carbon source and sulfate as the electron acceptor to enumerate PO-SRB. MPN estimates of SRB in the Cheesequake salt-marsh mat sample were included as a control. Results were scored after 30, 60, and 90 days of incubation (Table 3). All dilutions showed an increase in MPN estimates between 60

and 90 days of incubation, with the increase in PO-SRB being most pronounced, increasing from 2.2×10^3 to 9.3×10^3 cell mL^{-1} during that time interval. After 90 days, the PO-SRB (9.3×10^3 cells mL^{-1}) accounted for 33% of the total SRB counts (2.8×10^4 cells mL^{-1}) in the MAT sample. As expected, the MPN estimates for SRB at the MAT site were lower than those in the saltmarsh mat at all time intervals (Table 3).

As biomarkers of microbial sulfate reduction, the *dsrB* genes were detected in all MPN-positive tubes for the enrichment of SRB and for the 10^{-1} dilution of the PO-SRB series (Fig. 4). *dsrB* phylogeny (Fig. 5) based on the sequences of retrieved DGGE fragments showed that most sequences, seven of the total eight, from the SRB-MPN series formed a cluster closely grouped with *dsrB* from *Desulfovibrio desulfuricans* subsp. *desulfuricans* (AF273034) and *D. desulfuricans* isolate SRDQC (DQ450464), and were loosely affiliated with *D. desulfuricans* G200 (SRB6). These seven partial *dsrB* gene sequences were 90–95% similar to isolate SRDQC and their clustering together with reference *Desulfovibrio* spp. (Fig. 5) suggested that *Desulfovibrio*-like strains probably dominated the enriched MAT SRB communities. Three closely related sequences from the PO-SRB MPN enrichments branched at the base of the *Desulfovibrio* clade (Fig. 5), suggesting that they differed from those dominated in the SRB-MPN series. With the exception of one sequence from the 10^{-1} dilution series of the SRB-MPN, which was related to *dsrB* from SRB2 (*Desulfobulbus* spp.), none of the *dsrB* MAT sequences was affiliated with other groups of SRB. Thus, enrichments of SRB in the floating MAT sample were dominated by SRB6, and those enriched by propionate as a carbon source formed a unique clade.

Discussion

The concentrations of Hg and MeHg and potential Hg methylation activities in distinct habitats within the Sunday Lake watershed were examined in order to identify Hg sinks and sites of MeHg production. The highest MeHg concentrations (18.6–21.2 ng g^{-1}; Table 1) were found in *Sphagnum*-dominated sites (i.e. BOG-top, MAT) where high THg concentrations were also noted. In contrast, riparian soil samples (i.e. SURN, SURF), previously considered as a sink for THg and a source of MeHg to the Sunday Lake ecosystem (McLaughlin, 2003), had 26–90 times lower concentrations of MeHg than the BOG and MAT samples. The MeHg concentrations in the BOG-top and MAT samples were comparable to the concentrations in Berry's Creek sediment (32.3 ng g^{-1}), an industrially contaminated site (Schaefer *et al.*, 2004), although THg concentrations in the latter were 35–54 times higher than in Sunday Lake samples. These results suggest that MeHg production and/or accumulation occur in *Sphagnum* mats, which may serve as a source of MeHg for Sunday Lake and other northern forest lakes.

The THg concentrations in BOG-top and MAT (Table 1) in this study were higher than the range (7.6 ± 4.6–155.4 ± 65.8 ng g^{-1} dw) reported by McLaughlin (2003) in *Sphagnum* samples collected in the riparian site of Sunday Lake. Nonetheless, the values from *Sphagnum* samples in McLaughlin's study (2003) were generally the highest among various terrestrial and riparian vegetation examined and other sites in the Adirondacks (Selvendiran *et al.*, 2009). Unfortunately, McLaughlin (2003) did not collect samples near the lake-front (e.g. MAT, BOG), nor were MeHg concentrations determined for any of the samples in that study (McLaughlin *et al.*, unpublished data). Similar to our findings, Moore *et al.* (1995) reported that at a peatland lake in Ontario, CA, the highest concentrations of THg and MeHg in wetland plants were found in bryophytes, with THg ranging from 27 to 119 ng g^{-1} dw, 1.6–2.5 times lower

