MICROBIAL MERCURY METHYLATION AND DEMETHYLATION: BIOGEOCHEMICAL MECHANISMS AND METAGENOMIC PERSPECTIVES IN FRESHWATER ECOSYSTEMS

by

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ABSTRACT OF THE DISSERTATION

Microbial Mercury Methylation and Demethylation: Biogeochemical Mechanisms and Metagenomic Perspectives in Freshwater Ecosystems

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Elevated concentration of methylmercury (MeHg) in fish is a worldwide concern due to its detrimental effects on human health. Although Hg methylation is a key issue regarding MeHg contamination, neither abiotic nor microbial methylation mechanisms are well understood. The overall objective of this study was to link the potential for microbial methylation and demethylation to the molecular characterization of microbial communities in two typical freshwater ecosystems and to gain in-depth understanding of Hg methylation mechanisms by syntrophy.

Sunday Lake is a remote and "pristine" forest lake exposed to Hg mostly through atmospheric deposition in the Adirondack Mountains, New York. This study demonstrated that floating *Sphagnum* moss mats near the lake water front were hot spots for MeHg accumulation and microbial methylation, and sub-habitats where sulfate reducing bacteria (SRB) community was highly developed. SRB were identified as a major group of Hg methylators, as sulfate addition to the mat samples doubled the potential Hg methylation rates while molybdate significantly inhibited them. The dominant distribution of *Syntrophobacter* spp. in the *Sphagnum* mats led to the

investigation of syntrophy in Hg methylation. By incubating mono- or co-cultures of *Syntrophobacter* spp., *with Desulfovibrio* spp., this study was the first to demonstrate that a *Syntrophobacter-Desulfovibrio* coculture significantly increased growth of both syntrophic partners and stimulated MeHg synthesis compared to activities of *Desulfovibro* spp. monocultures. Syntrophy could stimulate MeHg synthesis by two pathways: *Desulfovibrio* growing with methanogens in sulfate-free environments, and *Desulfovibrio* growing with *Syntrophobacter* in sulfate-limited environments where sources of energy and carbon are limited.

In the South River, an industrially Hg-contaminated site in Virginia, high Hg methylation rates and low demethylation activities were observed in nine sites downstream from the contaminating source, partially explaining why fish in this river have high MeHg levels. 16S rRNA sequencing from sediment cDNA showed that at least three groups of SRB and one group of *Geobacter*-like iron reducing bacteria (IRB) that were closely affiliated to known Hg methylators, were active in the sediments. Further metabolic inhibition and stimulation experiments confirmed that both SRB and IRB were involved in the microbial methylation in South River sediments.

DEDICATION

To my father, in memory of his anticipation.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Bacteria

CHAPTER 1

GENERAL INTRODUCTION

1. 1. MERCURY CHEMISTRY – OVERVIEW

Mercury (Hg) is a naturally occurring element which originates from the earth's crust, oceans and atmosphere. Mercury in rocks and mineral deposits commonly occurs as cinnabar, a mercury sulfide (HgS) mineral (Barnes & Seward, 1997). The unique properties of Hg make it useful in many applications such as gold and silver mining, chlor-alkali manufacturing, reaction catalysis, biocide treatments, and the production of dental amalgams and pharmaceuticals (Fitzgerald & Lamborg, 2005).

As a transition metal, mercury has four main chemical forms, elemental Hg^0, mercurous Hg_2^{2+}, mercuric Hg^{2+}, and organic mercury and has a complex biogeochemical cycle (Clarkson & Magos, 2006). Elemental mercury is a silver-white metal that is a liquid at room temperature and easily vaporizes. Because of its low Henry's Law constant, Hg^0 is only slightly soluble in water and is relatively unreactive. Mercurous mercury is rare, and is typically found in calomel or as mercurous chloride (Hg_2Cl_2), while mercuric Hg accounts for most of ionic forms of Hg. Organic forms of Hg include methylmercury (MeHg or CH_3Hg) and dimethylmercury (Me_2Hg) (Akagi et al., 1975) with MeHg as the dominant form in natural environments. Organic mercury is the most toxic organic form of Hg and is most readily accumulated by aquatic organisms (NCR, 2000).

1. 2. BIOGEOCHEMICAL CYCLING OF MERCURY

Mason and Sheu (Mason & Sheu, 2002) estimated that, 36% of all Hg existing in the environment is released through natural processes including surface volcanic eruptions, deep sea vents and volcanic activity, hot springs, evaporation from the ocean basins, other water bodies and soils. However, the remaining 64% are the result of anthropogenic inputs of mercury to the environment (Mason & Sheu, 2002). In 2000, anthropogenic sources of Hg included about 65% from stationary fuel combustion, 11% from gold production, 7% from nonferrous metals production, 6% from cement production, and the rest from a variety of smaller source types (Pacyna et al., 2006). In the atmosphere, nearly 95% of total Hg is in the elemental form (Hg^0) which has a residence time on the order of a year (Morel et al., 1998). Elemental Hg could be oxidized slowly by ozone, bromine, and UV in the atmosphere (Fig. 1.1). Obrist et al. (2011) recently showed that bromine species were the primary oxidants of elemental Hg over the Dead Sea, and suggested that bromine-induced Hg oxidation may be an important source of Hg to the world's ocean.

Atmospheric deposition after long-range transport is the dominant source of Hg to most remote areas of the planet (Mason et al., 1994), resulting in the contamination of pristine water ecosystems such as forest lakes in the Adirondacks. Therefore, Hg is considered as a global pollutant. Once elemental Hg is oxidized in the atmosphere, Hg(II) is rapidly absorbed by rain, snow or particles, and precipitates onto water or land (Fig. 1.1). Newly formed Hg(II) is generally highly reactive and bioavailable for microbial transformations and directly or indirectly taken up by fish (Harris et al., 2007). Freshwater ecosystems are extremely sensitive to the impact of Hg precipitation. Ionic Hg in freshwater systems is mainly partitioned by sedimentation, and subjected to biological

and abiotic reduction to its elemental form (Morel et al., 1998). Biological reduction of Hg(II) could be mediated by the mercury resistance (*mer*) system in Hg resistant bacteria (Fig. 1.1 and 1.2), a specific detoxification mechanism encoded by the *mer* operon (Barkay et al., 2003). Microbial mercury methylation is a key process producing the neurotoxic substance MeHg, which occurs mostly in wetland soil, lake sediment, and floating mats or biofilms (Compeau & Bartha, 1985; Gilmour et al., 1992; Cleckner et al., 1999; Yu et al., 2010). These habitats are also major sinks for MeHg in aquatic systems (Barkay & Wagner-Döbler, 2005). Methylmercury is a lipophilic form of mercury that is highly toxic and readily biomagnified and bioaccumulated by aquatic food chains. For example, MeHg biomagnification occurred from a primary producer phototrophic green alga *Zygogonium* to primary consumers in geothermal springs (Boyd et al., 2009). MeHg concentrations in stratiomyid (Diptera: Stratiomyidae) larvae grazing on *Zygogonium* in microbial mats in Yellowstone National Park were 2-5 times higher than concentrations in the microbial mat biomass (Boyd et al., 2009). Concentrations of total Hg or MeHg in surface waters often do not correlate with the Hg content of fish as MeHg is the fraction of total mercury that is most efficiently transferred up the food chain (Wang & Wong, 2003; Driscoll et al., 2007). It has been suggested that more than 85 to 90% of Hg in fish, regardless of trophic level, is present as MeHg (Grieb et al., 1990). A 2004 National Listing of Fish Advisories by EPA showed that 35% of the US's lake acres (excluding the Great Lakes) and 24 % of the US's river miles were under a fish advisory (EPA, 2011). Human uptake of MeHg, which often occurs via consumption of fish high in MeHg, can cause damage to the nervous system, and is of special concern regarding fetal brain development. In the body, MeHg forms a complex with the amino acid

cysteine, which has a structural similarity to the large, neutral amino acid, methionine. Because of this similarity, MeHg can easily gain entry to the endothelial cells of the blood-brain barrier and acts as a neurotoxicant (Clarkson & Magos, 2006). Due to increased concerns about the health effects of Hg, the EPA and the FDA have established, respectively, the National Mercury Advisory and the Consumer Advisory on the consumption of fish.

1.3. MICROBIAL MERCURY METHYLATION AND METHYLATING MICROORGANISMS

In most aquatic ecosystems, the external supply of methylmercury is insufficient to account for MeHg accumulation in biota and sediments (Gilmour & Henry, 1991). Thus, *in situ* MeHg formation plays a key role in determining the amount of MeHg reaching higher trophic levels. The Hg methylation phenomenon in natural environments was first identified by Jensen and Jernelöv (1969). They observed that concentrations of MeHg significantly increased over days of incubation after spiking sediments samples taken from aquaria and a lake near Stockholm, Sweden, with Hg(II). Since this activity was abolished upon sterilization, the authors suggested that MeHg synthesized by microorganisms in sediments significantly contributed to the uptake and distribution of MeHg in lake fishes. It was subsequently shown that Hg methylation in sediments and soils was generally of biological rather than chemical origin (Berman & Bartha, 1986). However, specific microorganisms responsible for Hg methylation remained unknown for a long time. By using metabolic inhibitors, e.g., molybdate as a specific inhibitor for sulfate reduction and 2-bromoethane sulfonate (BES) as a methanogenesis inhibitor,

Compeau and Bartha (1985) clearly demonstrated that sulfate reducing bacteria (SRB) were responsible for 95% of Hg methylation, while methanogens were not involved in MeHg synthesis in saltmarsh sediments. These observations were contrary to previous hypotheses that attributed Hg methylation to methanogens (Wood et al., 1968; Wood, 1974). The nearly complete inhibition of Hg methylation by molybdate in freshwater reservoir sediments further supported the hypothesis that SRB were responsible for Hg methylation (Gilmour et al., 1992), leading to the conclusion that these bacteria are the principal producers of MeHg in sediments and soils.

In saltmarsh and marine sediments, a positive relationship between Hg methylation rates and sulfate reduction rates was exhibited (King & Garey, 1999; King et al., 2001). When no sulfate was present in the culture medium, no Hg methylation activities occurred for *Desulfobacterium* sp. strain BG-33, a complete oxidizer, and for *Desulfovibrio desulfuricans* ATCC 13541, an incomplete oxidizer (i.e., SRB that could not utilize acetate) (King et al., 2000). This strong correlation between sulfate and Hg methylation is probably relevant in coastal and marine sediments where sulfate is abundant and where sulfate reduction is favored as the terminal oxidation process in anoxic sediments. However, in freshwater environments, sulfate is often limited; therefore, fermentation and syntophic metabolism could be important mechanisms contributing to MeHg synthesis (Choi & Bartha, 1993; Pak & Bartha, 1998). The transport of Hg into microbial cells is a key process for Hg microbial methylation as methylation is thought to be an intracellular process. Benoit et al. (1999) showed that the availability of Hg for methylation in sediment porewater was strongly correlated with neutrally charged Hg-sulfur complex (HgS^0), rather than with Hg^{2+} or total dissolved Hg.

This author and coworkers (Benoit et al., 2001b) further tested this conclusion by using a pure bacterial strain, *Desulfobulbus propionicus* (1pr3), and reported that MeHg production was linearly correlated with the neutral dissolved complex HgS^0, and that this complex could be taken up by cells via passive diffusion. However, at high sulfide concentrations leading to formation of charged mercury-polysulfide, MeHg in cultures declined (Benoit et al., 2001a, b). Mercury-polysulfide in natural waters, while not satuarated with cinnabar, has a tendency to quickly shift from HgS^0 to charged complexes and thus reduces methylation rates (Jay et al., 2002).

The biochemical mechanism of Hg methylation was initially studied with *Desulfovibrio desufuricans* strain LS. Berman et al. (1990) showed that when adding ^{14}C-serine (^{14}C was labeled in the methyl group) to the culture medium, 95% of the ^{14}C was recovered as ^{14}C-MeHg by strain LS with tetrahydrofolate and cobalamin as two important metabolic intermediates. By using propyl iodide as an inhibitor of transmethylation, this study concluded that cobalamin contributed to methyl transfer during the process of Hg methylation. Serine is the methyl donor in the cobalamin methylamine process. Choi and Bartha (1993) further found that when ^{57}Co was added to strain LS cultures, Hg methylation was stimulated 2.5 fold and 97% of ^{57}Co was associated with cobalamin, consistent with earlier observations on the role of cobalamine in methylation. Although methylcorrinoid compounds are capable of abiotic MeHg synthesis (Ridley et al., 1977), Choi et al. (1994) demonstrated by using crude cell extracts of strain LS that Hg methylation was enzymatically catalyzed rather than a spontaneous transmethylation. Based on these findings, Choi et al. (1994) proposed that methylcobalamin is a key catalyst in the Hg methylation process, and that methylation of

Hg by *D. desulfuricans* LS proceeded through the acetyl-CoA sythase pathway. Understanding of the Hg methylation pathway was further extended by Ekstrom et al. (2003). Using chloroform as an inhibitor of the acetyl-CoA pathway, they concluded that only complete-oxidizer SRB (*Desulfococcus*, *Desulfosarcina*, and *Desulfobacterium*) and *D. desulfuricans* LS, an incomplete oxidizer, use the acetyl-CoA pathway for Hg methylation. Most Hg methylators that incompletely oxidize organic substrates (e.g., *Desulfovibrio africanus*, *Desulfobulbus propionicus*) do not actually have the acetyl-CoA pathway, which converts acetate into carbon dioxide by breakdown of acetate into CO and a methyl moiety, and subsequently oxidizes both into CO_2. For *D. africanus*, chloroform did not inhibit either growth or Hg methylation, suggesting that Hg methylation by this strain is independent of the acetyl-CoA pathway. *D. desulfuricans* LS is a unique incomplete oxidizer which could not degrade acetate but contains Acetyl-CoA pathway enzymes, presumably as a minor biosynthetic process (Ekstrom et al., 2003). MeHg synthesis by the complete oxidizer SRB, therefore, is mainly catalyzed by a B_{12} (or cobalamin)-containing methyltransferase, while MeHg synthesis by incomplete oxidizers like *D. africanus* is mediated by a B_{12}-independent methyltransferase (Ekstrom & Morel, 2008). Another biochemical pathway for Hg methylation includes the methionine synthase pathway in *Neurospora crassa* (Landner, 1971). However, MeHg synthesis by this aerobic fungus most likely accounts for for only a minor contribution of MeHg in the environment.

Recent studies reported that iron reducing bacteria (IRB) including *Geobacter* sp. strain CLFeRB, *Geobacter sulfurreducens*, and *Geobacter metallireducens* are active Hg methylators and suggested that iron reduction represents another major pathway

contributing to MeHg synthesis in aquatic ecosystems (Fleming et al., 2006; Kerin et al., 2006). Phylogenetically, these strains are affiliated with the Deltaproteobacteria (Kerin et al., 2006). However, the mechanism for Hg methylation by *Geobacter* spp. is largely unknown.

It is clear that microbial Hg methylation activity is a co-metabolic process and has diverse mechanisms. SRB strains with Hg methylating ability are scattered throughout the phylogenetic tree of the SRB (Benoit et al., 2003) indicating that phylogenetically similar organisms may have different Hg methylation capabilities. Previous studies demonstrated that bacteria including SRB and IRB which are involved in environmentally significant methylation are associated with Deltaproteobacteria (Ranchou-Peyruse et al., 2009). Hg methylation potentials seem neither genus- nor species-dependent, suggesting that community analysis by metagenomic sequencing of SRB and IRB and subsequent identification at the genus level may not be the only tool for identifying potential Hg methylators in natural environments (Ranchou-Peyruse et al., 2009).

1.4. MICROBIAL DEMETHYLATION OF METHYLMERCURY

MeHg is ubiquitous, and is found in many environments where the total ambient Hg concentration is low. MeHg in its cationic form (CH_3-Hg^+) is kinetically stable in water and is generally associated with anions (i.e. Cl^-, SO_4^{-2}) (Morel et al., 1998). Soil and sediment can be significant as either sources or sinks for MeHg in aquatic systems. Microbial degradation of MeHg (demethylation), a naturally occurring process of which little is known, plays a very important role in mercury biogeochemical cycling and detoxification.

Natural demethylation is usually caused either by microbial activity or light photoreduction (Morel et al., 1998). There are generally two types of microbial demethylation reactions. One is reductive demethylation, which degrades MeHg to Hg^0 and CH_4. Reductive demethylation is mediated by mercury resistance (*mer*) operon systems (see Figs. 1.1 and 1.2), dominating in more aerobic settings (e.g., oxic water layers), and may be induced by high Hg concentration (Oremland et al., 1991; Marvin-DiPasquale et al., 2000; Schaefer et al., 2004). Microbial *mer* system functions are encoded by specific *mer* genes that are arranged in an operon. One *mer* encoded enzyme, organomercurial lyase (MerB), cleaves the C-Hg bond of CH_3Hg to CH_4 and Hg^{2+} (Schottel, 1978) and another, mercuric reductase (MerA), then reduces Hg^{+2} to Hg^0 (Furakawa & Tonomura, 1972; Barkay et al., 2003). MerA was induced in polluted water only when the Hg concentration exceeds 50 pM (Morel et al., 1998). Another known pathway of Hg demethylation is oxidative whereby MeHg is degraded to Hg^{2+} and CO_2. Oxidative demethylation is an important degradation activity dominating in anaerobic environments with low levels of Hg (e.g., sediment or soil) (Marvin-DiPasquale et al., 2000; Schaefer et al., 2004).

MeHg can also be degraded abiotically in oxic waters of lakes and seawater via photodegradation (Suda et al., 1993; Sellers et al., 1996). This may be caused by singlet oxygen generated by photochemical reactions (Suda et al., 1993). Photodegradation is likely to be the main degradation pathway for MeHg in oxic water bodies with low mercury concentrations (<50 pM).

1.5. PURPOSE OF STUDY

The overall purpose of this study was to increase understanding of the biogeochemical mechanisms of microbial mercury methylation and demethylation, to characterize the microbial communities involved in Hg methylation by metagenomic approaches in two distinct freshwater ecosystems: a low Hg-contaminated forest lake peatland in Sunday Lake, Adirondack Mountains, NY, and a highly Hg-contaminated riverine ecosystem in the South River, VA, and to explore Hg methylation pathways by syntrophic associations among *Syntrophobacter* spp., *Methanospirillum hungatei*, and two Hg methylating SRB strains *Desulfovibrio desulfuricans* ND132 and *Desulfovibrio africanus* DSM 2603. To accomplish these goals, samples including water, soil or sediments were collected over a four-year period (2004 to 2007) in Sunday Lake, and a two-year period (2008 and 2010) in the South River.

Specific objectives for the Sunday Lake project:

1. To detect sulfate reducing bacteria by *dsrAB* genes and characterize the diversity of SRB in floating *Sphagnum* moss mats by *dsrB* genes;
2. To study the distribution of SRB in various habitats and characterize SRB in floating *Sphagnum* moss mats by nested PCR, DGGE and sequencing of 16S rRNA genes;
3. To determine potential methylation rates in various lake subhabitats (contributed by Dr. Mark Hines's lab, University of Massachusetts Lowell, as a part of a collaborative study);
4. To estimate abundance of SRB and propionate-oxidizing SRB in floating *Sphagnum* moss mats by MPN enrichments and characterize the *Syntrophobacter*-like bacteria in the enrichments;

5. To analyze Hg and MeHg and other geochemical parameters in different habitats in the Sunday Lake ecosystem (contributed by Dr. Charley Driscoll's lab, University of Syracuse, as a part of a collaborative study).
6. To study the Hg methylation mechanism by syntrophy through incubation of mono- and co-cultures of three representative strains of *Syntrophobacter* spp., one methanogen, and two Hg methylating *Desulfovibrio* spp.

Specific objectives for the South River project:

1. To determine potential methylation rates in sediment from nine sites along a 21-km river reach downstream of a Hg contamination source;
2. To determine potential demethylation rates in sediment from nine sites along a 21-km river reach downstream of a Hg contamination source;
3. To characterize the active microbial communities including SRB and IRB by 16S rRNA in RNA extracts of four river sediment samples collected at sites representing high potential methylation and demethylation activities;
4. To study the influence of metabolic inhibitors and stimulators on Hg methylation and MeHg degradation;
5. To reconstruct the phylogeny of potential Hg methylating SRB and IRB, and to determine the relationships of these groups to measured potential Hg methylation rates and other biogeochemical parameters;
6. To analyze Hg, MeHg, and other geochemical parameters in sediment from ten different sites along a 21-km river reach downstream from a Hg contamination source.

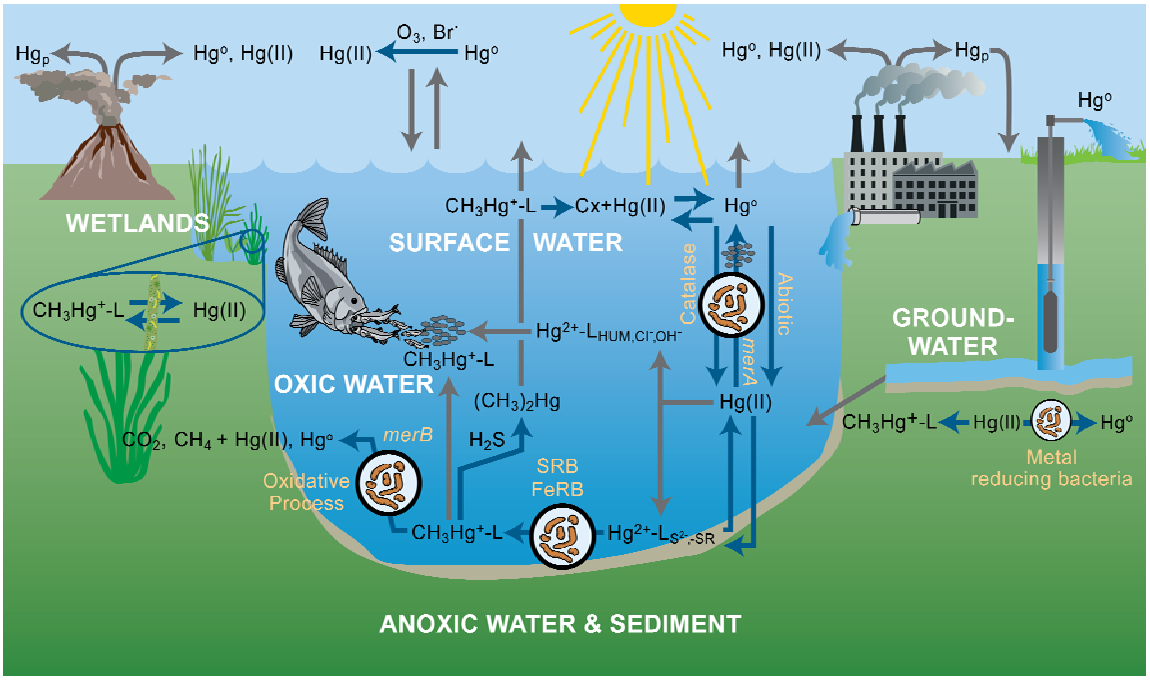

Fig. 1.1. The biogeochemical cycling of mercury in the environment (Lin et al, in press).

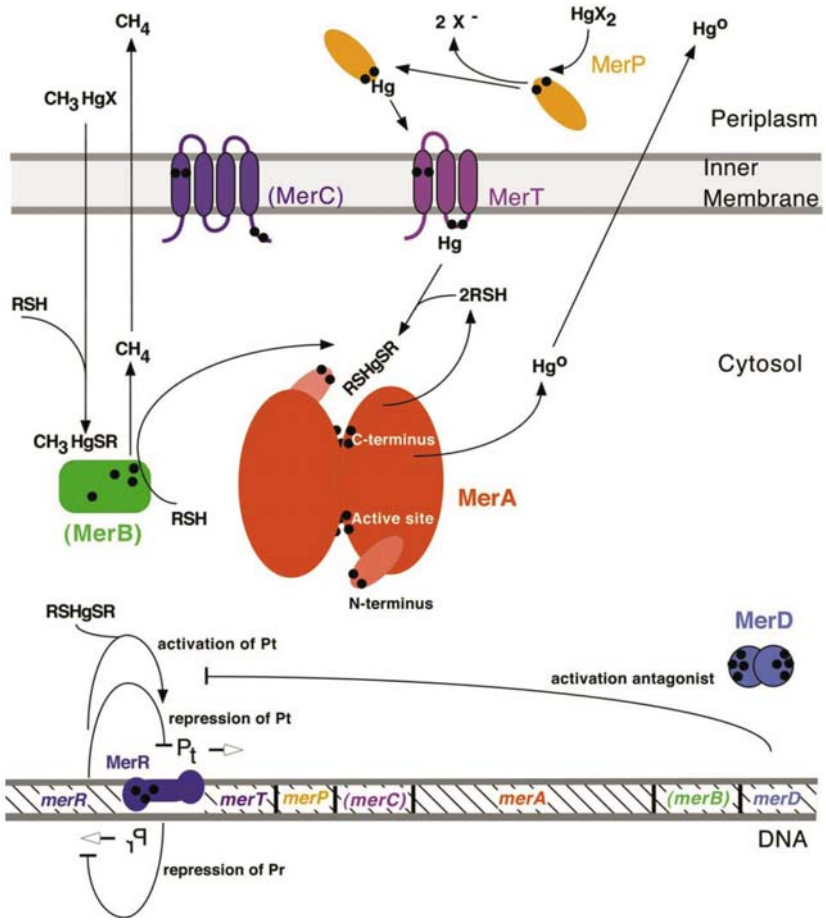

Fig. 1. 2. Model of a typical Gram-negative mercury resistance (*mer*) operon. The symbol • indicates a cysteine residue. X refers to a generic solvent nucleophile. RSH depicts low molecular mass, cytosolic thiol redox buffers such as glutathione (Barkay et al., 2003).

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CHAPTER 2

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, New York, 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 low biomass of sulfate reducing bacteria (SRB), 2.8×10^4 cells ml^{-1} in mat samples, evidence consisting of (i) a two fold stimulation of potential methylation by the addition of sulfate (ii) significant decrease in Hg methylation in the presence of the sulfate reduction inhibitor molybdate, and (iii) 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, oligiotrophic 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 and 2007). Sunday Lake watershed, for instance, experiences 8.1μg THg m^{-2} yr^{-1} and 0.04 μg CH_3Hg^+ m^{-2} yr^{-1} in wet deposition (Network, 2003; Demers et al., 2007; Selvendiran et al., 2009). In this remote lake, fish tissue contained an average of 1.0 $\pm$ 0.47 ng g^{-1} THg (ww), 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 likely involves complex biogeochemical processes. Previous studies in this ecosystem focused on the biogeochemical cycling and aquatic trophic transfer of Hg (Driscoll et al., 1998; McLaughlin, 2003; Demers et al., 2007; Driscoll 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; St. Louis, 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). Mercury biotransformations, primarily methylation and 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 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 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 employed to detect SRB by using hybridization with 16S rRNA oligoprobes (Devereux et al., 1992), PCR with primers specific to dissimilatory (bi)sulfite reductase (*dsrA*B) 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 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 the Hg methylation is expected in freshwater wetlands as compared to marine habitants. Freshwater sediments are characterized by low sulfate reduction rates due to low concentrations of sulfate (Bak & Pfennig, 1991a, 1991b). 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 to 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: (i) examining the potential for microbial Hg methylation in different microbial habitats within a wetland ecosystem, (ii) describing the diversity of SRB populations, and (iii) assessing the possible role of SRB in methylation.

Materials and methods

Site description and sample collection

The study site, Sunday Lake (44º20' N, 74º 18' W), is located in the southwestern Adirondack Mountains, New York. 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 (Fig. A-1 in Appendix A). 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* sp. sedge (Sedge), a bog dominated by *Sphagnum* 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* 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 hrs, and stored at -80 °C prior to 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* saltmarsh in Cheesequake State Park, New Jersey, 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, New Jersey, a superfund site highly contaminated with Hg (Schaefer et al., 2004).

Analytical methods

Sample pH was measured by 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 by the loss-on-ignition (LOI) method (Nelson, 1996).

Samples taken from Sunday Lake were freeze-dried prior to 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) by 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 gas chromatography (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 mercury 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 hour pre-incubation, 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 3N 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 three standard deviations. 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 prior to 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 gamma 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 acid [BES]), 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 (HSD) 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 three volumes of site water in pre-sterilized anaerobic tubes, fully filled and sealed immediately. A saltmarsh mat sample was similarly collected from Cheesquacke State Park, NJ, in Aug. 2007. The numbers of SRB and 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 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 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, *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 by 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 Table S2.1 for a primer list), $MgCl_2$ at 1.5 mM (final concentration) except for SRB4 where 1.35 mM was employed, 1× PCR buffer provided by the polymerase manufacturer, 0.2 nM of each deoxynucleoside triphosphate, 0.25 mg of bovine serum albumin ml^{-1}, 50 to 250 ng of purified DNA, and 0.025 U of *Taq* polymerase (Denville, NJ). PCR conditions were 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 sec (15 sec 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 six groups of classic SRB in Sunday Lake ecosystem, the nested PCR approach from Daly et al. (2000) was employed as described by Dar et al. (2005) with the exception that new primers for groups SRB5 and SRB6

were developed (Table S2.1). 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 by 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 by 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).

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 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 by QIA-quick Gel Extraction Kit (Qiagen, CA), and sequenced by GENEWIZ, Inc (North Brunswick, NJ).

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 to HQ148658, and FJ040921 to 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 2.1). In the former (SURN, Upland soil, and Sedge), the mineral matter consisted mostly of sand. In the later, 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 2.1). With the exception of SURF-top, sample sites which 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 2 orders of magnitude lower than the highly contaminated Berry's Creek sample (10273.2 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 2.1). 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, were the highest among all samples

analyzed. Concentrations of MeHg generally followed the patterns 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 to 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 less than 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. 2.1). 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).

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. 2.1; $p=0.0002$). For the MAT sample, potential Hg methylation rates were 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 seen (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 if 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. 2.2). 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 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. 2.2) 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. 2.1) with MAT and BOG samples positive for both. On the other hand, the absence of significant methylation activities in SURN (Fig. 2.1) 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 (data not shown). We therefore employed 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 likely the dominant anaerobic electron accepting processes, 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 employing six reference strains representing each SRB group (Fig. S2.1).

The nested PCR approach using SRB-specific 16S rRNA primers detected SRB1, 2, 5 (Fig. S2.1), and 6, but failed to detect SRB3 and 4, in all Sunday Lake samples (Table 2.2). SRB5, consisting of complete oxidizers (Daly et al., 2000), were present in

all samples. 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., was present. The lowest diversity of SRB was found in upland soil samples, which remained largely aerated year round, and where only SRB5 was 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 2.2). As this sample did not methylate Hg at any of the test conditions (Fig. 2.1), 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. 2.1). 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 populations.

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. 2.3) showed that, with two exceptions, MAT *dsrB* sequences formed large clusters which had no clear similarity to *dsrB* of cultured SRB. 41.1% of these clustered with an uncultured SRB

clone W23 (DQ855257) from metaliferous 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 (i) 11 and six clones that clustered with *dsrB* of *Desulfobulbus* spp. (SRB2) and *Syntrophobacter* spp., respectively, and (ii) two sequences, which clustered with *dsrB* from *Desulfovibrio aminophilus* (SRB6) and *Desulfobacca acetoxidans*. Sequences similar to Archaeal *dsrB* genes were not found.

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 by 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 saltmarsh mat sample were included as a control. Results were scored after 30, 60, and 90 days of incubation (Table 2.3). 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 2.3).

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. 2.4). *dsrB* phylogeny (Fig. 2.5) 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 *Desulfovibrio 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. 2.5) 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. 2.5), 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 PO-SRB, 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 to 21.2 ng g^{-1};

Table 2.1) 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 to 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 to 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 2.1) in this study were higher than the range (7.6±4.6 to 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 (McLaughlin, 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., submitted). 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 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 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 to SURN and BOG samples (Fig. 2.1). 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 a hot spot for Hg methylation, due to moderately anoxic conditions, availability of substrates for microbial growth provided as carbonaceous plant exudates, and presence of surfaces for biofilm formation (Cleckner et al., 1999; Hines et al., 1999; Guimaraes et al., 2000). Considering the large area which is dominated by *Sphagnum* moss in the Sunday Lake ecosystem, including the MAT and BOG sites, and methylation activities which were detected year round in samples collected at these sites (Adatto et al., in prep.), 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 microbes 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 mercury methylation in Sunday Lake is associated with sulfate reduction because incubation with sulfate stimulated potential methylation rates while molybdate addition inhibited them (Fig. 2.1). Based on previous observations that indicated low abundance of *Deltaproteobacteria* in SURN (data not shown), 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 2.2). Permanently saturated sites dominated by *Sphagnum*, BOG and MAT, contained the highest diversity of SRB, likely because *Sphagnum* and flooded conditions created diverse niches that supported growth and activities of SRB. Except for SRB 3 and 4, which generally dominate in coastal and marine environments (Dhillon et al., 2003; Bahr et al., 2005), our studies detected SRB 1, 2, 5, and 6 (Table 2.2). Within the four groups, SRB 1, 2, and 6 are incomplete oxidizers which 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 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 sequence analysis 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. 2.3) and those following enrichment in MPN tubes (Fig. 2.5). 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 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 source and sulfate as 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 provided MPN growth conditions. These SRB lineages

might also have been associated with the metabolism of other substrates including H_2/CO_2, formate, or ethanol. This result suggests that a much broader 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 2.2). 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 *Desulfobulbus propionicus* (1pr3) (Fig. 2.5), a strong Hg methylator (Benoit et al., 2001) related to SRB2. The occurrence of *Desulfobulbus* sp. was consistent with the *dsrB* cloning results (Fig. 2.3) and the detection of SRB2 in MAT and BOG by 16S rRNA gene analysis using the nested PCR approach (Table 2.2).

The sulfate reducing guild in the floating MAT, a hotspot for Hg and MeHg accumulation with a potential for Hg methylation, therefore, was 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 to 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 to 69.5µM (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 2.3) 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 (Chapter 3). 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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Table 2.1. Physical and chemical characteristics of study sites in Sunday Lake, New York (July 21, 2005), Cheesequake saltmarsh (July 23, 2007), and Berry's Creek, New Jersey (July 26, 2007)

Study site	Temp. (°C)[1]	pH	Sand (%)	Silt+Clay (%)	Organic matter content (%)	THg (ng g^{-1} dry wt)	MeHg (ng g^{-1} dry wt)	MeHg fraction in THg (%)
SURN-top[2]	17.3	4.00	80.4	19.6	18.71	60.4 (1.9)[3]	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[4]	<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[5]	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	10273.2 (363.0)	32.35 (5.79)	0.32

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

[2]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.

[3]Values in parentheses represent the standard deviations.

[4]Below detection limit.

[5]ND - Not determined.

Table 2.2. Distributions of SRB groups and effects of sulfate enrichment (2 mM) on this distribution in samples collected at various ecological niches within Sunday Lake wetland during July 2005 sampling[1]

Sites	SRB1	SRB2	SRB3	SRB4	SRB5	SRB6
SURN-top[2]	+	-	-	-	+	+
SURN-bottom	+	-	-	-	+	+
SURF-top	+	-	-	-	+	+
SURF-bottom	+	-	-	-	+	+
Upland soil	-	-	-	-	+	-
BOG-top	+	+	-	-	+	+
Sedge	+	-	-	-	+	+
MAT	+	+	-	-	+	+
SURN+SO_4^{2-}[3]	-	-	-	-	-	-
BOG+SO_4^{2-}	+/-	+	-	-	+	+
MAT+SO_4^{2-}	+	+	-	-	+	+

[1]+ and – indicate presence and absence, respectively, of PCR products specific for the indicated group.

[2]Samples were collected from the "top" 15 cm or the "bottom" 15 cm of the soil.

[3]Sample to which sulfate was added followed by a three weeks incubation prior to methylation assays and DNA extraction. Both SURN and BOG were from the top layer.

Table 2.3. MPN estimates (cells ml^{-1}) of SRB and propionate-oxidizing SRB (PO-SRB) in the MAT sample, Sunday Lake, NY, and of SRB in Cheesequake saltmarsh mat, NJ

Site	Group	30 and 60 d[1]		90 d	
		MPN	95% CL[2]	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)

[1]Period for anaerobic incubation at 28°C.

[2]Numbers in parentheses indicate the 95% confidence limits (CL) of MPN estimates.

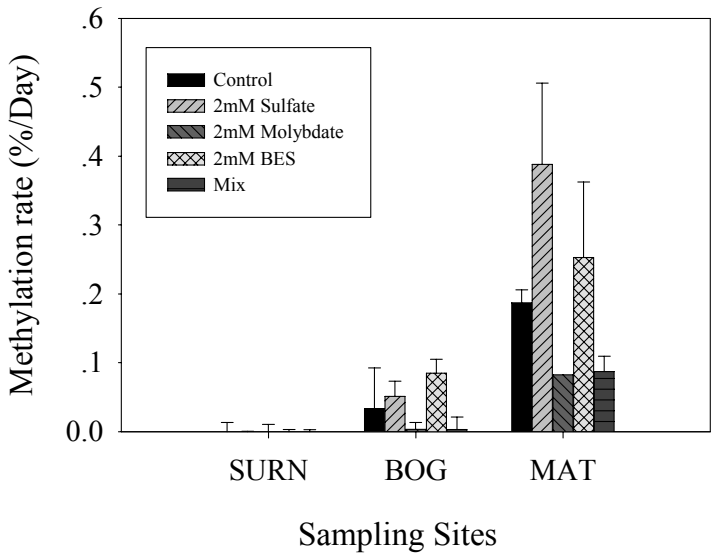


Fig. 2.1. Potential methylation rates of Sunday Lake samples and the impact of various treatments on these rates. The "Mix" treatment was amended with a combination of sulfate, molybdate, and BES at 2 mM each.

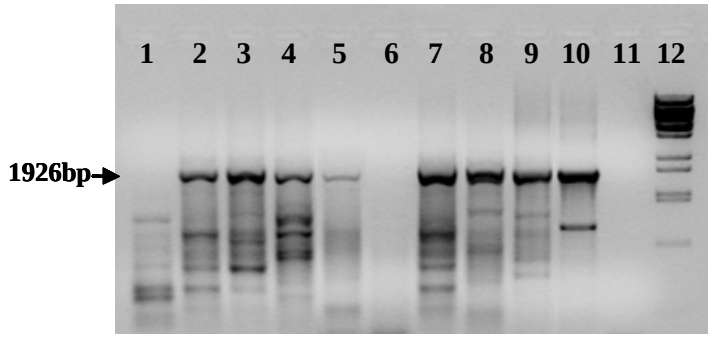


Fig. 2.2. Dissimilatory sulfite reductase (*dsrAB*) genes in the native and sulfate-enriched samples of Sunday Lake wetland. Lanes marked as: 1. SURN-Top; 2. BOG-Top; 3. BOG-Bottom; 4. Sedge; 5. MAT; 6. SURN-top-SO_4^{2-}; 7. BOG-top-SO_4^{2-}; 8. MAT-SO_4^{2-}; 9. Cheesequake (saltmarsh mat); 10. *Desulfovibrio desulfurican* G200 (positive control); 11. Blank; 12. Size marker - λ DNA-BstE II digest.