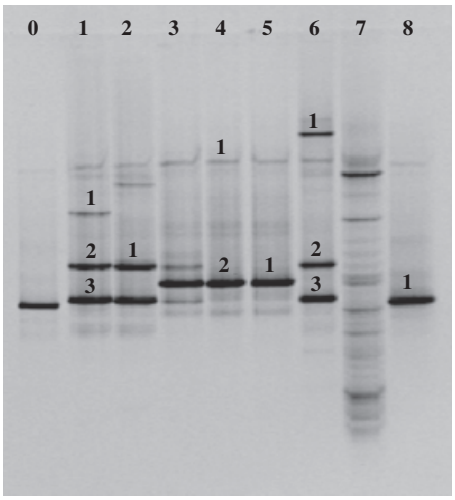

Fig. 4. DGGE of bacterial *dsrB* PCR products amplified from DNA extracts of MPN cultures that were set up with MAT samples. Lanes 0 and 8, *Desulfovibrio desulfurican* G200 (positive control). Lanes 1–5, cultures of the 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} dilutions of the SRB MPN series after 12 days of incubation. Lane 6, the 10^{-1} dilution of the PO-SRB series after 9 days of incubation. Lane 7, Cheesequake saltmarsh mat.

Table 3. MPN estimates (cells mL^{-1}) of SRB and PO-SRB in the MAT sample, Sunday Lake, NY, and of SRB in Cheesequake saltmarsh mat, NJ

		30 and 60 days*		90 days	
Site	Group	MPN	95% CL†	MPN	95% CL
Sunday Lake	SRB	2.2×10^4	(0.7–7.3)	2.8×10^4	(0.9–9.2)
	PO-SRB	4.3×10^3	(1.0–18.0)	9.3×10^3	(1.0–18.0)
Cheesequake	SRB	7.0×10^6	(2.1–23.1)	1.1×10^7	(0.3–3.6)

*Period for anaerobic incubation at 28 °C.
†Numbers in parentheses indicate the 95% confidence limits (CL) of MPN estimates.

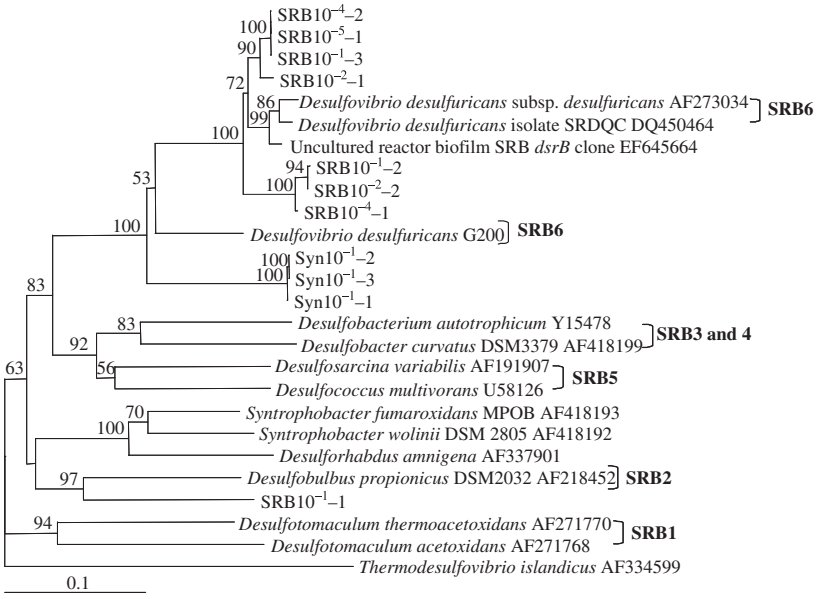

Fig. 5. Phylogenetic analysis of *dsrB*-based DGGE fragments of SRB and syntrophic PO-SRB from MPN series dilution cultures of the MAT sample. The neighbor-joining method was used and bootstrap values were shown at branch points. The scale bar indicated 10% sequence difference. Clone designation denotes the MPN enrichment medium (the initial three letters of the clone; SRB for SRB-MPN medium or Syn for PO-SRB medium), culture dilution (10^{-1}–10^{-5}), and the number of DGGE band shown in Fig. 4 (last digit).