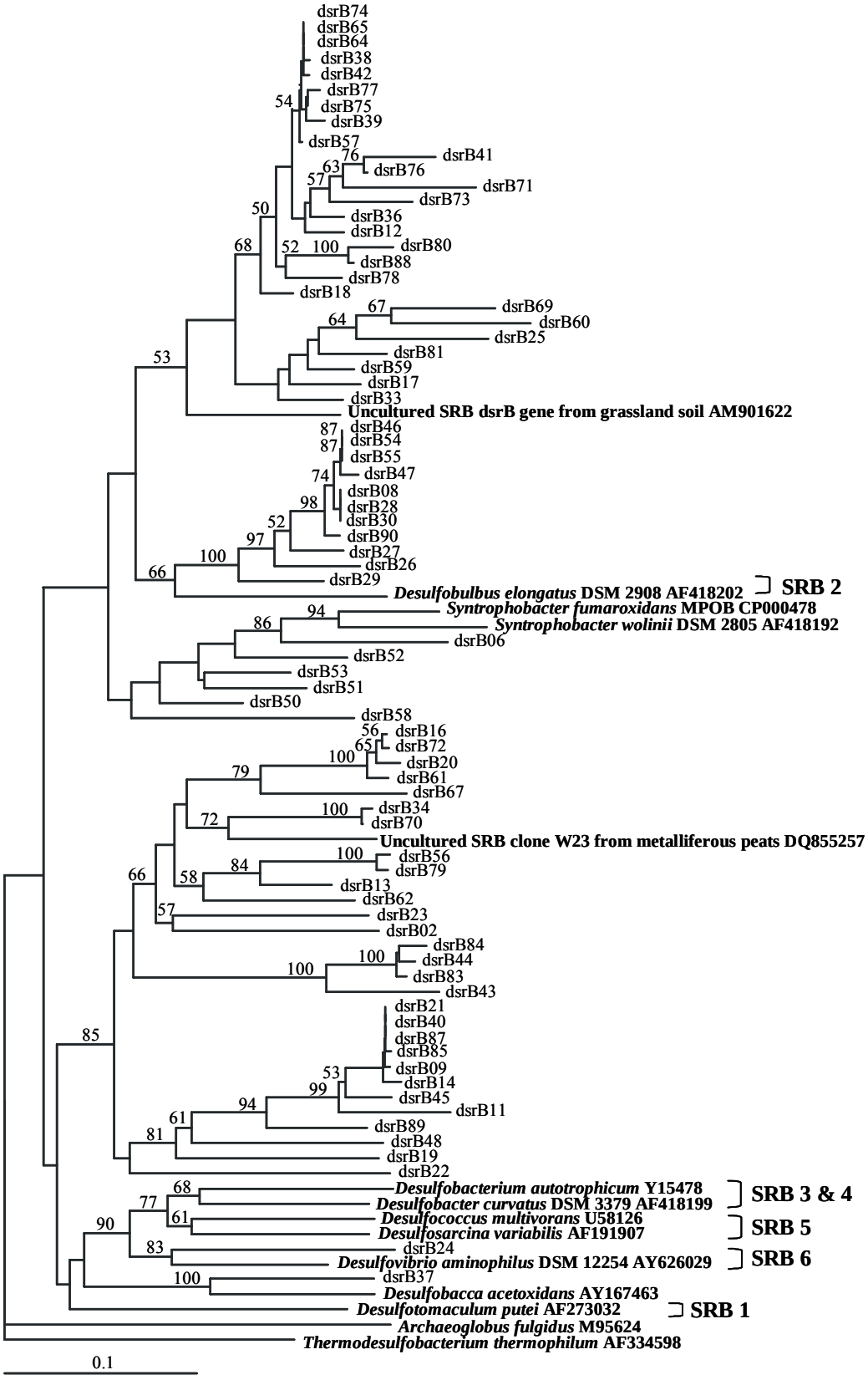

dsrB74
dsrB65
dsrB64
dsrB38
dsrB42
dsrB77
dsrB75
dsrB39
dsrB57
dsrB41
dsrB76
dsrB71
dsrB73
dsrB36
dsrB12
dsrB80
dsrB88
dsrB78
dsrB18
dsrB69
dsrB60
dsrB25
dsrB81
dsrB59
dsrB17
dsrB33
Uncultured SRB dsrB gene from grassland soil AM901622
dsrB46
dsrB54
dsrB55
dsrB47
dsrB08
dsrB28
dsrB30
dsrB90
dsrB27
dsrB26
dsrB29
Desulfobulbus elongatus DSM 2908 AF418202
SRB 2
Syntrophobacter fumaroxidans MPOB CP000478
Syntrophobacter wolinii DSM 2805 AF418192
dsrB06
dsrB52
dsrB53
dsrB51
dsrB50
dsrB58
dsrB16
dsrB72
dsrB20
dsrB61
dsrB67
dsrB34
dsrB70
Uncultured SRB clone W23 from metalliferous peats DQ855257
dsrB56
dsrB79
dsrB13
dsrB62
dsrB23
dsrB02
dsrB84
dsrB44
dsrB83
dsrB43
dsrB21
dsrB40
dsrB87
dsrB85
dsrB09
dsrB14
dsrB45
dsrB11
dsrB89
dsrB48
dsrB19
dsrB22
Desulfobacterium autotrophicum Y15478
Desulfobacter curvatus DSM 3379 AF418199
SRB 3 & 4
Desulfococcus multivorans U58126
Desulfosarcina variabilis AF191907
SRB 5
dsrB24
Desulfovibrio aminophilus DSM 12254 AY626029
SRB 6
dsrB37
Desulfobacca acetoxidans AY167463
Desulfotomaculum putei AF273032
SRB 1
Archaeoglobus fulgidus M95624
Thermodesulfobacterium thermophilum AF334598
0.1

Fig. 2.3. 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.

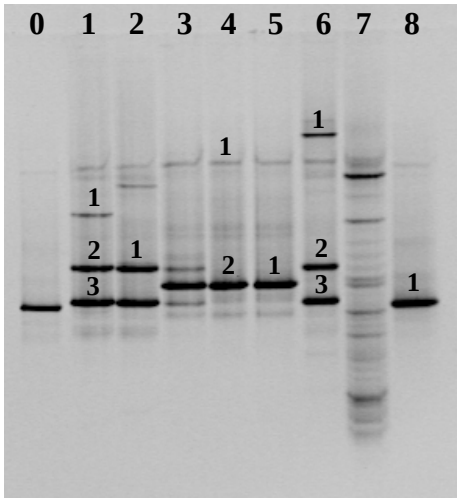


Fig. 2.4. DGGE of bacterial *dsrB* PCR products amplified from DNA extracts of MPN cultures that were set up with MAT samples. Lanes 0 and 8, *Desulfovibrio desulfurican* G200 (positive control). Lanes 1-5, dilution cultures of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} dilution of the SRB MPN series 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.

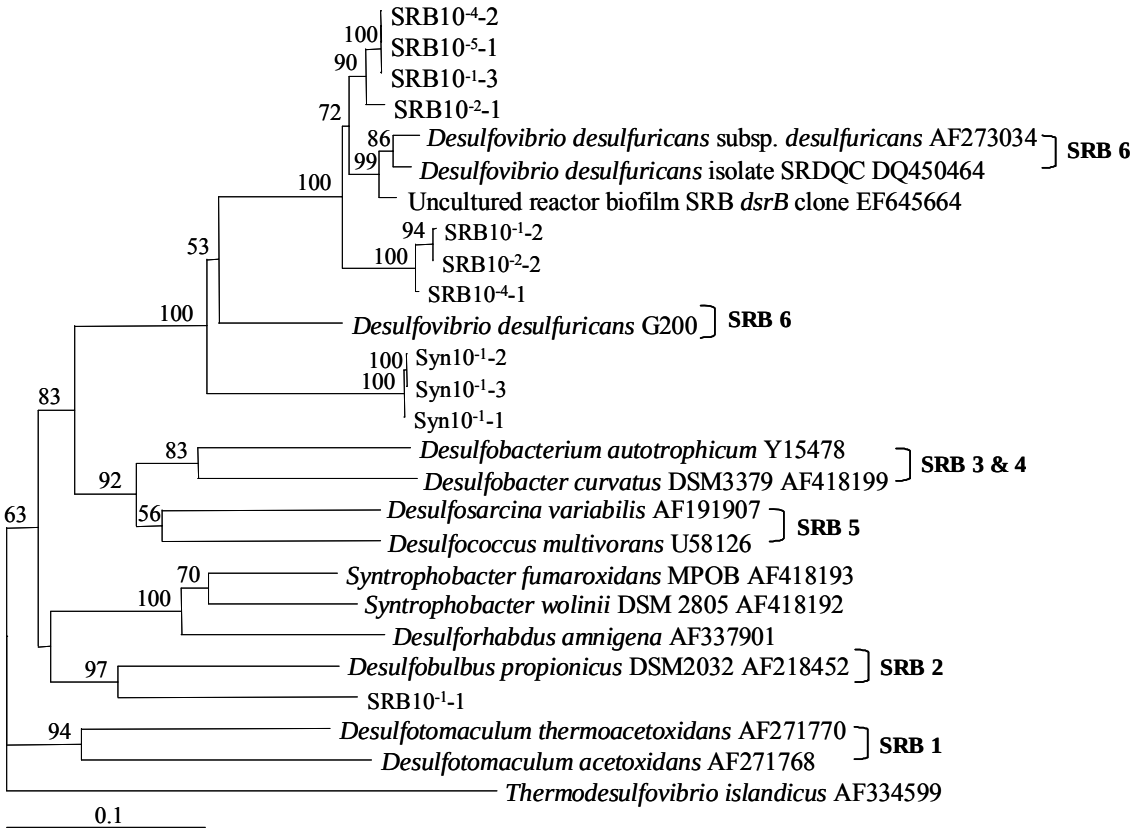
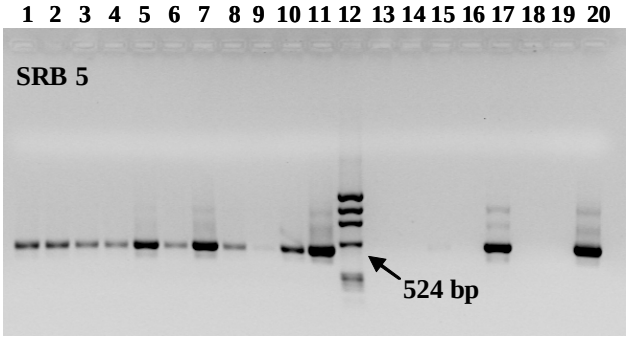


Fig. 2.5. Phylogenetic analysis of *dsrB*-based DGGE fragments of sulfate reducing bacteria (SRB) and Syntrophic Propionate-oxidizing SRB from MPN series dilution cultures of the MAT sample. The neighbor-joining method was used and bootstrap values were shown at branch points. The scale bar indicated 10% sequence difference. Clone designation denotes the MPN enrichment medium (the initial three letters; SRB for SRB or Syn for PO-SRB medium), culture dilution (10^{-1}-10^{-5}), and the number of DGGE band shown in Fig.4 (last digit).

Supplemental Table S2.1. Primers and PCR conditions that were used in this study

Targeted taxa/genes	Primer	Sequence 5'-3'	Annealing T_m (°C)	Reference
Most bacteria	GM3F	AGA GTT TGA TCM TGG C	45	Muyzer et al. 1995
	GM4R	TAC CTT GTT ACG ACT T		
SRB 1	DFM140	TAG MCY GGG ATA ACR SYK G	58	Daly et al. 2000
	DFM842	ATA CCC SCW WCW CCT AGC AC		
SRB 2	DBB121	CGC GTA GAT AAC CTG TCY TCA TG	66	Daly et al. 2000
	DBB123	GTA GKA CGT GTG TAG CCC TGG TC		
SRB 3	DBM169	CTA ATR CCG GAT RAA GTC AG	64	Daly et al. 2000
	DBM1006	ATT CTC ARG ATG TCA AGT CTG		
SRB 4	DSB127	GAT AAT CTG CCT TCA AGC CTG G	60	Daly et al. 2000
	DSB1273	CYY YYY GCR RAG TCG STG CCC T		
SRB 5	DC5F	ATKTGATACTGTSGGACTTGAGTATGG	54	This study
	DC5R	ATCTYYAGAGTYCCCAMCYDWAWTG ATGGTAACTRAAGA		
SRB 6	DV6F	CTGCWDDRCTWGAGTYCRGGAGAGG GTG	61	This study
	DV6R	GACGSRYTTTTTGGGATTRGCKYSACC TC		
dsrAB	DSR1F	ACS CAC TGG AAG CAC G	54	Wagner et al.1998
	DSR4R	GTG TAG CAG TTA CCG CA		
dsrB	DSRp206	CAA CAT CGT YCA YAC CCA GGG	55	Geets et al.

	0F			2006
	DSR4R	GTG TAG CAG TTA CCG CA		
DGGE-*dsrB*	GC-DSRp206 0F	CGCCCGCCGCGCGCGGCGGGCGGGGC GGG GGCACGGGGGGCAACATCGTYCAYAC CCA GGG	55	Geets et al. 2006
	DSR4R	GTG TAG CAG TTA CCG CA		



Supplemental Fig. S2.1. PCR amplification products indicating the presence of 16S rRNA genes homologous to those of SRB5 in Sunday Lake samples. Lane 1-8: native samples from SURN-Top, SURN-Bottom, SURF-Top, SURF-Bottom, BOG-Top, Sedge, MAT, Upland soil, respectively. Lane 9-11, the sulfate-enriched (2mM) samples (2 wks) from SURN-Top, BOG-Top, and MAT, respectively. Lane 13-18: pure culture controls for Group 1 (*D. nigrificans*), Group 2 (*D. propionicus*), Group 3 (*D. autotrophicum*), Group 4 (*D. curvatus*), Group 5 (*D. variabilis*), and Group 6 (*D. desulfuricans*), respectively. Lane 12, 19, and 20 were size marker (ΦX174 DNA Hae III), blank, and Cheesequake saltmarsh mat, respectively.

CHAPTER 3

PRESENCE OF *SYNTROPHOBACTER* SPP. IN HG METHYLATING *SPHAGNUM* MOSS MATS AND TWO PROPOSED PATHWAYS FOR THE STIMULATION OF METHYLATION BY SYNTROPHIC INTERACTIONS

Abstract

Under methanogenic conditions, syntrophic associations stimulate the growth of both methanogens and SRB, suggesting that this association may also enhance microbial Hg methylation. This possibility was supported by the detection of 16S rRNA and *dsr* genes (encoding dissimitory sulfite reductase) that were most similar to those of *Syntrophobacter* spp. in native samples or enrichments of *Sphagnum* moss mats with significantly elevated Hg methylation activities. I tested the role of syntrophy in Hg methylation by setting up coculture incubations among propionate-utilizing *S. wolinii*, *S. sulfatireducens*, and *S. fumaroxidans*, a methanogen *Methanospirillum hungatei,* and Hg methylating SRB, strains *Desulfovibrio desulfuricans* ND132 and *Desulfovibrio africanus* DSM 2603 which cannot grow with propionate. *S. wolinii* was the only syntroph with a low rate of Hg methylation activity (0.67 pg CH_3Hg μg initial protein^{-1} day^{-1}) when grown with propionate (15.6 mM) and sulfate (19.7 mM). This activity was also demonstrated when *S. wolinii* grew in a coculture with *M. hungatei* (2.00 pg CH_3Hg μg initial protein^{-1} day^{-1}) in the absence of sulfate. When strains ND132 and DSM 2603 were grown with *M. hungatei* in a sulfate-free lactate medium, methylation was stimulated by 2.1 to 3.3 fold and 6.1 to 19.4 fold, respectively, as compared with methylation by the fermenting monocultures. Statistical comparison showed that, CH_3Hg synthesis (pg per μg

protein) by syntrophy of *M. hungatei* with a weak Hg methylator (*D. africanus*) was 2.1 to 3.5 fold higher than the syntrophy with a strong methylator (ND132). In a propionate (15.6 mM)-sulfate (3.94 mM) medium, a coculture of *S. sulfatireducens* with ND132 significantly increased CH_3Hg production by 1.3 to 1.6 fold as compared to methylation by ND132 monoculture, while the syntrophy with DSM 2603 stimulated Hg methylation by 1.8 to 2.0 fold over DSM 2603 monoculture. Cell counts using flow cytometry showed that this association significantly increased the growth of the two syntrophic partners, strains ND132 (by 4.3 fold) and DSM 2603 (3.3 fold), and of *S. sulfatireducens* (3.1 fold), while *Desulfovibrio* monocultures of strains ND132 and DSM 2603 did not grow in a propionate amended medium. Similar stimulation of Hg methylation by syntrophy was observed in medium amended with sulfate at 0.39 to 19.71 mM. This study demonstrated for the first time that the syntrophy between *S. sulfatireducens* and *Desulfovibrio* spp. significantly stimulate Hg methylation. These results suggest that *S. sulfatireducens* by oxidizing propionate provided hydrogen and a carbon source to support growth of methylating *Desulfovibrio* spp. In summary, syntrophy may enhance Hg methylation by *Desulfovibro* spp. growing with methanogens in sulfate-free environments and by *Desulfovibrio* growing with *Syntrophobacter* spp. in sulfate limited environments where sources of energy and carbon are limiting.

Introduction

Syntrophic bacteria degrade propionate, long-chain fatty acids, alcohols, and some amino acids and aromatic compounds by a mutual relationship with H_2 or formate-utilizers, especially methanogens, under anaerobic conditions and a limited supply of terminal electron acceptors (Schink, 1997; McInerney et al., 2008). By oxidizing these substrates to acetate, H_2, formate, and other methanogenic substrates, and eventual conversion of CO_2 to CH_4, syntrophy plays an essential role in recycling of organic matter and the global carbon cycle. Phylogenetic analyses have shown that most syntrophs are affiliated with the Deltaproteobacteria and the low G+C gram-positive bacteria. Within the Deltaproteobacteria, syntrophic bacteria include species that represent the genera *Syntrophus*, *Syntrophobacter*, *Desulfoglaeba*, *Geobacter*, *Desulfovibrio*, and *Pelobacter* (de Bok et al., 2004; McInerney et al., 2008).

Mercury methylation is an activity that is mostly attributed to bacteria that belong to the Deltaproteobacteria, including various species of *Desulfovibrio* among the sulfate reducing bacteria (SRB), and several species of *Geobacter* among the iron reducing bacteria (IRB) (Compeau & Bartha, 1985; Fleming et al., 2006; Kerin et al., 2006; Ranchou-Peyruse et al., 2009). Mechanisms of microbial Hg methylation have been widely investigated with most studies focusing on pure cultures of single species (Choi et al., 1994a, b; Benoit et al., 2001; Ekstrom et al., 2003; Schaefer & Morel, 2009). Numerous studies addressing the microbial guilds which are involved in methylation within the context of mixed microbial communities, pointed out that SRB methylate Hg in freshwater environments where sulfate concentrations likely limited the importance of sulfate reduction as a major terminal electron accepting pathway (Gilmour et al., 1992; Oremland et al., 1995; Yu et al., 2010). This observation led to

the suggestion that SRB growing by interspecies H_2 transfer, i.e., syntrophy, with methanogens may methylate Hg. Indeed, methylation was observed when the robust methylators *Desulfovibrio desulfuricans* strains ND132 and LS were grown with lactate in the absence of sulfate as co-cultures with the methanogen *Methanococcus maripaludis* (Pak & Bartha, 1998). To date, a role for syntrophy in methylation has not been reported for mixed microbial communities under environmental conditions.

In anaerobic environments in the absence of sulfate as a terminal electron accepter, *Desulfovibrio* spp. may consume pyruvate by fermentation, and oxidize lactate and ethanol in syntrophic association with hydrogen-scavenging methanogens by interspecies H_2 transfer (Bryant et al., 1977). Under sulfate-limited conditions, SRB may compete with each other for available sulfate during the complete oxidation of organic matter. Under such conditions syntrophic associations are established whereby one SRB species ferments the substrate (e.g., lactate) by proton reduction and another utilizes the hydrogen for sulfate reduction (Muyzer and Stams, 2008). On the other hand, syntrophs originally described for their growth on propionate with H_2 utilizing methanogens (Boone & Bryant, 1980; de Bok et al., 2004) may grow as monocultures with sulfate as a terminal electron acceptor (Wallrabenstein et al., 1994; Harmsen et al., 1993) and may form syntrophic associations with SRB that cannot oxidize propionate whereby H_2 and acetate that are produced during propionate oxidation by *Syntrophobacter* spp. serve as energy and carbon source, respectively, for the SRB (Muyzer and Stams, 2008). During syntrophic growth of *Desulfovibrio vulgaris* Hildenborough on lactate with one methanogen, Walker et al. (2009) demonstrated by comparative transcriptional analysis that numerous genes involved in electron transfer and energy generation were upregulated compared with their expression in sulfate-limited monoculture, and further suggested that syntrophic

growth and sulfate respiration in *D. vulgaris* use largely independent energy generation pathways. These variable metabolic options clearly show the physiological flexibility of both SRB and syntrophic bacteria (Scholten et al., 2007; Muyzer & Stams, 2008). With multiple lifestyles for *Desulfovibrio* and *Syntrophobacter* species, syntrophy not only increases survival opportunities and adaptation to environmental change, but also greatly promotes the recycling of organic matter (Walker et al., 2009). Although syntrophy in its various permutations is central to organic matter degradation under anaerobic conditions and *Syntrophobacter* spp. belong to the Deltaproteobacteria, the same class of the Proteobacteria to which the majority of the known Hg methylators belong (Kerin et al., 2006; Ranchou-Peyruse et al., 2009), their role in Hg methylation remains largely unknown.

In this study, I report a high abundance of *Syntophobacter*-like 16S rRNA sequences in a *Sphagnum* moss mat where MeHg accumulation and high rates of Hg methylation were documented (Chapter 2 or Yu et al., 2010). These results led us to hypothesize that syntrophic associations were involved in Hg methylation. To test this hypothesis, we set up mono- and co-culture incubations of *Syntrophobacter* spp., a methanogen, and methylating *Desulfovibrio* spp. with various electron donors and acceptors. The results extend the known diversity of the organisms and metabolic pathways that may contribute to Hg methylation in anoxic environments.

Materials and Methods

PCR amplification, Denaturing Gradient Gel Electrophoresis (DGGE) analysis, and DNA sequencing of 16S rRNA genes from environmental samples. Samples were collected from floating *Sphagnum* moss mats (MAT) in Sunday Lake (44º20' N, 74º 18' W), a Hg-contaminated forest peatland located in the southwestern

Adirondack Mountains, New York, and from a *Spartina* saltmarsh mat in Cheesequake State Park, New Jersey, for DNA extraction and PCR amplification of 16S rRNA genes as described previously (Yu et al., 2010). To detect the presence of SRB in the samples, six sets of SRB group-specific primers (Daly et al., 2000) and a nested PCR approach (Dar et al. 2005) were used as previously described (Yu et al., 2010).