than the values reported here in Sunday Lake, and MeHg from 0.2 to 77 ng g^{-1} dw in *Sphagnum*, more than three times higher at the maximum level than the highest value (21.2 ng g^{-1} dw) in our study. Grigal (2003) proposed that peatlands are a sink for Hg. In boreal forest wetland ecosystems, *Sphagnum* mosses have high growth rates and large standing crop biomass, and readily form thick mats where dead tissues quickly accumulate in submersed bottom layers. The spongy-like tissues with innumerable enlarged porous dead cells (i.e. hyaline cells) can hold 20 times their dry weight in water, and strongly absorb cations due to a high cation exchange capacity, large specific surface area (> 200 m^2 g^{-1}), and their porous structures (Schofield, 1985; McLelland & Rock, 1988). These properties are conducive to sorption of Hg by submerged *Sphagnum* mats. Consistent with this hypothesis, Bargagli *et al.* (2007) showed that planktonic and benthic moss-dominated mats were the main sinks for Hg in the summer meltwater period in polar regions.

Incubation experiments with native samples from Sunday Lake indicated significantly higher Hg methylation potentials in the MAT sample as compared with SURN and BOG samples (Fig. 1). Floating macrophyte mats and periphyton were found to be the most important sites for Hg methylation in Amazonian floodplain lakes and in the Florida Everglades (Cleckner *et al.*, 1999; Guimaraes *et al.*, 2000; Mauro *et al.*, 2002). Submerged vegetation is considered to be a hot spot for Hg methylation, due to moderately anoxic conditions, availability of substrates for microbial growth provided as carbonaceous plant exudates, and the presence of surfaces for biofilm formation (Cleckner *et al.*, 1999; Hines *et al.*, 1999; Guimaraes *et al.*, 2000). Considering the large area that is dominated by *Sphagnum* moss in the Sunday Lake ecosystem, including the MAT and BOG sites, and methylation activities that were detected year round in samples collected at these sites (Adatto *et al.*, unpublished data), we propose that not only are *Sphagnum* mosses an important sink for the accumulation of Hg but they are also environments conducive for the production of MeHg and therefore a source of MeHg to Sunday Lake.

Opelt *et al.* (2007) showed that *Sphagnum* mosses represent ecological niches that harbor uncharacterized microbial communities. The sponge-like structures in the submerged portion of the *Sphagnum* mat may serve as niches for microbial colonization leading to the formation of biofilms (Richardson, 1981; Solheim *et al.*, 2004). Therefore, it is possible that microorganisms with the ability to methylate Hg colonize the moss tissue. The availability of anaerobic niches and sorption of Hg by the moss tissue may create conditions favorable for Hg methylation by these moss-associated microbial communities.

Our results suggest that Hg methylation in Sunday Lake is associated with sulfate reduction because incubation with sulfate stimulated potential methylation rates, while molybdate addition inhibited them (Fig. 1). Based on previous observations that indicated low abundance of *Deltaproteobacteria* in SURN (Yu *et al.*, unpublished data), a nested PCR approach specifically designed for the detection of SRB at low abundance (Daly *et al.*, 2000) detected SRB in all Sunday Lake samples (Table 2). Permanently saturated sites dominated by *Sphagnum*, BOG, and MAT contained the highest diversity of SRB, possibly because *Sphagnum* and flooded conditions created diverse niches that supported the growth and activities of SRB. Except for SRB3 and 4, which

generally dominate in coastal and marine environments (Dhillon *et al*., 2003; Bahr *et al*., 2005), our studies detected SRB1, 2, 5, and 6 (Table 2). Within these four groups, SRB1, 2, and 6 are incomplete oxidizers that metabolize organic matter into acetate. Group 5 are complete oxidizers. These observations are in agreement with similar studies that used nested PCR approaches to show the presence of *Desulfovibrio–Desulfomicrobium* (i.e. SRB6) and *Desulfococcus–Desulfonema–Desulfosarcina* (i.e. SRB5), but the absence of *Desulfobacterium* (i.e. SRB3) in floating macrophyte rhizospheres from the floodplain of an Amazonian lake where high Hg methylation rates were noted (Acha *et al*., 2005).