Purified PCR products of 16S rRNA genes obtained with primer sets specific to SRB groups 1, 2, 5, and 6 (Daly et al., 2000) from the MAT DNA extracts were further amplified by touchdown PCR with the DGGE primer set GC-341F and 518R (Muyzer et al., 1993). The annealing temperature in the touchdown program was decreased by 0.5 °C per cycle for 20 cycles from 65 to 55 °C. PCR products (250-350 ng) of these reactions were separated by DGGE as described by Muyzer and Smalla (1998) using DCode^{TM} Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA). DGGE gels contained 6% polyacrylamide and a 20 to 80% denaturant gradient established using a stock solution consisting of 7 M urea and 40% (v/v) formamide. Electrophoresis was performed in 1× TAE buffer at 50 V for 15 h at 60 °C. The gels were stained, gel slices containing DNA bands were cut, DNA was extracted from the gel slices, and its purity was confirmed as described previously (Yu et al., 2010). The purified DNA bands were sequenced by GENEWIZ, Inc (Piscataway, NJ).

In order to confirm the presence and characterize the diversity of *Syntorphobacter*-like bacteria in MAT samples, *Syntrophobacteraceae*-specific primers (SYBAC_282F and SYBAC1427R), (Loy et al., 2004) were used to amplify 16S rRNA genes from DNA extracts of MAT enrichment cultures. Enrichments consisted of MPN dilution cultures (Yu et al., 2010) of MAT samples established with

a freshwater SRB medium (28.1 mM Na_2SO_4, and sodium salts of acetate, propionate, butyrate, and lactate at 4.0 mM each) modified from Widdel and Bak (1992). 16S rRNA genes of *Syntrophobacter*-like bacteria were also amplified from the DNA extracts of the native Cheesequake saltmarsh sample. Purified PCR products were ligated into pGEM®-T Easy vectors (Promega, Madison, 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 and plasmid DNA was extracted using a Concert™ 96 Plasmid Purification System as described by the manufacturer (Invitrogen, Carlsbad, CA). The presence and size of 16S rRNA gene inserts were verified by EcoRI restriction digests (New England BioLabs, MA) and gel electrophoresis. The NEBcutter v2.0 software *in silico* (New England Biolabs, MA) was employed to design a restriction fragment length polymorphism (RFLP) approach that grouped clones according to their restriction patterns. RFLP analysis by the enzyme XmnI was then performed by digesting purified PCR products (7µl) with 10 U of XmnI following the manufacturer (Promega) protocol for 11 h, and band patterns were checked on 1.5% agarose gels. Inserts representing distinct RFLP patterns were selected and sequenced using the primer SYBAC+282F and the BigDye Terminator v 3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and an ABI 3100 Genetic Analyzer (Applied Biosystems).

Phylogenetic analyses. Sequences of excised DGGE fragments, clone libraries, and reference sequences of 16S rRNA genes obtained from GenBank (http://www.ncbi.nih.gov/) were edited using Contig Express (Vector NTI Advance™ 10; Invitrogen) and Chromas (http://www.technelysium.com.au/chromas.html), and aligned by ClustalW (Thompson et al., 1994). Phylogenetic trees were constructed by

PAUP* (version 4.0 beta 10; Sinaur Associates, Sunderland, MA, USA) and ClustalX (Thompson et al., 1997). The statistical support for tree topologies was tested by bootstrap resampling (1000 iterations).

Nucleotide sequence accession numbers. All cloned 16S rRNA sequences obtained in this study were deposited in GenBank. Sequences from DGGE analysis of native MAT were deposited under accession numbers FJ040892 to FJ040920, those from enriched MAT communities under FJ040932 to FJ040960, and those from the native Cheesquake saltmarsh under FJ040961 to FJ040986.

Microorganisms and culture conditions. Strains tested in this study included *Syntrophobacter wolinii* DSM 2805, *Syntrophobacter sulfatireducens* DSM 16706, *Syntrophobacter fumaroxidans* DSM 10017, *Methanospirillum hungatei* DSM 864, and *Desulfovibrio africanus* DSM 2603. These cultures were purchased from the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH [DSMZ]) and were grown in their respective media as recommended by the DSMZ at 37 °C. The exception was *D. africanus,* which was grown at 28 °C and in the medium (containing 21.4 mM lactate as sole organic substrate and 28.2 mM Na_2SO_4) described by Widdel and Bak (1992) for methylation tests in order to reduce the formation of black FeS precipitation with DSMZ Medium 63. In addition, *Desulfovibrio desulfuricans* ND132, received from Cynthia Gilmour, was grown at 28 °C in a same medium as for *D. africanus*. Prior to Hg methylation experiments, the purity of all strains was confirmed by DNA extraction and sequencing of the 16S rRNA genes. The archaeal primer set, Ar4F (Hershberger et al., 1996) and Ar958R (DeLong, 1992) was used for *M. hungatei* and the universal primer set, 27f and 910r (Lane, 1991) was used for the bacterial strains. All manipulations were performed under strictly anaerobic conditions using O_2-free

gases obtained by passage through a reduced, hot copper column for bench handling, or in an anaerobic glovebox (Coy Laboratory Products Inc., Grass Lake, MI) with a gas mixture of 95% nitrogen (N_2) and 5% hydrogen (H_2).

All media were reduced with 0.25 mM titanium(III)-nitrilotriacetic acid (100 μM) (TiNTA) in order to minimize the influence of sulfide as a reductant on Hg speciation and methylation (Jay et al., 2002), and contained 1 mg L^{-1} of resazurin as a redox indicator. A filter sterilized TiNTA stock (25 mM $TiCl_3$ in 100 mM NTA) by a 0.2 μm syringe filter (Whatman) was prepared under anaerobic conditions by adding 10% $TiCl_3$ (Fisher Sci.) to saturated Na_2CO_3 with NTA, adjusting the pH to 7.0 (Moench & Zeikus, 1983). Rumen fluid to support growth of *S. wolinii* and *S. sulfatireducens* was collected from a fistulated cow available at the Cook College Farm, Rutgers University (New Brunswick, NJ). The raw rumen fluid was stored at 4 °C overnight to remove large solids, and the aqueous phase was then centrifuged (SLA-1500 rotor in the Sorvall RC-5B plus centrifuge) twice at 5, 000 rpm for 10 min to remove fine solids. The clarified rumen fluid that was obtained after the second spin was autoclaved and stored at room temperature, and added to growth media preparations when needed after 0.2 μm filtration. Sludge for Medium 119 of *M. hungatei* was taken from the sewage treatment facility of Western Monmouth Utilities Authority (Englishtown, NJ).

Three types of syntrophic associations on each specific co-culture medium were studied including a methanogen with *Syntrophobacter* spp., a methanogen with *Desulfovibrio* spp., and *S. sulfatireducens* with *Desulfovibrio* spp. A medium for co-culture of *M. hungatei* with *S. wolinii* or *S. sulfatireducens* was modified from DSMZ Medium 307 (*S. wolinii* medium) by including 15.6 mM sodium propionate as the sole electron donor and removing sulfate, glucose and butyric acid (Table S3.1). A co-

culturing medium for *M. hungatei* with *S. fumaroxidans* was modified from DSMZ Medium 684 (*S. fumaroxidans* medium), by adding 20 mM sodium propionate (Harmsen et al., 1998) and 0.3 g L^{-1} Cysteine-HCl.H_2O, removing sodium fumarate since previous studies showed that *S. fumaroxidans* could grow on propionate when it was co-cultured with *M. hungatei* (Stams et al., 1993), and replacing sodium sulfide with TiNTA as a reducing agent (Table S3.2). The co-culturing media were prepared under an 80% N_2+ 20% CO_2 atmosphere at pH 7.0 for modified DSMZ 307 and at 7.2 for modified DSMZ 684. The modified DSMZ Medium 307 containing various concentrations of sulfate was used for co-culturing of *S. sulfatireducens* with *D. desulfuricans* ND132 or *D. africanus* (Table S3.1). Sulfate concentrations included 0.39, 1.97, 3.94, and 19.71 mM (corresponding to 50, 10, 5, and 1 folds dilutions, respectively, of the original sulfate concentration recommended for Medium 307) were set up to test how sulfate concentration affected Hg methylation during syntrophic growth. The medium described by Pak and Bartha (1998) (Table S3.3) was used to co-culture *M. hungatei* with *D. desulfuricans* ND132 or *D. africanus*, and was prepared under 100% N_2 at pH 7.0 and contained TiNTA. Growth of mono- and cocultures with the three types of coculture media was tested spectrophotometrically at OD_{660} prior to Hg methylation assay.

Mercury Methylation Experiments. Individual cultures for methylation experiments were pre-grown for 4-5 days until they reached exponential phase. For Hg methylation assays by each strain alone, 7 ml (10%) of pre-grown culture was added to 63 ml of fresh medium in a 130 ml serum bottles in triplicate. For co-culture experiments in which electron acceptors and donors were different from those used during growth of individual strains, pre-grown cells were washed twice with the co-culturing medium under strictly anaerobic conditions using tightly sealed 50 ml

centrifuge tubes. Washed cells were then resuspended in the co-culturing medium and used to inoculate at roughly 1 to 10 ratio of inoculum to fresh medium. An equal amount of inocula of each individual strain (roughly 10%) was used in the co-culture treatment. Experiments were performed in triplicate (duplicate for co-cultures of *S. sulfatireducens* with strains ND132 or DSM 2603). Heat killed abiotic controls were prepared by treating cultures at 80 °C for 1 h (Wiatrowski et al., 2006) and media blanks were included in all methylation experiments. All glassware used in methylation experiments was acid-cleaned.

Potential Hg methylation rates were measured by spiking $^{203}HgCl_2$ (in 0.1 N HCl) purchased from Eckert & Ziegler Isotope Products (Valencia, CA). The radioisotope was injected into each serum bottle at 166.7 kBq L^{-1} (corresponding to 29.5 nM or ~5.9 μg L^{-1} Hg). The final concentration of Hg in these incubation was similar to or lower than concentrations in previous methylation studies (Pak & Bartha, 1998; Benoit et al., 2001; Kerin et al., 2006; Schaefer & Morel, 2009). A volume of 0.1 N NaOH similar to that of the injected ^{203}Hg substrate was immediately injected into the serum bottles to neutralize the added acid, and the cultures were thoroughly mixed by vortexing. Cultures were then statically incubated at either 28 or 37 °C in the dark. Ten ml aliquots were withdrawn with a syringe in daily intervals for 5 days and a similar volume of O_2-free 100% N_2 was added to the bottles to maintain constant pressure.

Newly synthesized $CH_3{}^{203}Hg$ in cultures was separated from unreacted $^{203}HgCl_2$ by a toluene extraction method which was modified from methods used in previous studies (Guimaraes et al., 1995; Marvin-DiPasquale et al., 2003; Hines et al., 2006). The 10 ml sampled aliquots were transferred into 50 ml glass centrifuge tubes, mixed with 5 ml saturated KCl (in 0.6 M H_2SO_4) and 0.5 ml saturated $CuSO_4$, vortexed, and

heated to 70-75 °C for 30 min followed by the addition of 5 ml of toluene and vortexing for 1 min. Tubes were then centrifuged by a GLC-2 general laboratory centrifuge (Sorvall Instruments, FL) to separate the organic toluene and aqueous phases at 2,500 rpm for 5 min. The top toluene phase was transferred to a second centrifuge tube, which was capped quickly to minimize toluene evaporation, and the aqueous phase was extracted again with a fresh volume (5 ml) of toluene as above. The toluene phases were then combined and dehydrated with anhydrous Na_2SO_4 (ca. 0.5 g). An aliquot (5 ml) of the toluene extract was mixed with a scintillation fluid (ScintiSafe Econo F, Fisher Sci.) and $CH_3{}^{203}Hg$ was quantified by scintillation counting using a Beckman LS-6500 Counter (Beckman Instruments, Fullerton CA). Samples were extracted on the same day of sampling to minimize the effect of ^{203}Hg decay. Recoveries of MeHg ranged from 90 to 98% as determined by using cell cultures to which 2 ml of 3N HCl were added to terminate microbial activities followed by the addition of ~0.1 uCi ^{14}C-CH_3HgCl (specific activity, 60 mCi/mmol; radiochemical purity, 95.2%; Amersham Corp., Buckinghamshire, England), and employing the same extraction protocol. Potential Hg methylation rates (pg CH_3Hg per μg protein day^{-1}) were calculated from the initial linear range of lines describing $CH_3{}^{203}Hg$ concentrations vs. time. Results of radioactive counts were converted to mass of CH_3Hg using the known specific activity of the isotope. The initial concentration of cell proteins (see below) at inoculation was used in calculations to allow comparison with results that had been obtained by others (e.g., Pak & Bartha, 1998).

Protein assay. Cell growth was quantified as total protein concentration using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA). During sampling for Hg methylation, 1 ml cell culture was taken from each serum bottle at each time

point for protein assays. Cultures were centrifuged at 13,000 g for 10 min to completely remove supernatant, and the cell pellets were stored at -20 °C until analysis. Thawed cell pellets were resuspended in 0.3 ml of 0.22 μm filtered MiliQ water and 0.1 ml of 0.5 N NaOH and suspensions heated to 85 °C for 30 min for cell breakage (Hemme, 2004). Standards were similarly prepared using BSA. Processed samples were then mixed with the dye reagent according to the protein assay kit instructions for 96 well microtiter plates and absorbance at 595 nm was measured after 20 min incubation by a Tecan Sunrise Microplate Readers (Phenix Research Products, Candle, NC) with an advanced Magellan^{TM} data reduction software.

Fluorescence staining and flow cytometry (FC). In order to estimate growth of the individual cultures during co-culturing, 1 ml of cell cultures taken at each time point, were preserved by adding 143 μl 48% betaine stock (Cleland et al., 2004) and stored at -20 °C until analysis. Upon thawing, preserved cells were stained with SYBR Gold concentrated stock (Molecular Probes, Invitogen, Eugene, OR) at a final dilution of $1x10^{-4}$ for 20 min in the dark at room temperature. A sterile phosphate-buffered saline solution served as the sheath fluid during FC. Individual strains in co-cultures were distinguished based on differences in light diffraction likely due to different cell properties and counted separately by a BD Influx Mariner 209s Flow Cytometer and High Speed Cell sorter (BD Biosciences, Rockville, MD). All signals were collected with logarithmic amplification and were triggered on the fluorescence channel. Samples were analyzed in triplicate. The ability of the FC protocol to distinguish the cells of each individual strain in co-cultures was confirmed by FC analysis of pure culture (Fig. S3.1 and S3.2) and the counting accuracy was confirmed by using an internal bead standard (Polysciences, Warrington, Pa.).

Statistical analysis. Results of CH_3Hg synthesis were analyzed by repeated

two-way ANOVA. Overall effects of the two dimensions of treatments (incubation time and different strains or cultures) were shown by results of Type 3 Tests. Specific comparisons among different treatments were performed by Tukey's honest significant difference (HSD) or Tukey-Kramer test using SAS software (SAS Institute, Cary, NC).

Results

Identification and phylogeny of SRB in MAT. The *Sphagnum*-dominated MAT sample was selected for detailed analyses of the SRB community because this site was characterized by high MeHg concentrations and potential methylation rates, and where the SRB community was highly developed (Yu et al., 2010). DGGE PCR products of the desired size (193 bps), amplified with the general bacterial DGGE primers of Muyzer et al. (1993) from group-specific 16S rRNA genes of SRB as templates, were analyzed. A total of 50 DGGE bands representing native and SO_4-enriched MAT samples were retrieved, and 41 of them were sequenced. Of these, 24 represented the native (unamended) samples and 17 were retrieved from sulfate enrichments (Table S3.4). 58.3% of the sequences from native samples were most closely related (88 to 93% similarity) to 16S rRNA genes of *Syntrophobacteraceae*-like bacterium or to *S. wolinii*, while only 8.3% were most similar to the classic SRB groups described by Daly et al. (2000) (e.g., uncultured *Desulfobacteraceae* bacterium). 12.5% of the excised DGGE fragments were most similar to unidentified *Deltaproteobacterium* clones. Among the 17 bands representing SO_4-enriched samples, 29.4% were most closely related (87 to 92% similarity) to sequences of *Syntrophobacteraceae,* while 11.7% were tightly affiliated (93 to 97% similarity) with

SRB (e.g., *Desulfocapsa* spp. family *Desulfobulbaceae*). The additional 23.5% were affiliated with an unidentified *Deltaproteobacterium*. In addition, native and SO_4-enriched MAT samples contained 16S rRNA genes with similarity to those of *Bacillus* spp. and *Acidocella* spp.

The SRB-like and *Syntrophobacter*-like sequences of the partial 16S rRNA genes were aligned with those of reference SRB and *Syntrophobacteraceae* species for phylogenetic analysis (Fig. 3.1). The tree clearly shows the separation of environmental clones from reference strains. Twenty seven of a total of 29 clones were found in three clusters exclusive to MAT sequences (A-C in Fig. 3.1) that were separated with a significant, yet modest, boot support, from a cluster, defined here as the *Sytrophobacter-Desulforhabdus* group, which consisted of reference sequences representing four syntrophic organisms and one SRB, *Desulforhabdus aminigena*. Together with cluster A-C, this cluster was separated from the rest of the tree by a boot support value of 85 (Fig. 3.1).

Sequences of Cluster A were most similar (88 to 93%) to an uncultured *Syntrophobacteraceae* bacterium clone SbIISybac3-2 (accession number AY167459) from acidic fen soil or to *S. wolinii* (X70906) (Table S3.4). Cluster B was most closely related (91-100%) to uncultured *Deltaproteobacterium* clones (e.g., DQ294024) and *S. wolinii* (Table S3.4). Sequences of Cluster C included those that were most similar (91-92%) to uncultured *Syntrophobacteraceae* bacterium clones (e.g. SbIISybac3-2) or *S. wolinii* (Table S3.4). Two clones clustered with a *Desulfocapsa* sp. clone (Cluster D in Fig. 3.1) and were most closely affiliated (93-97%) with *Desulfocapsa* sp. (DQ831556). While this cluster was well supported (bootstrap value of 96), its relationship to the rest of the tree cannot be deciphered as there is no bootstrap support for the node separating this lineage from the rest of the

tree. The results of this analysis clearly show the uniqueness of the SRB-like microbes in the microbial community of the MAT site and their close affiliation with *Syntrophobacter*-like organisms.

Molecular characterization of *Syntrophobacter*-like bacteria from the enriched MAT SRB consortia.

Further characterization of syntrophs in MAT and saltmarsh samples was conducted by cloning and sequencing of PCR products obtained with primers specific to "*Syntrophobacteraceae*" 16S rRNA genes (Fig. 3.2). A total of 29 clones were sequenced from the MAT enrichment with a SRB medium (Table S3.5). 75.9% of the clones were 95-98% similar to uncultured acid fen soil clones (SbIISybac 11-2, 3-2, 12-1, 25-1, and 5-2) from a *Sphagnum*-covered fen in Germany (Loy et al., 2004). The second largest group included 17.2% of the library and was 86-96% similar to the 16S rRNA gene of *S. wolinii*. The remaining two clones were related to *S. sulfatireducens* and an unidentified SRB from rice root at 92% and 93% similarity, respectively. The clone library representing the native Cheesequake saltmarsh mat community consisted of 26 clones (Table S3.5), and included a group (30.8% of the clones) bearing 93-96% similarity to environmental clones from a mature mat collected at an evaporative lagoon in Puerto Rico (Isenbarger et al., 2008), a group (11.5% of the clones) at similarity ranging from 89 to 94% to uncultured *Syntrophaceae* bacterium clones from a tar-contaminated subsurface soil in Germany (Winderl et al., 2008), and four singlet clones most closely related to an uncultured *Syntrophobacteraceae* bacterium clone SbISybac13 (Loy et al., 2004), to *Syntrophobacter pfennifii* (93% similarity), to *Desulfobacterium cetonicum* and to a novel SRB strain (95-97% similarity). The remaining 11 clones in the saltmarsh mat library were most similar to unidentified Deltaproteobacterium phylotypes from

miscellaneous habitats including coastal harbor sediment, hydrocarbon seep sediment, mangrove soil, and freshwater ponds.

Phylogenic analysis showed that all MAT and three saltmarsh mat clones clustered with the *Syntrophobacter-Desulforhabdus* group with a 100% bootstrap value (Fig. 3.2). The majority of the MAT clones, clones SMSyn 25 and SMSyn17 being the exceptions, formed a well-supported cluster (bootstrap value of 99) with uncultured phylotype clones from a *Sphagnum* fen in Germany and with *S. wolinii*. Within this cluster, 37.9% of the total were grouped to the uncultured *Syntrophobacteraceae* clone SbIISybac11-2 and 3-2, 17.2% grouped to the uncultured *Syntrophobacteraceae* clone SbIISybac25-1 or to the uncultured clone D14217 from a flooded paddy soil, and 10.3% grouped with *S. wolinii*. The majority of the saltmarsh mat clones grouped separately from the MAT clones in three clusters, which included no cultured syntrophs or SRB (Fig. 3.2). The largest cluster, which was not boostrap supported, was composed of two subclusters, one (accounting for 50% of the total sequences) which was associated with an uncultured clone MAT-CR-P1-D07 from a lagoon and an uncultured clone VHS-B1-47 from coastal harbor sediment, and another minor subcluster (7.7%) related to the uncultured *Syntrophaceae* clone D15_36. One of the remaining two clusters on the bottom of tree was probably related to the well-characterized SRB (19.2% of the saltmarsh mat clones), and the other (11.5% of clones), clustering with an uncultured clone MSB-3A9 from mangrove soil, was not affiliated with any other cluster. The results showed that the "*Syntrophobacteraceae*"-related clones from the MAT sample from Sunday Lake and from Cheesquake's mat were related to cultured and uncultured syntrophic bacteria. The separation of the clusters representing each library suggests different syntrophic communities in the two sites.

Hg methylation by individual strains. In order to test the role of syntrophic associations in Hg methylation, three representative *Syntrophobacter* spp. which were highly similar to those present in the MAT sample form Sunday Lake, one methanogen, and two *Desulfovibrio* spp., strains DSM 2603 and ND132, weak and strong Hg methylators, were selected for the experiments, respectively. Incubations of mono- and co-cultures were examined for growth and Hg methylation.

To investigate Hg methylation by monocultures of each strain prior to coculturing experiments, each strain was inoculated in its recommended growth medium, and grown for 5 days when an increase of 2.2 to 7.6 fold in biomass, as measured by protein concentration, was noted for *S. wolinii*, *S. sulfatireducens*, *S. fumaroxidans*, *M. hungatei*, and *D. africanus*, respectively ($p<0.01$; Fig. 3.3B). A statistically significant change in biomass over time was not observed for the heat-killed *S. wolinii* and the blank ($p>0.68$, data not shown). The trace amount of protein (2.3 to 3.0 μg ml^{-1}) exhibited in the blank was likely from the rumen fluid added to the *S. wolinii* medium (data not shown).

The only known Hg methylator to be included in the experiment (Fig. 3.3A), *D. africanus* DSM 2603 synthesized the highest amount of CH_3Hg (1.88 pg CH_3Hg μg $protein^{-1}$) with a potential methylation rate of at least 1.11 pg CH_3Hg μg $protein^{-1}$ day^{-1} calculated for the first two days of incubation, following which CH_3Hg accumulation leveled off. The only other culture to methylate Hg was *S. wolinii* with a methylation rate of 0.67 pg CH_3Hg μg $protein^{-1}$ day^{-1} on Day 2 followed by a decline in CH_3Hg accumulation in subsequent days. Statistical analyses showed that Hg methylation by the live *S. wolinii* was significantly higher than the CH_3Hg accumulation by the blank and the heat killed *S. wolinii* control ($p<0.01$), as well as the CH_3Hg accumulation by the other two *Syntrophobacter* spp. ($p<0.0001$) and *M*

hungatei (p=0.01). *S. sulfatireducens*, *S. fumaroxidans*, and *M. hungatei* did not synthesize CH_3Hg since their monocultures contained CH_3Hg concentrations similar to that of the blank ($p>0.05$) although they had significantly higher protein concentrations than the blank and were growing during the five days of the experiment (Fig. 3.3B).

Methylation by cocultures of *M. hungatei* with *Syntrophobacter* spp. The three cocultures of *M. hungatei* with *Syntrophobacter* spp., grown with propionate produced significantly higher protein concentrations than when grown as mono-cultures under the same conditions (Fig. 3.4D-F, $p<0.0001$ for each coculture). After five days of incubation, cocultures of *M. hungatei* with *S. wolinii*, *S. sulfatireducens*, or *S. fumaroxidans* grew by 2.1 ($p<0.05$), 2.4 ($p<0.0001$), and 1.8 ($p<0.01$) fold, respectively. Monocultures of *S. wolinii* and *S. sulfatireducens* in coculture media which did not contain sulfate grew by 1.5 ($p<0.05$) and 1.9 ($p<0.0001$) fold, respectively, but growth was not noted for the monoculture of *S. fumaroxidans* ($p>0.07$). The protein concentrations in *M. hungatei* monocultures on the coculture medium modified from DSMZ Medium 307 (*S. wolinii* medium) significantly increased by 2.6 times ($p<0.001$) and 1.9 times ($p=0.003$) respectively over five days incubation, while on the coculture medium for *S. fumaroxidans* the protein level showed no obvious changes ($p=0.11$).

For the coculture of *M. hungatei* with *S. wolinii*, the difference in CH_3Hg synthesis between *M. hungatei* and the killed control was insignificant ($p>0.05$, Fig. 3.4A), suggesting that *M. hungatei* was not able to produce CH_3Hg in this medium. However, methylation by the coculture was significantly higher than methylation by other treatments ($p<0.0001$), reaching a highest potential methylation rate of 2.00 pg μg protein^{-1} day^{-1} on Day 2 and the *S. wolinii* monoculture methylated Hg as well at a

rate of 0.96 pg μg initial protein^{-1} day^{-1} calculated with data on Day 2 ($p<0.01$). The coculture of *M. hungatei* with *S. sulfatireducens* produced low levels of CH_3Hg with a discernable increase from Day 1 to later time points ($p<0.0001$) (Fig. 3.4B). Statistical analysis indicated that this production was significantly higher than those of the heat killed control and of the monocultures of all strains ($p<0.0001$). Neither *S. sulfatireducens* nor *M. hungatei* alone formed CH_3Hg as their levels were similar to that of the killed control. Surprisingly, a monoculture of *M. hungatei,* growing in the co-culture medium prepared for supporting its syntrophic growth with *S. fumaroxidans*, methylated Hg ($p<0.0001$ for treatments comparison and $p<0.01$ for the increase in CH_3Hg concentrations with time; Fig. 3.4C). Even more surprising is the observation that CH_3Hg synthesis by the coculture of *M. hungatei* and *S. fumaroxidans* was similar to that of the killed controls, suggesting that methylation by the methanogen was inhibited while grown with the syntroph. *S. fumaroxidans* grown in mono- or co-culture did not methylate Hg.

Methylation by syntrophic associations of *M. hungatei* with *Desulfovibrio* spp. There was no statistically significant growth for the monoculture of *M. hungatei* on the sulfate-free lactate coculture media in five days of incubation ($p>0.59$, Fig. 3.5C and D). Monocultures of *D. desulfuricans* ND132 and *D. africanus* DSM 2603 showed statistically significant but limited growth over time ($p<0.05$ and $p<0.01$, respectively) since the overall protein concentrations of these cultures were only slightly higher than those of the killed control ($p=0.03$ and $p=0.14$, respectively). Growth of cocultures of *M. hungatei* with *D. desulfuricans* and with *D. africanus*, however, was highly significant ($p<0.0001$ for both) with 7.9 and 5.1 fold increase in protein concentrations in five days, respectively.

A monoculture of *M. hungatei* did not methylate Hg in the coculture medium

(Fig. 3.5A and B). A monoculture of strain ND132 in the coculture medium had a significantly higher specific rate of CH_3Hg synthesis ($p=0.003$), while methylation by *D. africanus* DSM 2603 was barely statistically different ($p=0.051$) as compared with the killed control and the blank. After 1 day of incubation for ND132 and 2 days for DSM 2603, however, no significant increases of CH_3Hg were observed ($p>0.31$ and >0.37, respectively). This low level of methylation by the monocultures may be due to fermentative growth on lactate. Syntrophic growth with *M. hungatei* greatly stimulated Hg methylation by both strains ($p<0.0001$) in comparison to methylation as monocultures. For strain ND132, syntrophy enhanced methylation by 2.1 to 3.3 fold after Day 2 with 2.6 to 10.1% of the spiked $^{203}Hg(II)$ methylated by Day 5. For strain DSM 2603 and as compared to methylation by the monoculture, syntrophy increased methylation by 6.1 to 19.4 fold ($p<0.0001$) after Day 1, with 9.2 to 13.7% of the spiked $^{203}Hg(II)$ being methylated in 5 days. Interestingly, CH_3Hg synthesis by a coculture of *M. hungatei* with *D. africanus* DSM 2603, a weak Hg methylator, was 2.1 to 3.5 fold higher ($p>0.0001$) than methylation by the coculture of *M. hungatei* with *D. desulfuricans* ND132, a strong methylator.

Methylation by syntrophic associations of *S. sulfatireducens* with *Desulfovibrio* spp. Results of methylation assays in cocultures of the syntroph *S. sulfatirducens* with two Hg methylating *Desufovibrio* spp. in media containing 3.94 mM sulfate and 15.6 mM propionate at 28 °C are presented in Fig. 3.6A and B. The coculture with *D. desulfuricans* ND132, synthesized significantly more CH_3Hg (pg CH_3Hg per μg protein) than the ND132 monoculture; this increase amounted to 1.3 to 1.6 fold after the first day of incubation ($p<0.0001$, Fig. 3.6A). This increased Hg methylation by the coculture relative to the monoculture was also seen when results were expressed as CH_3Hg concentration (pM) in culture media or as % methylation of

the initial spiked Hg (II). Between days 4 and 5, 22.2 to 24.4% of the spiked ^{203}Hg(II) was methylated by the coculture in comparison with 15.0 to 18.5% by the monoculture (data not shown). Over the incubation period, a significant increase in Hg methylation was observed for the mono- and the cocultures of ND132 ($p<0.0001$). Methylation by monocultures of *S. sulfatireducens* was similar to that of the heat killed control ($p=0.42$).

The effect of sulfate concentration on Hg methylation by the syntrophic association between *S. sulfatireducens* and *D. desufuricans* ND132 was studied by varying sulfate concentrations from 0.39 to 19.71 mM and at two different temperatures (28 °C and 37 °C). At all sulfate levels including 0.39, 1.97, 3.94, and 19.71 mM, coculturing significantly stimulated Hg methylation ($p<0.01$, 0.0001, 0.0001, and 0.0001, respectively) at 28 °C (Fig. S3.3) and 37 °C (data not shown) as compared to the ND132 monoculture. Protein assays (data not shown) indicated that at all sulfate levels both coculture and the *S. sulfatireducens* monoculture grew significantly over time while the ND132 monoculture failed to grow possibly because it was unable to utilize propionate as sole carbon and energy source.

For the syntrophy of *S. sulfatireducens* with *D. africanus* DSM 2603, the coculture stimulated CH_3Hg synthesis compared with the monoculture of DSM 2603 by 1.8 to 2.0 fold after Day 1 ($p<0.0001$, Fig. 3.6B). Significantly higher positive effects of coculturing on Hg methylation (by more than 2 fold after Day 1) were observed when results were expressed as concentrations of CH_3Hg (pM) or as % methylation of spiked Hg(II) (data not shown). Hg methylation by the mono- and the coculture of DSM 2603 significantly increased over five days of the incubation ($p<0.0001$) while no CH_3Hg accumulation was noted in incubations of the *S. sulfatireducens* monoculture or the heat killed control and the blank.

Methylation by strain DSM 2603 was stimulated by coculturing with *S. sulfatireducens* at a range of sulfate cocnentrations (0.39 to 19.71 mM) as compared to monoculture methylation (Fig. S3.4) and 1.3 to 2.4 fold increases in protein concentrations were recorded for cocultures and for *S. sulfatireducens* monocultures, but not for *D. africanus* DSM2603 monocultures, during the 5 days of the incubation period (data not shown).

Growth and sulfate utilization during coculture and monoculture growth of *S. sulfatireducens* with *Desulfovibrio* spp.

Flow cytometery (FC) was used to follow growth of each strain during coculture and monoculture incubations of *S. sulfatireducens* DSM 16706 with methylating *D. desufuricans* spp. with propionate and 3.94 mM sulfate (Fig. 3.6C and D). SYBR gold stained cells of the two strains partaking in coculture growth were distinguished by the FC based on differences in cell properties leading to unique patterns of light scattering (Fig. S3.1 and S3.2). Strain ND132 failed to grow when incubated alone in the propionate-sulfate coculturing medium (Fig. 3.6C, $p>0.78$). When cocultured with *S. sulfatireducens*, however, its cell numbers increased 4.3 fold in five days ($p<0.0001$). After 1 day, the cell density of ND132 in the coculture was 7.7 to 18.7 fold higher than its density in the monoculture ($p<0.0001$). Both mono- and cocultures of strain DSM 16706 grew significantly by 1.6 and 2.6 fold, respectively, during the incubation period ($p<0.03$ and $p<0.0001$, respectively). In the coculture, the proportion of strains ND132 to strain DSM 16706 after one day of incubation ranged from 1.0:2.9 to 1.0:4.9. The growth rate constant calculated for the five days of growth for *S. sulfatireducens* in coculture (0.189 day^{-1}) was significantly higher ($p<0.0001$) than its growth rate as a monoculture (0.091 day^{-1}).

No growth was noted in the *D. africanus* monoculture (Fig. 3.6D), while its cell

density increased by 3.3 fold when cocultured with the syntroph for 5 days ($p<0.03$) and was significantly higher, by 1.8 to 3.4 fold, than its growth as a monoculture ($p<0.05$) after one day of incubation. The cell density ratios between *D. africanus* DSM 2603 and *S. suflatireducens* DSM 16706 in the coculture ranged from 1.0:1.1 to 1.0:1.4. The calculated five days growth rate constant for *S. sulfatireducens* in coculture (0.252 day^{-1}) was also significantly higher ($p<0.0001$) than its growth rate as a monoculture (0.180 day^{-1}). Thus, the syntroph grew faster in cocultures with either *Desulfovibrio* spp. partner than as a monoculture, suggesting that for this strain, syntrophic growth with a *Desulfovibrio* sp. was more efficient than growth by sulfate reduction.

Sulfate consumption was followed during coculture and monoculture growth of the syntrophic associations (Fig. 3.6E and F). For *D. desulfuricans* ND132 monoculture, sulfate levels in monoculture were not significantly different from the blank with no obvious consumption during the incubation period (Fig. 3.6E, $p>0.24$) indicating that ND132 could not consume sulfate in the coculture medium. Sulfate concentrations in five days, however, decreased significantly ($p<0.0001$) by a coculture of ND132 with *S. sulfatireducens* (50.8%) and by a monoculture of the syntroph alone (34.5%) and statistical comparison illustrated that sulfate reduction by the coculture was more significant than that by the monoculture ($p<0.0001$). Similarly, sulfate concentrations in the monoculture of *D. africanus* were unchanged when compared with the blank ($p>0.05$). 61.8% of the sulfate was reduced by the coculture, which was significantly higher than by the monoculture of the syntroph (51.3%; $p<0.0001$; Fig. 3.6F).

Influence of sulfate concentration on Hg methylation by *Desulfovibrio* spp. during syntrophic growth with *S. sulfatireducens*.

The maximum net increase in Hg methylation (pg CH_3Hg per µg initial protein contents), calculated from data collected on Day 4 of the incubations (Fig. S3.3 and S3.4), by differentiating CH_3Hg synthesis of syntrophic cocultures with that from monocultures of *Desulfovibrio* spp., was positively correlated with the initial concentration of sulfate in the coculturing medium. This correlation was stronger for cocultures of *D. africanus* (p=0.002) than for strain ND132 (p=0.157) with *S. sulfatireducens* (Fig. 3.7A), and suggested that sulfate concentration had a strong influence on Hg methylation by the syntrophic associations, i.e., the higher the initial sulfate concentrations, the larger was the stimulation of CH_3Hg production by cocultures relative to the monocultures of the methylating SRB.

When Hg methylation in cocultures and monocultures was plotted as concentrations (pM) of CH_3Hg in media vs. sulfate amendment, it is obvious that the initial sulfate concentration had a strong negative effect on CH_3Hg production by the coculture of the syntroph strain DSM 16706 with strain ND132, while the effect of sulfate concentration on CH_3Hg production by the ND132 monoculture was not significant (Fig. 3.7B, p=0.24). In contrast, increased sulfate concentrations from 0.39 to 19.71 mM significantly enhanced CH_3Hg production in both the co- and monocultures of *D. africanus* (Fig. 3.7C, p=0.0004 and 0.008, respectively).

Discussion

Syntrophs and SRB in the floating *Sphagnum* MAT. Extensively acidic *Sphagnum* peat wetlands dominate boreal forest ecosystems in northern hemisphere (Dedysh et al., 1998; Brauer et al., 2006). Yet, little is presently known about the microbial communities of these ecosystems and their ecological functions. By specifically

targeting SRB as a group known to be involved in mercury methylation, we retrieved 16S rRNA gene fragments that were more closely related to 16S rRNA genes of syntrophic bacteria than to those of SRB (Fig. 3.1 and Table S3.4). Among sequences that were retrieved from DGGE gels following amplification of DNA extracts from the MAT sample with the group-specific SRB primer sets, only 8.3% were most similar to classic SRB (e.g., uncultured *Desulfobacteraceae*), while 58.3% were related to *Syntrophobacter* spp. The latter, however, were only 91 to 92% similar to the 16S rRNA gene sequence of *S. wolinii* (Table S3.4) and these sequences constituted a novel cluster in the phylogenetic tree of the SRB (Fig. 3.1). Phylogenetic analyses showed that this cluster of *Syntrophobacter* spp. was related to SRB, most closely to *Desulforhabdus amnigenus* (Fig. 3.1) (Harmsen et al., 1993). The amplification of *Syntrophobacter*-related sequences by SRB group-specific primer sets may be explained by the high sequence similarity of 16S rRNA genes between *Syntrophobacter* spp. and SRB which both belong to the *Deltaproteobacteria*, and by low abundance of SRB in the MAT sample.

Among the *Syntrophobacter*-like sequences, 50.0% were 88-93% similar to the uncultured *Syntrophobacteraceae* bacterium clones SbIISybac3-2 and 12-1, obtained from the water saturated and acidic *Sphagnum*–covered fen soil in Germany (Loy et al., 2004). Loy et al. (2004) used microarray and clone sequencing approaches showing that the *Syntrophobacter*-like consortia, loosely affiliating with *S. wolinii* (3.6 to 5.5% of sequence dissimilarity), were common in the fen SRB community. In Sunday Lake, 42.9% of retrieved MAT *Syntrophobacter*-like sequences from DGGE were related to *S. wolinii* with 90-92% of similarity, while 7.1% of them were similar to the uncultured *Syntrophobacter* sp. clone X3Ba04 belonging to the rice soil cluster that was described by Lueders et al. (2004). Using stable-isotope probing with ^{13}C-

propionate as a substrate, the authors showed that this *Syntrophobacteraceae*-cluster, active in propionate oxidation under methanogenic conditions, constituted 29% of all clones. Similarly, novel *Syntrophobacter-Desulforhabdus* groups were discovered in a low sulfate *Sphagnum* peat bog in West Siberia (Sizova et al., 2003), freshwater wetland soil in the Florida Everglades (Chauhan et al., 2004), rice rhizosphere (Scheid & Stubner, 2001), and saltmarsh sediments in New England (Bahr et al., 2005). Thus, *Syntrophobacter*-like bacteria, originally described among municipal sewage digester isolates (Boone & Bryant, 1980), are widely distributed in freshwater wetlands and other water logged habitats including saltmarshes.

The overall sequence similarity of the DGGE fragments to the 16S rRNA genes of *Syntrophobacteraceae*-like bacteria was only 88-93%, possibly due to the short DGGE fragment amplified by the DGGE primers employed (193 bp). On the other hand, high similarities (95-98%) of 16S rRNA genes were found for the clone libraries of the detected *Syntrophobacteraceae*-like bacteria from the enriched MAT consortia (Table S3.5). A majority, 75.9% of all clones obtained from MAT samples that had been enriched under sulfate reducing conditions were 95-98% similar to the same *Syntrophobacteraceae*-like sequences that had been obtained from the *Sphagnum*-covered fen by Loy et al. (2004). These results are consistent with the results of the nested PCR-DGGE analysis (see above), and are a strong indication for the presence of *Syntrophobacter* spp. in the floating *Sphagnum* moss mats in Sunday Lake. A clone library constructed from MAT enrichments with propionate and sulfate (Yu et al., 2010) consisted of 74.1% clones that were affiliated with *Syntrophobacteraceae*-like bacteria (Fig. A-1 in Appendix A). Thus, *Syntrophobacter* spp.-like bacteria were enriched under classical sulfidogenic conditions as well as in propionate-sulfate medium from *Sphagnum* moss, consistent with the documented

ability of some *Syntophobacter* spp. to grow as sulfate reducers in the presence of sulfate (Wallrabenstein et al., 1994).