The presence of SRB was also confirmed by the detection of *dsrAB* PCR products in all water-saturated sites (i.e. BOG, MAT, Sedge) and the sequencing results of the *dsrB* genes from native samples and MPN enrichments in the MAT site. Interestingly, different *dsrB* genes were detected in native MAT samples (Fig. 3) and those following enrichment in MPN tubes (Fig. 5). The *dsrB* clone library of the native MAT sample consisted mostly of sequences that were related to other environmental *dsrB* and had weak affiliation with any cultured SRB. These, therefore, could belong to novel groups of SRB, not likely to have been detected by the six 16S rRNA gene primer sets that targeted known SRB (Daly *et al*., 2000). On the other hand, *dsrB* genes from the MPN enrichments were dominated by *Desulfovibrio*-like *dsrB* sequences, i.e., related to SRB6. This difference in dominant phylotypes obtained by two different molecular approaches could be due to different environmental conditions during sampling in Sunday Lake, pH 5.0, in July 2005 when the native MAT sample was collected, and pH 6.0 in July 2007, when samples were collected for MPN. It is, however, likely that a shift in community structure was caused by the enrichment conditions (Gittel *et al*., 2009) with the four common organic substrates as carbon and energy sources and sulfate as the electron acceptor. Thus, it seems that the majority of the MAT populations which carry *dsrB* gene homologs might not be able to utilize the four substrates or could not compete with the 'classical' SRB (e.g. *Desulfovibrio* spp.) under the MPN growth conditions provided. These SRB lineages might have also been associated with the metabolism of other substrates including H_2/CO_2, formate, or ethanol. This result suggests that a broad diversity of SRB exists in natural environments such as *Sphagnum* moss mats.

The MPN–DGGE–*dsrB* sequencing approach showed a dominance of *Desulfovibrio*-like sequences (SRB6) in the enriched community. Nested PCR results also showed this group to be one of two most widely detected in the Sunday Lake wetland (Table 2). Many species of *Desulfovibrio* have been confirmed as strong Hg methylators, including *D. desulfuricans* LS (Choi *et al*., 1994) and *D. desulfuricans* ND132 (Jay *et al*., 2002). The only *dsrB* phylotype ($SRB10^{-1}$-1) that was not affiliated with SRB6 was most similar to *dsrB* of *D. propionicus* (1pr3) (Fig. 5), a strong Hg methylator (Benoit *et al*., 2001) related to SRB2. The occurrence of *Desulfobulbus* sp. was consistent with *the dsrB* cloning results (Fig. 3) and the detection of SRB2 in MAT and BOG by 16S rRNA gene analysis using the nested PCR approach (Table 2).

The sulfate-reducing guild in the floating MAT, a hotspot for Hg and MeHg accumulation with a potential for Hg methylation, therefore, were dominated with uncultured *dsrB* lineages, *Desulfobulbus*-like (SRB2), *Desulfovibrio*-like (SRB6), and *Syntrophobacter*-like bacteria. The known phylotypes all represent SRB that incompletely oxidize organic substrates. Thus, it is likely that methylation in the MAT sample is the result of the activity of these incomplete oxidizers. In contrast, King *et al*. (2000) showed that for pure cultures and amended microcosms of subtropical salt marsh sediments, Hg methylating activities were higher among the complete oxidizers, which utilized acetate as compared with the incomplete oxidizers, which utilized lactate. Thus, there may be a clear distinction between members of the SRB guild which methylate Hg in subtropical marine sediments and in northern *Sphagnum* moss-dominated wetlands. Further studies are needed to test this hypothesis.

Observations of low sulfate in Sunday Lake, 27.6–69.5 μM (McLaughlin, 2003), might undermine the role of SRB in Hg methylation. However, it is possible that SRB metabolizing in syntrophy (Pak & Bartha, 1998) or by fermentation methylated Hg. The high proportion of PO-SRB accounting for about one-third of all SRB in the MPN enrichments (Table 3) and the evidence that *Syntrophobacter*-like genes represented 6.7% of the native MAT *dsrB* clone library support the former. PO-SRB (e.g. *Syntrophobacter*s) are known to engage in syntrophic relationships with methanogens (Muyzer & Stams, 2008), and indeed, the *mcr* gene was readily detected by PCR in the MAT DNA extract (R.-Q. Yu, unpublished data). These results and observations highlight the need for a thorough investigation of the role of syntrophy in Hg methylation in northern wetlands.

Our work clearly indicated that *Sphagnum*-dominated niches within the wetland were a sink for THg and a source of MeHg, and strongly suggests that floating *Sphagnum* moss mats are a hot spot for MeHg production and accumulation. Considering the immense biomass of *Sphagnum* moss mats in boreal peatlands, this habitat may play a critical role in the production and accumulation of MeHg in high-altitude forested ecosystems.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. PCR amplification products indicating presence of 16S rRNA genes homologous to those of SRB5 in Sunday Lake samples.
Table S1. Primers and PCR conditions that were used in this study.

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