While applying the same *Syntrophobacteraceae*-specific primers for the cloning and sequencing for DNA extract from the saltmarsh mats in Cheesequake, NJ, this study indicated that the retrieved 16S rRNA genes sequences formed novel bacterial clusters in the phylogeny, and mostly differed from the Sunday Lake MAT *Syntrophobacteraceae*-like bacteria (Fig. 3.2 and Table S3.5). The dominant Cheesequake sequences (30.8%) were highly related to 16S rRNA genes of uncultured bacteria from a lagoon mat in Puerto Rico amplified by a new 10-nucleotide "miniprimer" which could target more novel sequences than the standard primer methods from the environmental samples (Isenbarger et al., 2008).

Propionate conversion is a key intermediate process in organic matter mineralization under methanogenic conditions. Under anaerobic conditions, the *Syntrophobacter*-like group degrades propionate either by syntrophy with methanogens (Boone & Bryant, 1980; de Bok et al., 2004), or in pure cultures with sulfate. Indeed, most members of this guild carry *dsrAB* genes encoding dissimitory sulfite reductase and express them in the presence of sulfate (Harmsen et al., 1998; Friedrich, 2002; de Bok et al., 2004). However, the role of this association in ecosystem processes in wetland habitats is largely unknown. This study showed that *Syntrophobacter*-like bacteria dominated SRB communities in floating *Sphagnum* moss mats, a habitat where both total Hg and CH_3Hg accumulated and where potential Hg methylation rates were high (Yu, et al., 2010). Methanogens were present and active in MAT samples as confirmed by PCR detection of *mcr* genes (encoding for methyl coenzyme M reductase) in cDNA that was obtained from MAT RNA extracts (Fig. B-1 in Appendix B). Together, these results highlight the role of

syntrophy in the terminal oxidation of organic matter in *Sphagnum* moss-dominated mats and brings up the possibility that syntrophic associations contribute to the accumulation of MeHg and the high potential for Hg methylation previously reported for MAT samples (Yu et al., 2010).

Syntrophic associations among *Syntrophobacter* spp., a methanogen, and two Hg methylating *Desulfovibrio* strains and their relationships with Hg methylation. All *Syntrophobacter* species isolated so far by definition of phylogenic taxonomy belong to the Deltaproteobacteria. Because *Syntrophobacter* spp. are phylogenetically affiliated with SRB (Harmsen et al., 1993) and some of them can grow by sulfate reduction (Wallrabenstein et al., 1994), it was important to determine whether representative strains of this guild methylated Hg. Table 3.1 summarized the enhanced methylation activities by mono- and co-cultures of syntrophic associations tested in this study. This study demonstrated that *S. wolinii* in monoculture growing with propionate and sulfate had a limited ability to methylate Hg (Fig. 3.3A), while the other two *Syntrophobacter* spp. did not produce CH_3Hg. The lack of Hg methylation in *S. fumaroxidans* is consistent with a previous report showing that this strain did not methylate Hg when grown with fumarate without sulfate (Ranchou-Peyruse et al., 2009).

Syntrophic propionate oxidation by *Syntrophobacter* sp. with *M. hungatei* is an important pathway under methanogenic conditions, consisting of two steps: an initial endergonic process whereby propionate is oxidized to acetate and hydrogen by the syntroph, and the following exergonic process in which H_2 is utilized by the methanogenic partner (Boone & Bryant, 1980; Muyzer & Stams, 2008). The reactions are listed as follows:

(1). An acetogenic reaction by *Syntrophobacter* spp.:

$Propionate^- + 3H_2O \rightarrow Acetate^- + HCO_3^- + H^+ + 3H_2$

$\Delta G^{0'} = +$ 76.1 kJ per mol propionate

(2). A methanogenic reaction by *M. hungatei*:

$4\ H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3\ H_2O$

$\Delta G^{0'} =$ - 151.9 kJ per mol carbon reduced

Mercury methylation by *S. wolinii* was observed when grown in a coculture with *M. hungatei* and as a monoculture, likely by the fermentation of propionate (Fig. 3.4A). Cocultures of *M. hungatei* with *S. sulfatireducens* or with S. *fumaroxidans* had very low methylation activities (Fig. 3.4B and C, respectively). Hg methylation of *M. hungatei* varied among different culture media. *M. hungatei* synthesized CH_3Hg when the monoculture was grown in its coculture medium with *S. fumaroxidans* containing propionate plus yeast extract (Fig. 3.4A and Table S3.2), likely by resting cells as growth of *M. hungatei* was not observed over five days of incubation in this medium (Fig. 3.4F). *M. hungatei* failed to methylate Hg in DSMZ medium or in coculture medium with *S. wolinii* or *S. sulfatireducens* which only contained propionate (Table S3.1). Pak and Bartha (1998) showed that *Methanococcus maripaludis* was unable to synthesize CH_3Hg either in sulfate-free lactate medium or in a complete methanogen medium although the protein yield of this strain reached 38 $\mu g\ ml^{-1}$.

Desulfovibrio spp. are fast-growing incomplete oxidizers in sulfate reducing enrichments and strong competitors among SRB (Laanbroek et al., 1984; Bødtker et al., 2008). This group also includes most Hg methylators described so far. Hg methylating *Desulfovibrio* spp. grew slightly in the sulfate-free lactate medium (Fig. 3.5C and D). However, in association with H_2-utilizing methanogen, *Desulfovibrio* spp. oxidize lactate to acetate, bicarbonate, and H_2 by fermentation. Methanogens

such as *M. hungatei* may then use the H_2, bicarbonate or acetate for methanogenesis (Bryant et al., 1977; Pak & Bartha, 1998; Muyzer & Stams, 2008). The two half reactions described below greatly stimulated coculture growth (Fig. 3.5C and D) by interspecies H_2 transfer:

(1). An acetogenic reaction by *Desulfovibrio* spp.:

$$Lactate^- + 2\ H_2O \rightarrow Acetate^- + HCO_3^- + H^+ + 2\ H_2$$

$\Delta G^{0'}$ = - 4.2 kJ per mol lactate

(2). A methanogenic reactions by *M. hungatei*:

$$4\ H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3\ H_2O$$

$\Delta G^{0'}$ = - 151.9 kJ per mol carbon reduced

When *M. hungatei* was incubated with *D. desulfuricans* ND132 or *D. africanus* DSM 2603, syntrophy significantly stimulated Hg methylation compared with the monocultures of *Desulfovibrio* spp., which was consistent with the results of Pak and Bartha (1998) showing enhanced methylation by syntrophy between *Methanococcus maripaludis* with *D. desulfuricans* strains LS or ND132. These results, therefore, confirm a synergistic stimulation of CH_3Hg synthesis by syntrophy of Hg-methylating *Desulfovibrio* spp. with H_2-scavenging methonagens. Pak and Bartha (1998) reported higher specific CH_3Hg synthesis per μg initial protein compared to rates reported in this study possibly due to a higher concentration of added Hg (II) (1 mg L^{-1}), a 169 fold higher than the level used in my experiments (5.9 μg L^{-1}). Previous studies showed that there was a strong positive correlation between the added Hg(II) concentration and extent microbial Hg methylation (Gilmour & Riedel, 1995; Ranchou-Peyruse et al., 2009). This study further demonstrated that syntrophy of *M. hungatei* with *D. africanus*, a weak Hg methylator, more significantly enhanced methylation than its syntrophy with the strong methylator, strain ND132 (Fig. 3.5A

and B), even though the effect of syntrophy on growth of the later association was more pronounced than with the former over the incubation period (Fig. 3.5C and D). This trend was also confirmed when Hg methylation results were expressed as % methylation of the spiked Hg (II). The proportion of cell density between two syntrophic partners in each coculture was not quantified in this study. Based on microscopic examinations, Pak and Bartha (1998) concluded that the two strains in the *M. maripaludis* and *Desulfovibrio* spp. cocultures were growing roughly in equal rates. Thus, it seems that the contribution of syntrophy to CH_3Hg synthesis is not necessarily proportional to the Hg methylation ability of the Hg methylating partner (i.e., *Desulfovibrio* spp.) as determined during monoculture growth with sulfate. A weak Hg methylator when associated with methanogens may produce the same or even more CH_3Hg in the natural environments than a strong methylator. These observations clearly suggest that our perspectives on the contributions of specific microbial guilds and methylating pathways to CH_3Hg production, gleaned from studies of pure cultures, need to take into account the numerous and complex interactions of methylating microbes in the environment where CH_3Hg is produced.

Propionate oxidization could occur through different metabolic pathways in the presence or absence of sulfate. Muyzer and Stams (2008) speculated that when sulfate was present but not enough for complete oxidation by sulfate reduction, hydrogen-utilizing methanogens were most likely replaced by hydrogen-utilizing sulfate reducers. Syntrophs such as *S. wolinii* could couple with hydrogen-utilizing sulfate reducers to degrade organic acids including propionate. In the sulfate-limited *Syntrophobacter-Desulfovibrio* cocultures, propionate could probably be degraded by two processes shown below (Boone & Bryant, 1980; Muyzer & Stams, 2008):

(1). An acetogenic reaction by *Syntrophobacter* spp.:

$Propionate^- + 3H_2O \rightarrow Acetate^- + HCO_3^- + H^+ + 3H_2$

$\Delta G^{0'} = +\ 76.1$ kJ per mol propionate

(2). A sulfate reduction by *Desulfovibrio* spp.:

$4H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4H_2O$

$\Delta G^{0'} = -\ 151.9$ kJ per mole sulfur reduced

Since H_2 is a powerful electron donor for anaerobic respiration, in a syntrophic interaction, H_2 is generally maintained below 10^{-4} atm due to the quick consumption by H_2-consumers (Madigan et al., 2009). At such low levels of H_2, oxidation by *Syntrophobacter*, a reaction that is endergonic under standard conditions (1 atm pressure and 1 M concentration at pH 7) as shown in the equation, becomes exergonic (Madigan et al., 2009) and makes the propionate fermentation and thus growth of the *Syntrophobacter* partner possible. *S. wolinii*, the first strain to be identified as a syntrophic propionate degrader, was initially isolated as a defined mixed culture with *Desulfovibrio* G11 (later identified as *Desulfovibrio vulgaris* G11) from an anaerobic sewage digester (Boone & Bryant, 1980). This coculture had been deposited in culture collections for over a decade before the *S. wolinii* was finally isolated as a pure culture under sulfate reduing conditions in 1994 (Wallrabenstein et al., 1994; de Bok et al., 2004). By providing 20 mM propionate as a sole energy and carbon source and 20 mM sulfate in a basal medium, later formulated as DSMZ medium 307 after adjustment for *S. wolinii*, Boone and Bryant (1980) showed that *S. wolinii* and *D. vulgaris* G11 could be well maintained as a coculture that grew better than the syntrophy of *S. wolinii* with *M. hungatei* on the same medium when sulfate was not added. Other fatty acids such as acetate, butyrate, caproate, and palmitate did not support the growth of the *S. wolinii-Desulfovibrio* sp. coculture. *S. wolinii* in the coculture could not grow if substrates were replaced by lactate with sulfate or

pyruvate in the absence of sulfate since strain G11 by itself could efficiently oxidize these substrates by sulfate reduction or fermentation and its growth overwhelmed that of *S. wolinii*. Thus, it seems that *S. wolinii* and *D. vulgaris G11* could form a syntrophic associatiation only when the medium contained propionate with sulfate. This syntrophic relationship has been little studied since 1980. By coculturing *S. wolinii* with *M. hungatei* on a propionate-sulfate medium, Wallrabenstein et al. (1994) showed that *S. wolinii* had preferred to couple propionate oxidation with proton consumpsion by *M. hungatei* producing a significant amount of CH_4, instead of reducing sulfate. The reasons why *S. wolinii* has strong tendency to transfer electrons to hydrogenotrophic partners even in the presence of sulfate in the coculture with *Desulfovibrio* or *M. hungatei* were unclear (Boone & Bryant, 1980; Wallrabenstein et al., 1994).

This study shows for first time that syntophic cocultures of *S. sulfatireducens* with *Desulfovibrio* spp., an association that greatly increased the growth of *D. desulfuricans* ND132 and *D. africanus* relative to their growth as monocultures in the same medium (Fig. 3.6C and D), significantly stimulated Hg methylation (Fig. 3.6A and B). Similar stimulation of CH_3Hg synthesis by a coculture of *S. wolinii* with *D. desulfuricans* ND132 was also observed (Yu, unpublished data). The growth of *S. sulfatireducens* in the two cocultures was also significantly stimulated as indicated by higher growth rate constants in comparison to those calclulated from the monocuture of this strain. Sulfate consumption was faster in cocultures of *S. sulfatireducens* with either strain ND132 or DSM 2603 (Fig. 3.6E and F), as compared with *S. sulfatireducens* monoculture, suggesting a more efficient sulfate utilization by the cocultures (Fig. 3.6C and D). Although the cell density of *S. sulfatireducens* in the cocultures was significantly and slightly higher than those of strains ND132 and DSM

2603, respectively, it is difficult to judge which partner consumed more sulfate in the coculture incubations. Muyzer and Stams (2008) proposed that, when *S. wolinii* was present in a coculture with *Desulfovibrio* spp., sulfate reduction by *S. wolinii* would be suppressed and the strain would tend to grow as an acetogen by fermentation. So far this hypothesis has not been tested. Laanbroek et al. (1984) indicated that *Desulfovibrio* spp. had the highest affinity for sulfate among the several SRB species including *Desulfobulbus* spp. and *Desulfobacter* spp., suggesting that *Desulfovibrio* spp. are strong competitors for sulfate. Thus, it is possible that when sulfate is present, *Desulfovibrio* spp. outcompete *Syntrophobacter* spp. and lead to the establishment of the syntrophic relationships. If this hypothesis is corerct, it raises interesting questions regarding the modes by which *Desulfovibro* spp. suppress *Syntrophobacter* spp. and what sulfate concentrations affect sulfate reduction by *Syntrophobacter* spp.

In the *Syntrophobacter-Desulfovibrio* coculture, *Desulfovibrio* exhibit unique adaptations in life styles amid changing environmental conditions. *Desulfovibrio* are able to grow on lactate by interspecies H_2 transfer with hydrogen utilizers such as methanogens (Bryant et al., 1977). Odom and Peck (1981) suggested that, in the presence of sulfate, *Desulfovibrio* can also function as the H_2-utilizing "sink" in mixed cultures with H_2 producers (e.g., *S. wolinii* in this study). By the use of its membrane-associated hydrogenases which could efficiently produce or consume H_2, *Desulfovibro* spp. make the respective syntrophic associations with methanogens and *Syntrophobacter* spp. possible. My study shows that these two modes of syntrophic interactions also stimulate Hg methylation by coupling with Hg methylating sulfate reducers.

Positive correlations were clearly observed between the concentration of sulfate in the coculturing media and the net increase in CH_3Hg production synthesis that was

stimulated by the *Syntrophobacter-Desulfovibrio* cocultures in comparison with the *Desulfovibrio* monocultures (Fig. 3.7A). This result strongly suggested that sulfate was a key factor in maintaining this type of syntrophy resulting in the stimulation of Hg methylation. Boone and Bryant (1980) showed that in the absence of sulfate, *S. wolinii* failed to coculture with *D. vulgaris* G11 as indicated by overall growth.

A preliminary assay showed that no CH_3Hg was formed when *Syntrophobacter* spp. were grown with *D. desulfuricans* G200, a strain that does not methylate Hg (data not shown), indicating that stimulation of Hg methylation by the coculture probably only occurs when one partner of the syntrophic relationship methylates. Therefore, a likely mechanism for the stimulation of Hg methylation by this syntrophy is that, during syntrophic growth of *D. desulfuricans* ND132 or *D. africanus* with *S. sulfatireducens*, *Desulfovibrio* suppresses the sulfate reduction of *S. sulfatireducens*. The propionate oxidation by the later results in the production of acetate, CO_2, and hydrogen which are then used by the Hg methylating *Desulfovibrio* spp. during sulfate respiration. This association supports the growth of both partners and stimulates the CH_3Hg synthesis. *Syntrophobacter* spp. are a group of syntrophs that are widely distributed in natural environments including freshwaster *Sphagnum* moss mats and salt marshs (Loy et al., 2004; Bahr et al., 2005). As an important intermediate in the mineralization of organic matter to CO_2 and methane (de Bok et al., 2004), propionate could be an available substrate in these wetland habitats to support syntrophic growth. Thus, the *Syntrophobacter-Desulfovibrio* syntrophy described here could be important in the contribution of MeHg synthesis in these wetland habitats while sulfate is available but energy and carbon sources are limiting. This contribution maybe even more important for salt marsh than for freshwater wetlands where sulfate levels are generally high and relatively recalcitrant substrates

such as propionate accumulate while degradable ones are depleted by the abundant SRB that occurr in this habitat.

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Table 3.1. Enhanced methylation activities by mono- and co-cultures of syntrophic associations tested in this study[1]

Methylating species	Mono- and co-cultures	Methylation
S. wolinii	Monoculture	+
	Coculture with *M. hungatei*	+
D. desulfuricans ND132	Monoculture	NA
	Coculture with *M. hungatei*	++
	Coculture with *S. sulfatireducens*	++
D. africanus DSM 2603	Monoculture	+
	Coculture with *M. hungatei*	++
	Coculture with *S. sulfatireducens*	+

[1]+ and ++ indicate weak and strong Hg methylation activities, respectively, of mono- and co-cultures;

NA means not available.

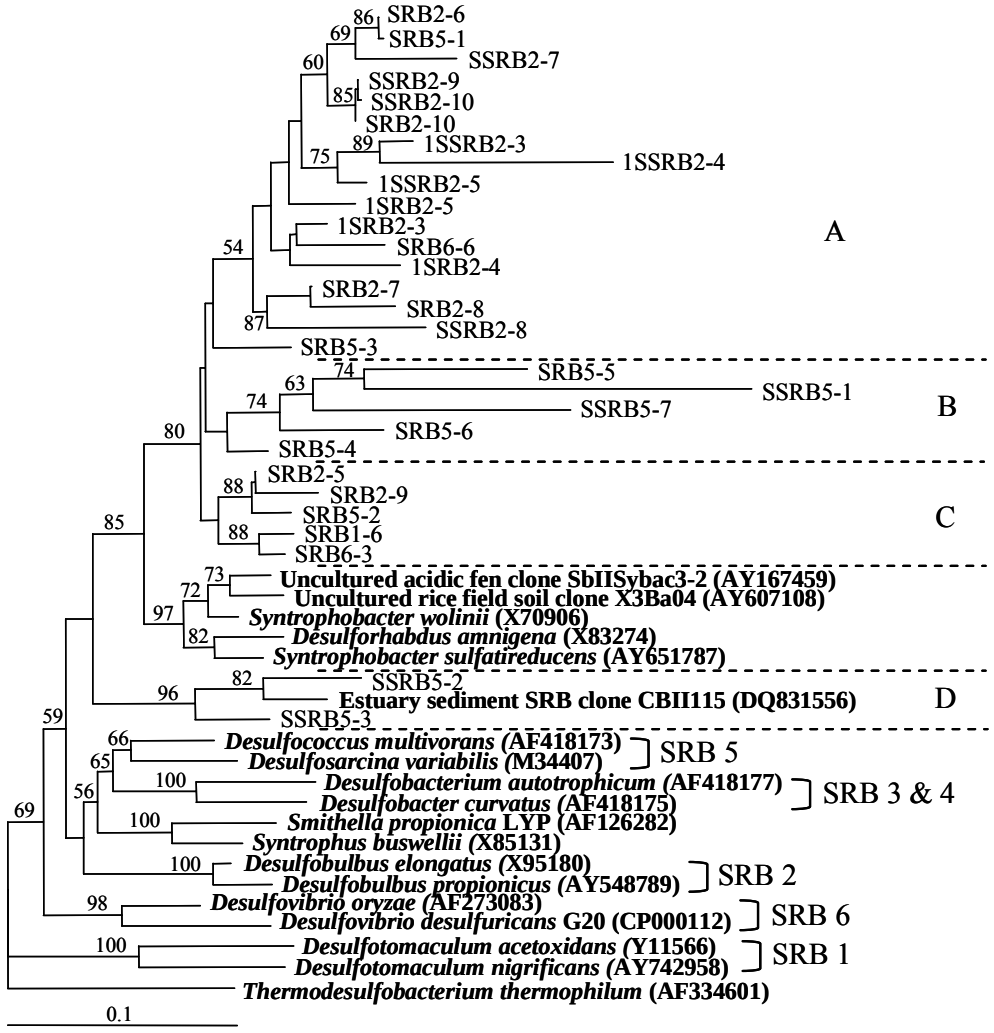


Fig. 3.1. Phylogenetic tree of 16S rRNA genes (179 bp) obtained by DGGE of PCR amplification products using four group-specific SRB primer sets from DNA extracts of floating *Sphagnum* moss mats in Sunday Lake, Adirondacks, NY. The tree was constructed using the neighbor-joining method. MAT sequences are grouped as Clades A-D. Bootstrap values are shown at branching points. The scale bar shows 10% sequence difference.

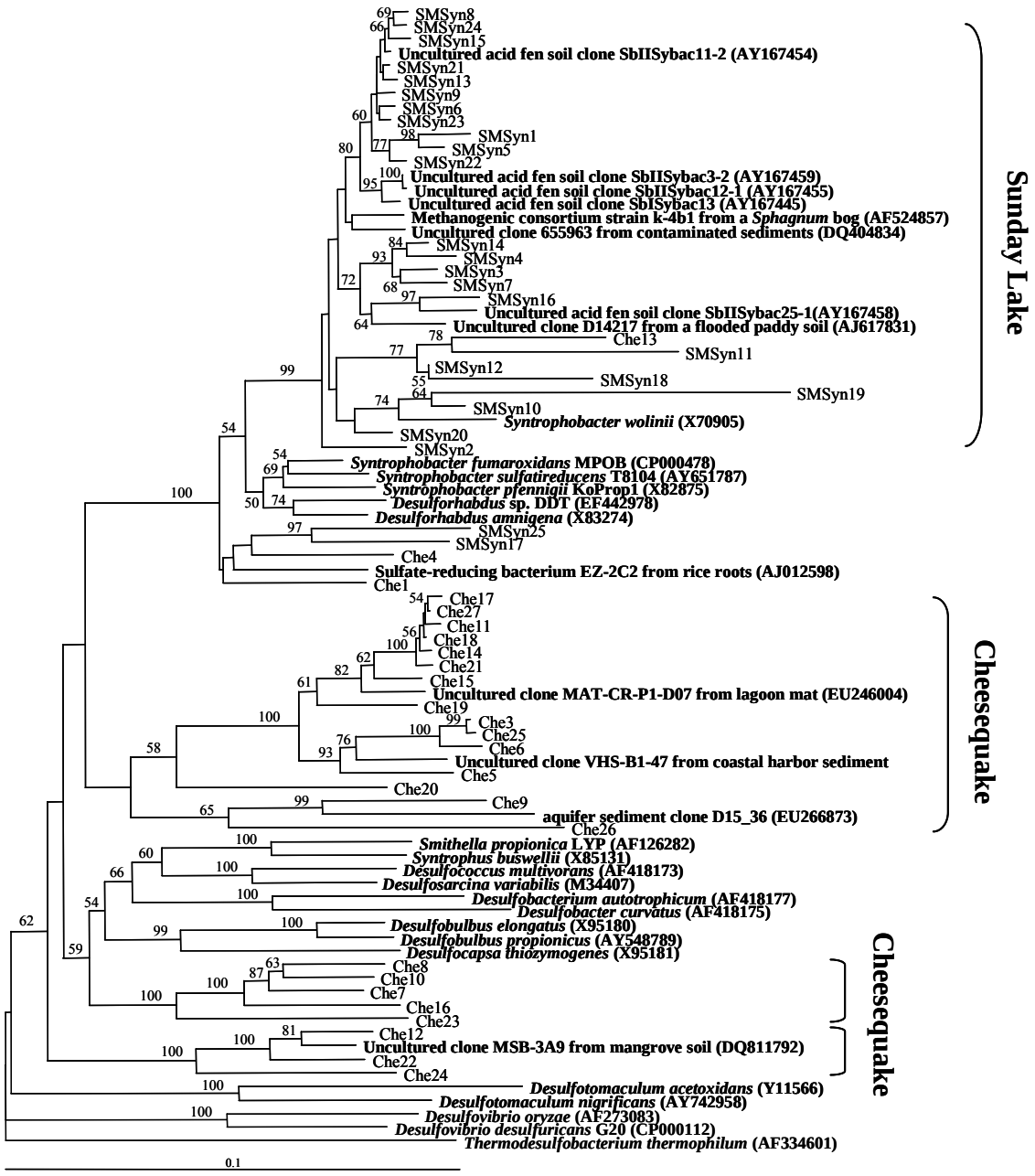


Fig. 3.2. Phylogenetic tree of 16S rRNA gene sequences (1100bp) obtained using PCR with *Syntrophobacteraceae*-specific primers from DNA extracts of SRB medium enrichments established with a floating *Sphagnum* mat sample, Sunday Lake (SMSyn entries in the tree), NY, and the DNA extract of a native mat sample from Cheesequake saltmarsh (Che entries in the tree), NJ. The tree was constructed using the neighbor-joining method and bootstrap values are shown at branch points. The scale bar indicates 10% sequence difference.

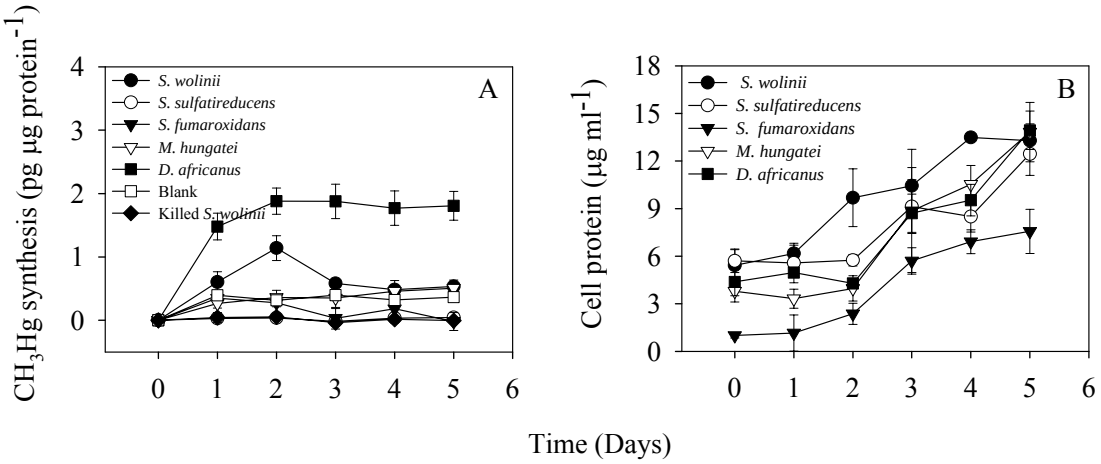


Fig. 3.3. Synthesis of CH_3Hg (pg of initial protein) (A) and change in protein contents (B) of monocultures of *S. wolinii* DSM 2805, *S. sulfatireducens* DSM 16706, *S. fumaroxidans* DSM 10017, *M. hungatei* DSM 864, and *D. africanus* DSM 2603.

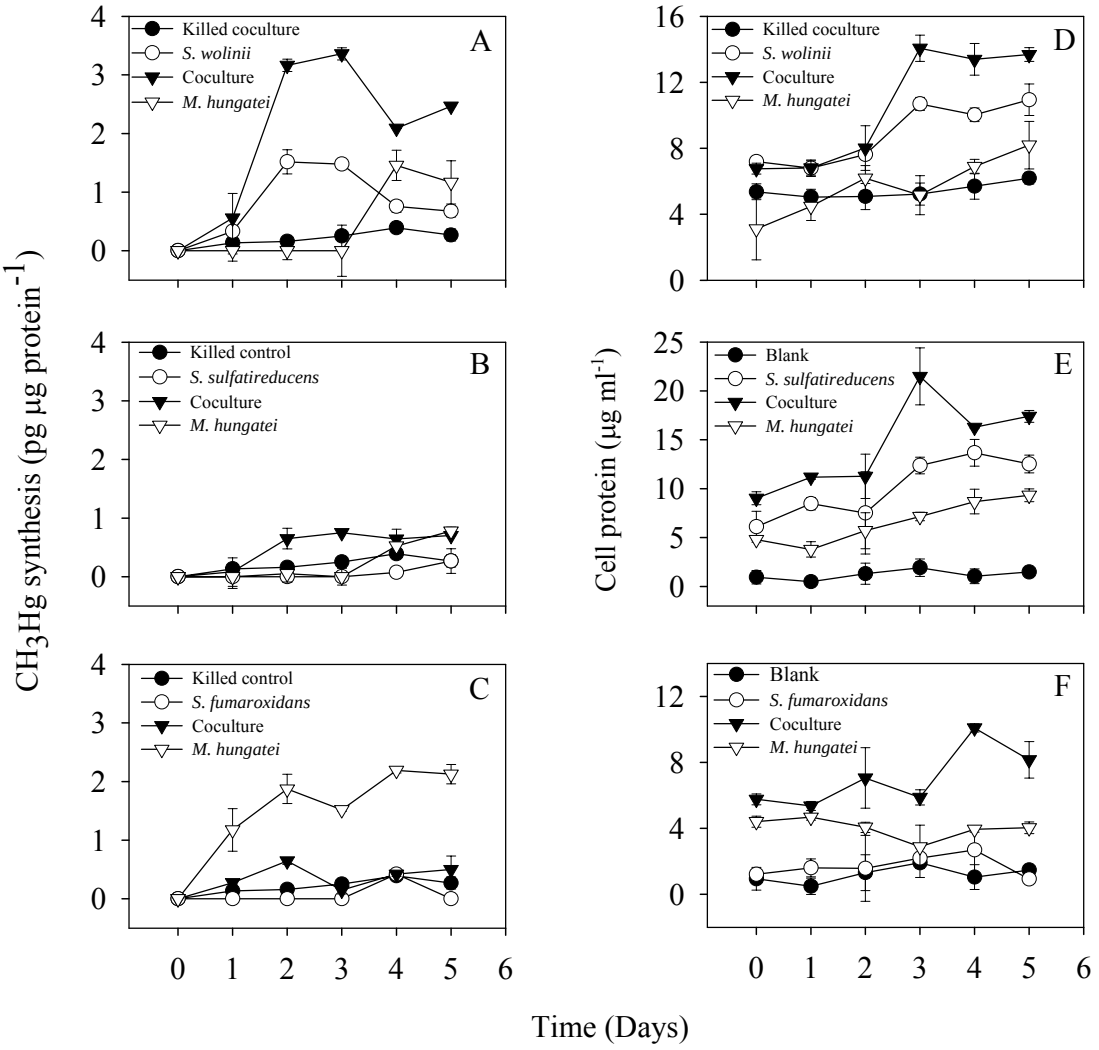


Fig. 3.4. Synthesis of CH_3Hg (pg of initial protein) and change in protein content in mono- and cocultures. (A) and (D): *S. wolinii* DSM 2805 and *M. hungatei* DSM 864; (B) and (E): *S. sulfatireducens* DSM 16706 and *M. hungatei*; (C) and (F): *S. fumaroxidans* DSM 10017 and *M. hungatei* in sulfate-free propionate media.

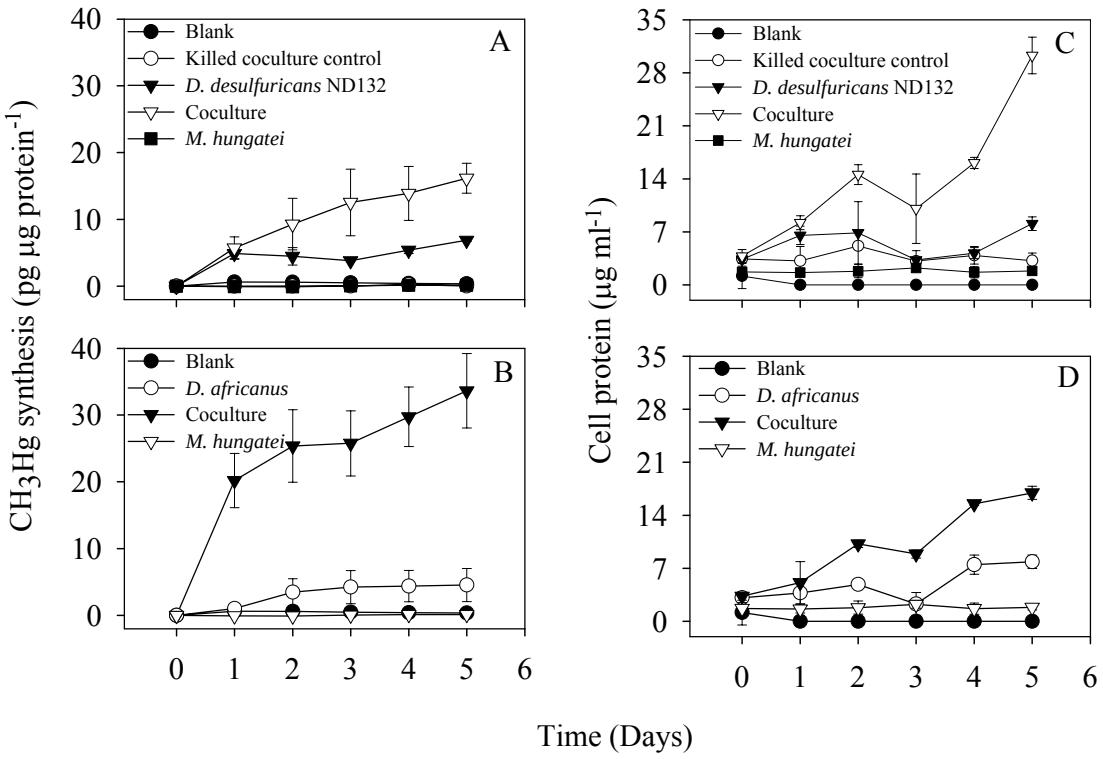


Fig. 3.5. Synthesis of CH_3Hg (pg per inital protein) and change in protein content in mono- and cocultures. (A) and (C): *M. hungatei* with *D. desulfuricans* ND 132, and (B) and (D): *M. hungatei* with *D. africanus* in a sulfate-free lactate medium.

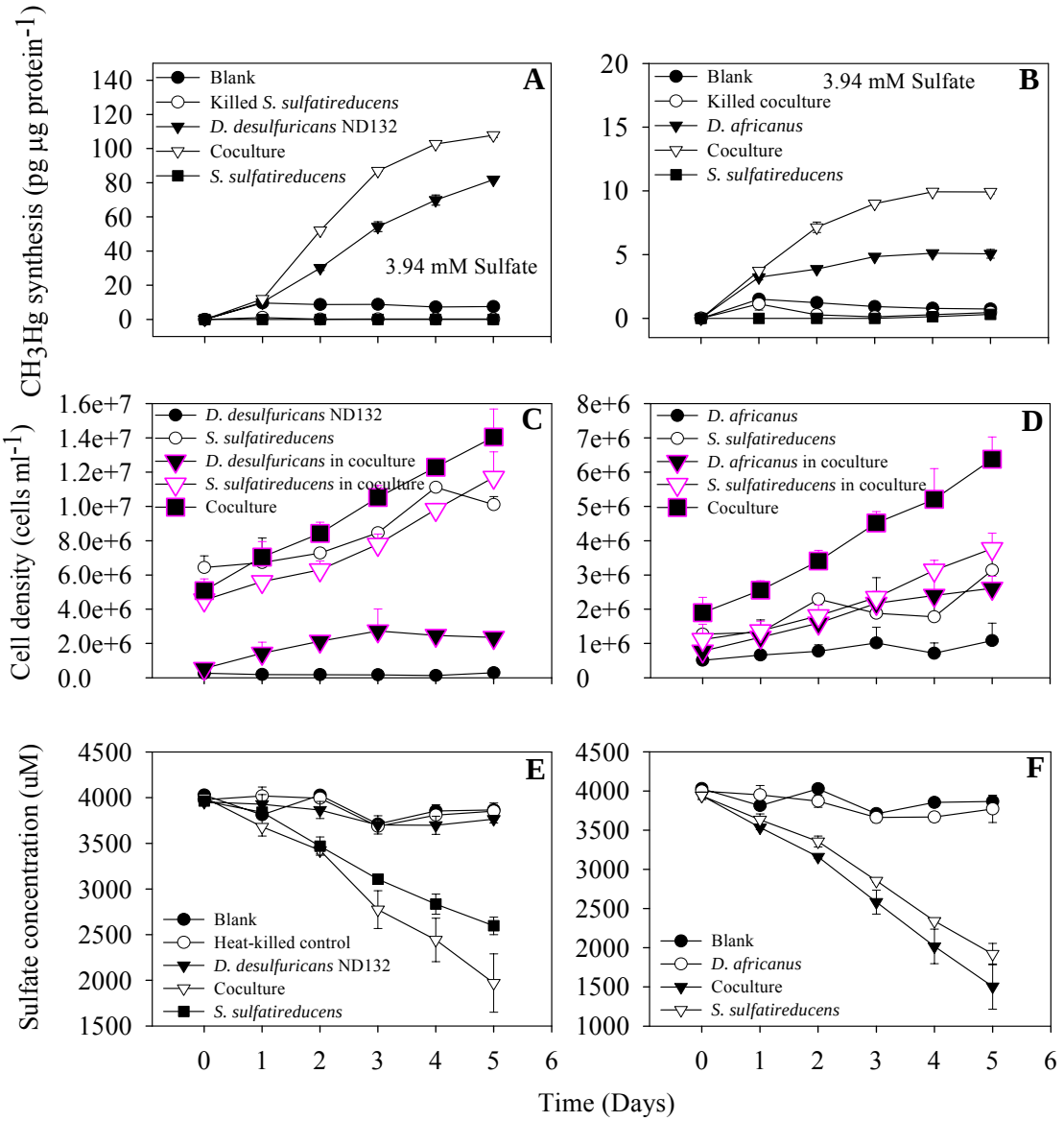


Fig. 3.6. CH_3Hg synthesis (pg of µg initial protein), (A) and (B); change in cell numbers, (C) and (D); and change of sulfate, (E) and (F) in mono- and cocultures of *S. sulfatireducens* with *D. desulfuricans* ND132 (left column), and *S. sulfatireducens* with *D. africanus* (right column) in a propionate-sulfate (3.94 mM) medium.

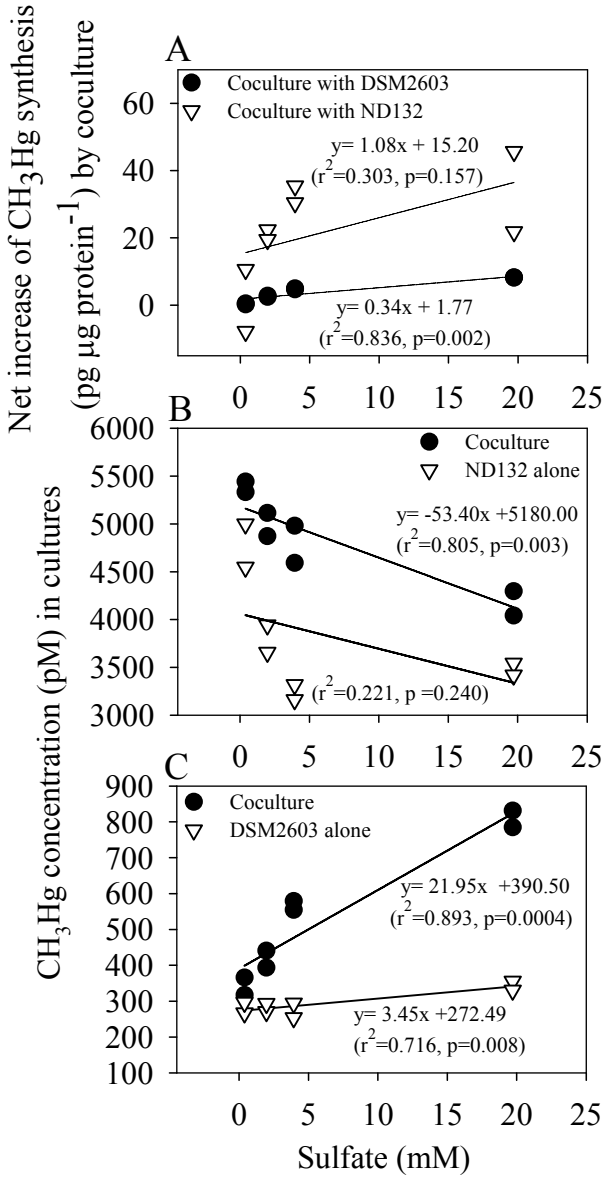


Fig. 3.7. Effect of sulfate concentration on Hg methylation during syntrophic growth of *S. sulfatireducens* DSM 16706 with *D. africanus* DSM 2603, or *D. desulfuricans* ND 132, respectively. (A) Maximum net increase (calculated on Day 4) in CH_3Hg synthesis (pg µg initial protein^{-1}) by the cocultures in comparison with monocultute; (B) CH_3Hg concentrations in a monoculture of ND132 and its coculture with strain DSM16706; (C) CH_3Hg concentrations in a monoculture of DSM 2603 and its coculture with DSM 16706.

Supplemental Table S3.1. Coculture medium for *M. hungatei* with *S. wolinii* or *S. sulfatireducens*, and for *S. sulfatireducens* with *D. desulfuricans* ND132 or *D. africanus* (modified from DSMZ Medium 307, *S. wolinii* medium)

Solution A:		
Mineral solution (see below)	50.00	ml
Trace element solution SL-10 (see medium 320)	1.00	ml
Rumen fluid, clarified	50.00	ml
Trypticase (BBL)	1.00	g
Vitamin solution (see below)	5.00	ml
Sodium propionate	1.50	g
Resazurin	1.00	mg
Distilled water	810.00	ml
Solution B:		
$NaHCO_3$	3.50	g
Distilled water	70.00	ml
Solution C:		
Cysteine-HCl × H_2O	0.30	g
Distilled water	10.00	ml
Solution D:		
25 mM $TiCl_3$ (in 100 mM NTA) solution	10.00	ml

Final pH 7.0-7.2. Gas atmosphere: 80% N_2 + 20% CO_2.

Mineral solution:		
KH_2PO_4	10.00	g
$MgCl_2 \times 6\ H_2O$	6.60	g
NaCl	8.00	g
NH_4Cl	8.00	g
$CaCl_2 \times 2\ H_2O$	1.00	g
Distilled water	1000.0	ml
Vitamin solution:		
Biotin	0.25	mg
Nicotinic acid	2.50	mg
Thiamine-HCl × 2 H_2O	1.25	mg
p-Aminobenzoic acid	1.25	mg
Pantothenic acid	0.62	mg
Pyridoxine-HCl	6.20	mg
Distilled water	1000.0	ml

Dissolve ingredients of solution A in the amount of water indicated, adjust pH to 7.2, boil for a few minutes, cool to room temperature under a stream of 80% N_2 + 20%

CO_2 gas mixture, then distribute under the same gas and autoclave. Solution B is filter-sterilized and then equilibrated with the gas mixture by gassing for at least 15 minutes. Solutions C and D are autoclaved under 100% nitrogen. To complete the medium, appropriate amounts of B, C, and D are added to solution A.

Supplemental Table S3.2. Coculture medium for *S. fumaroxidans* with *M. hungatei* (modified from DSMZ Medium 684-MPOB MEDIUM)

Sodium propionate	1.92	g
$Na_2HPO_4 \times 2\ H_2O$	0.53	g
KH_2PO_4	0.41	g
NH_4Cl	0.30	g
$CaCl_2 \times\ 2\ H_2O$	0.11	g
$MgCl_2 \times 6\ H_2O$	0.10	g
NaCl	0.30	g
Trace element solution (see medium 320)	1.00	ml
Selenite/tungstate solution (see medium 385)	1.00	ml
Yeast extract	0.20	g
Resazurin	0.50	mg
$NaHCO_3$	4.00	g
Vitamin solution (see medium 141)	10.00	ml
Cysteine-HCl $\times\ H_2O$	0.30	g
25 mM $TiCl_3$ (in 100 mM NTA) solution	10.00	ml
Distilled water	1000.0	ml

Prepare the medium anaerobically under 80% N_2 + 20% CO_2. Prepare separate anaerobic sterile stock solutions of sodium bicarbonate (outgassed with N_2 + CO_2), vitamin solution (N_2), Cysteine-HCl $\times\ H_2O$ (N_2), and $TiCl_3$-NTA (N2). After adding to the autoclaved basal medium, the pH of the completed medium should be 7.0 - 7.2. After inoculation pressure culture bottles to 0.7 bar N_2 + CO_2 overpressure.

Supplemental Table S3.3. Coculture medium for *M. hungatei* with *D. desulfuricans* ND132 or *D. africanus* DSM 2603 (modified from Pak and Bartha, 1998)

Solution A (Under 100% N_2):

KCl ····· 0.33 g
$MgCl_2 \cdot 7H_2O$ ····· 2.75 g
NH_4Cl ····· 0.25 g
$CaCl_2 \cdot 2H_2O$ ····· 0.14 g
K_2HPO_4 ····· 0.14 g
NaCl ····· 2.25 g
Yeast extract ····· 0.25 g
Sodium lactate ····· 6.0 g
Trace element SL-6····· 3.0 ml
Resazurin ····· 1.0 g
Distilled water ····· 920.0 ml

Solution B (flush with N_2 and autoclave in serum bottle):

$NaHCO_3$ ····· 5.00 g
Distilled water ····· 70.00 ml

Solution C (add chemicals to flushed water, autoclave under N_2):

Cysteine ····· 0.25 g
Sodium Ascorbate ····· 0.1 g
Thioglycolate ····· 0.1 g
Distilled water ·····10.00 ml

Note: a. Modify NaCl from 18 g to 2.25 g (ATCC medium 1043, *Methanosarcina* medium) since *D. africanus* is not saltwater strain; b. add 3 ml for trace element following ATCC medium 1043 (Pak and Bartha (1998) did not indicate the volume to add); c. prepare $NaHCO_3$ and reducing agents separately.

Modified Trace Elements Solution SL-6 in ATCC medium 1043 (Pak and Bartha 1998):

$ZnCl_2$ ····· 0.10 g
$MnCl_2 \cdot 4H_2O$ ····· 0.03 g
H_3BO_3·····0.30 g
$CoCl_2 \cdot 6H_2O$····· 0.20 g
$CuCl_2 \cdot 2H_2O$····· 0.01 g
$NiCl_2 \cdot 6H_2O$····· 0.02 g
$Na_2MoO_4 \cdot 2H_2O$····· 0.03 g
Ferric ammonium citrate ····· 0.50 g
Distilled water ····· 1.0 L

Supplemental Table S3.4. Sequence similarity of selected excised DGGE fragments of *Deltaproteobacteria* amplified from the MAT sample (*Sphagnum* moss) before and after sulfate enrichment[1]

SRB Clones	Most similar organism before sulfate enrichment (Accession number)	Similarity (%)	SRB Clones	Most similar organism after sulfate enrichment (Accession number)	Similarity (%)
SRB1 (5)*			**SRB1 (5)**		
SRB1-6	*Syntrophobacter wolinii* (X70906)	91			
SRB2 (9)			**SRB2 (7)**		
1SRB2-3	*Syntrophobacter wolinii* (X70906)	90	**1SSRB2-3**	*Syntrophobacter wolinii* (X70906)	87
1SRB2-4	Uncultured *Desulfobacteraceae* bacterium cLaKi-JM30 (AJ582688) (**SRB**)	93	**1SSRB2-4**	Uncultured bacterium HOClCi16 (AY328565)	92
1SRB2-5	Uncultured *Syntrophobacteraceae* bacterium SbIISybac3-2 (AY167459)	88	**1SSRB2-5**	Uncultured *Syntrophobacteraceae* bacterium SbIISybac3-2 (AY167459)	88
SRB2-5	Uncultured *Syntrophobacteraceae* bacterium clone SbIISybac3-2 (AY167459)	92	**SSRB2-7**	Uncultured *Syntrophobacteraceae* bacterium clone SbIISybac3-2 (AY167459)	90
SRB2-6	Uncultured *Syntrophobacteraceae* bacterium clone SbIISybac12-1 (AY167459)	92	**SSRB2-8**	Uncultured bacterium clone E62 (AJ966602)	96
SRB2-7	*Syntrophobacter wolinii* (X70906)	91	**SSRB2-9**	Uncultured *Syntrophobacteraceae* bacterium SbIISybac12-1 (AY167455)	92
SRB2-8	Uncultured *Syntrophobacter* sp. clone X3Ba04 (AY607108)	88	**SSRB2-10**	Uncultured *Syntrophobacteraceae* bacterium clone SbIISybac3-2 (AY167459)	90
SRB2-9	Uncultured *Syntrophobacteraceae* bacterium SbIISybac3-2 (AY167459)	91			
SRB2-10	Uncultured *Syntrophobacteraceae* bacterium SbIISybac3-2 (AY167459)	93			
SRB5 (7)			**SRB5 (4)**		
SRB5-1	Uncultured *Syntrophobacteraceae* bacterium SbIISybac3-2 (AY167459)	92	**SSRB5-1**	Uncultured *δ-Proteobacterium* clone E48G06cD (DQ109937)	97
SRB5-2	Uncultured *Syntrophobacteraceae* bacterium SbIISybac3-2 (AY167459)	92	**SSRB5-2**	Uncultured *Desulfocapsa* sp. clone CBII115 (DQ831556) (**SRB**)	93
SRB5-3	Uncultured *Desulfobacteraceae* bacterium clone cLaKi-JM30	92	**SSRB5-3**	Uncultured *Desulfocapsa* sp. clone SB1_88(AY177798) (**SRB**)	97
			SSRB5-7	Uncultured *δ-*	100

	(AJ582688) (**SRB**)			*Proteobacterium* clone	
SRB5-4	*Syntrophobacter wolinii* (X70906)	91		BRIC4 (DQ294024)	
SRB5-5	Uncultured *δ-Proteobacterium* clone JG36-TzT-168 (AJ534629)	100			
SRB5-6	Uncultured *δ-Proteobacterium* clone 05D2Z46 (DQ397430)	98			
SRB5-7	Uncultured *δ-Proteobacterium* clone (DQ294024)	96			
SRB6 (3)			**SRB6 (1)**		
SRB6-3	*Syntrophobacter wolinii* (X70906)	92			
SRB6-6	*Syntrophobacter wolinii* (X70906)	91			

[1]Entries are organized according to the group-specific primer set (SRB1, SRB2, SRB5, and SRB6) that was used to amplify the MAT metagenome prior to its reamplification with DGGE primers.

[*]The number in parenthesis next to group designation is the total number of excised and sequenced DGGE fragments.

Supplemental Table S3.5. Sequence similarity of 16S rRNA genes obtained with *Syntrophobacter*-specific primers from DNA extract of MAT SRB-MPN incubations and Cheesequake mat

Syn Clones from MAT(10^{-1} series)	Most similar organism (Accession number)	Similarity (%)	Clones from Cheese quake soil DNA	Most similar organism (Accession number)	Similarity (%)
SMSyn1	Uncultured *Syntrophobacteraceae* bacterium clone SbIISybac11-2 from acidic fern soil (AY167454)	95	**1-Che1**	Uncultured *Syntrophaceae* bacterium clone D15_18 (EU266858)	94
SMSyn2	Uncultured *Syntrophobacteraceae* bacterium clone SbIISybac3-2 from acidic fern soil (AY167459)	96	**2-Che3**	Uncultured bacterium clone VHS-B1-47 from coastal harbor sediment (DQ394922)	94
SMSyn3	Uncultured *Syntrophobacteraceae* bacterium clone SbIISybac11-2 from acidic fern soil (AY167454)	96	**3-Che4**	*Syntrophobacter pfennigii* 16S rRNA gene, strain KoProp1 (X82875)	93
SMSyn4	Uncultured *Syntrophobacteraceae* bacterium clone SbIISybac11-2 from acidic fern soil (AY167454)	96	**4-Che5**	Uncultured hydrocarbon seep *Deltaproteobacteria* bacterium BPC065(AF154094)	94
SMSyn5	Uncultured *Syntrophobacteraceae* bacterium clone SbIISybac11-2 from acidic fern soil (AY167454)	97	**5-Che6**	Uncultured *Deltaproteobacterium* clone MVP-18 from freshwater pond (DQ676442)	93
SMSyn6	Uncultured *Syntrophobacteraceae* bacterium clone SbIISybac11-2 from acidic fern soil (AY167454) (or *S. wolinii*, X70905 with 95% similarity)	98	**6-Che7**	Uncultured *Deltaproteobacterium* clone GoM HDB-20 from the Gulf of Mexico Gas Hydrates (AY542205)	96
			7-Che8	Sulfate-reducing bacterium strain mXyS1 similar to *Desulfobacterium cetonicum* (AJ006853)	97
SMSyn7	Uncultured *Syntrophobacteraceae* bacterium clone SbIISybac12-1 from acidic fern soil (AY167455)	96			
SMSyn8	Uncultured *Syntrophobacteraceae* bacterium clone SbIISybac11-2 from acidic fern soil (AY167454) (or *S. wolinii*, X70905 with 95% similarity)	98	**8-Che9**	Uncultured *Syntrophaceae* bacterium clone D15_36 from a distant redox zone (EU266873)	91
			9-Che10	A novel type of marine sulfate-reducing bacterium strain EbS7 (AJ430774)	95
SMSyn9	Uncultured *Syntrophobacteraceae* bacterium clone SbIISybac11-2 from acidic fern soil (AY167454) (or *S. wolinii*, X70905 with 94% similarity)	98	**10-Che11**	Uncultured *Deltaproteobacterium* clone MVP-18 (DQ676442)	92
			11-Che12	Uncultured *Deltaproteobacterium* clone VHS-B3-88 from coastal harbor sediment (DQ394969)	97
SMSyn10	*S. wolinii* DSM 2805 (X70905)	95			
SMSyn11	Uncultured *Syntrophobacteraceae* bacterium clone SbIISybac25-1 (AY167458)	96	**12-Che13**	Uncultured *Syntrophobacteraceae* bacterium clone SbISybac13 from low-sulfate, acidic terrestrial fens (AY167445)	97
SMSyn12	Uncultured *Syntrophobacteraceae* bacterium clone SbIISybac5-2 (AY167460)	96	**13-Che14**	Uncultured organism clone MAT-CR-P1-D07 from lagoon mat (EU246004)	96
SMSyn13	Uncultured *Syntrophobacteraceae* bacterium clone SbIISybac11-2 from acidic fern soil (AY167454) (or *S. wolinii*, X70905 with 95% similarity)	98	**14-Che15**	Uncultured organism clone MAT-CR-P1-D07 from lagoon mat (EU246004)	96
			15-Che16	Uncultured organism clone MAT-CR-M5-H02 from lagoon mat (EU245750)	94
SMSyn14	Uncultured *Syntrophobacteraceae* bacterium clone SbIISybac11-2 from acidic fern soil (AY167454)	96	**16-Che17**	Uncultured organism clone MAT-CR-P1-D07 from lagoon mat	96
SMSyn15	Uncultured *Syntrophobacteraceae*	98			

	bacterium clone SbIISybac11-2 from acidic fern soil (AY167454) (or *S. wolinii*, X70905 with 95% similarity)			(EU246004)	
			17-Che18	Uncultured organism clone MAT-CR-P1-D07 from lagoon mat (EU246004)	96
SMSyn16	Uncultured *Syntrophobacteraceae* bacterium clone SbIISybac25-1 (AY167458) (or *S. wolinii*, X70905 with 93% similarity)	96	**18-Che19**	Uncultured organism clone MAT-CR-P1-D07 from lagoon ma t(EU246004)	93
			19-Che20	Uncultured *Syntrophaceae* bacterium clone from distinct redox zone D15_18 (EU266858)	89
SMSyn17	*S. sulfatireducens* T8104 (AY651787)	92			
SMSyn18	Uncultured *Syntrophobacteraceae* bacterium clone SbIISybac11-2 from acidic fern soil (AY167454) (or *S. fumaroxidans* MPOB, CP000478 with 94% similarity)	97	**20-Che21**	Uncultured organism clone MAT-CR-P1-D07 from lagoon mat (EU246004)	95
			21-Che22	Uncultured *Deltaproteobacterium* clone MSB-3A9 from mangrove soil (DQ811792)	96
SMSyn19	*S. wolinii* DSM 2805 (X70905)	86			
SMSyn20	Uncultured *Syntrophobacteraceae* bacterium clone SbIISybac11-2 from acidic fern soil (AY167454) (or *S. wolinii*, X70905 with 95% similarity)	96	**22-Che23**	Uncultured *Deltaproteobacterium* clone GoM GC234 621E from the Gulf of Mexico(AY211680)	93
			23-Che24	MSB-3A9 from mangrove soil (DQ811792)	90
SMSyn21	Uncultured *Syntrophobacteraceae* bacterium clone SbIISybac11-2 from acidic fern soil (AY167454) (or *S. wolinii*, X70905 with 95% similarity)	98	**24-Che25**	Uncultured bacterium clone VHS-B1-47 from coastal harbor sediment (DQ394922)	95
			25-Che26	Uncultured bacterium clone GN01-8.058 from hypersaline microbial mat (DQ154855)	86
SMSyn22	Uncultured *Syntrophobacteraceae* bacterium clone SbIISybac11-2 from acidic fern soil (AY167454) (or *S. wolinii*, X70905 with 95% similarity)	98	**26-Che27**	Uncultured organism clone MAT-CR-P1-D07 from lagoon mat (EU246004)	96
SMSyn23	Uncultured *Syntrophobacteraceae* bacterium clone SbIISybac11-2 from acidic fern soil (AY167454) (or *S. wolinii*, X70905 with 95% similarity)	98			
SMSyn24	Uncultured *Syntrophobacteraceae* bacterium clone SbIISybac11-2 from acidic fern soil (AY167454) (or *S. wolinii*, X70905 with 95% similarity)	98			
SMSyn25	*S. wolinii* DSM 2805 (X70905)	94			
SMSyn26	Sulfate-reducing bacterium EZ-2C2 from rice roots and soil (AJ012598)	93			
SMSyn27	*S. wolinii* DSM 2805 (X70905)	96			
SMSyn28	*S. wolinii* DSM 2805 (X70905)	96			
SMSyn29	Uncultured *Syntrophobacteraceae* bacterium clone SbIISybac11-2 from acidic fern soil (AY167454) (or *S. wolinii*, X70905 with 95% similarity)	98			

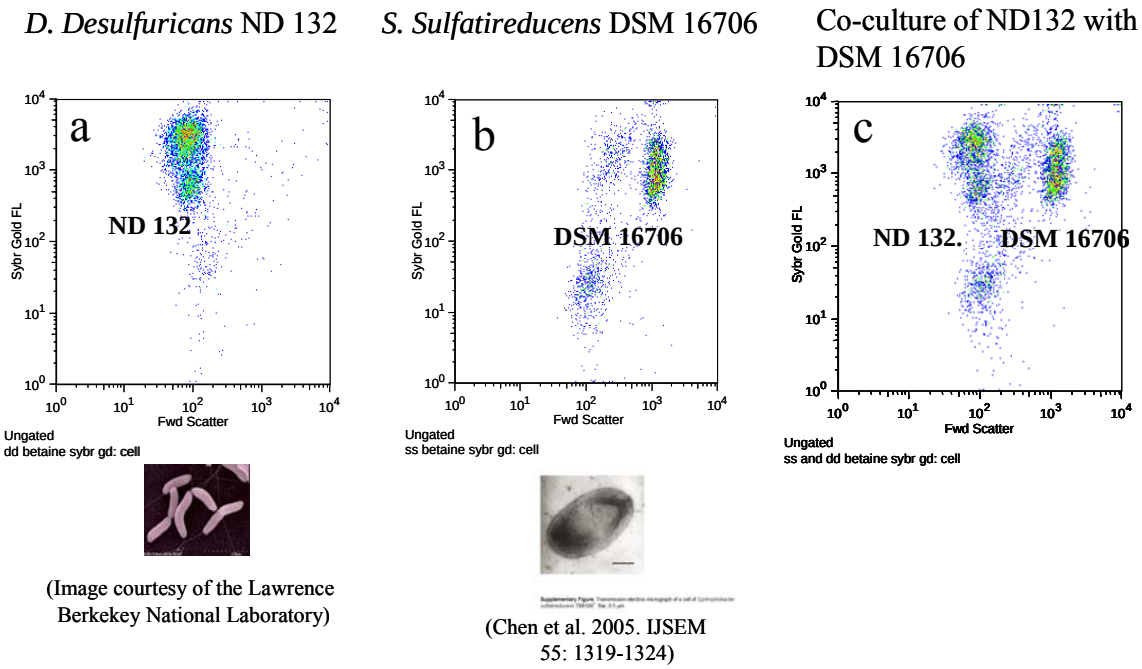


Fig. S3.1. Light scatter distributions of pure strains (a and b) and co-cultures (c) in the associations of *Syntrophobacter sulfatireducens* DSM 16706 with *Desulfovibrio desulfovibrio* ND 132 obtained with a BD Influx Mariner 209s Flow Cytometer and High Speed Cell sorter (FCSS) after SYBR Gold staining of cellular DNA.

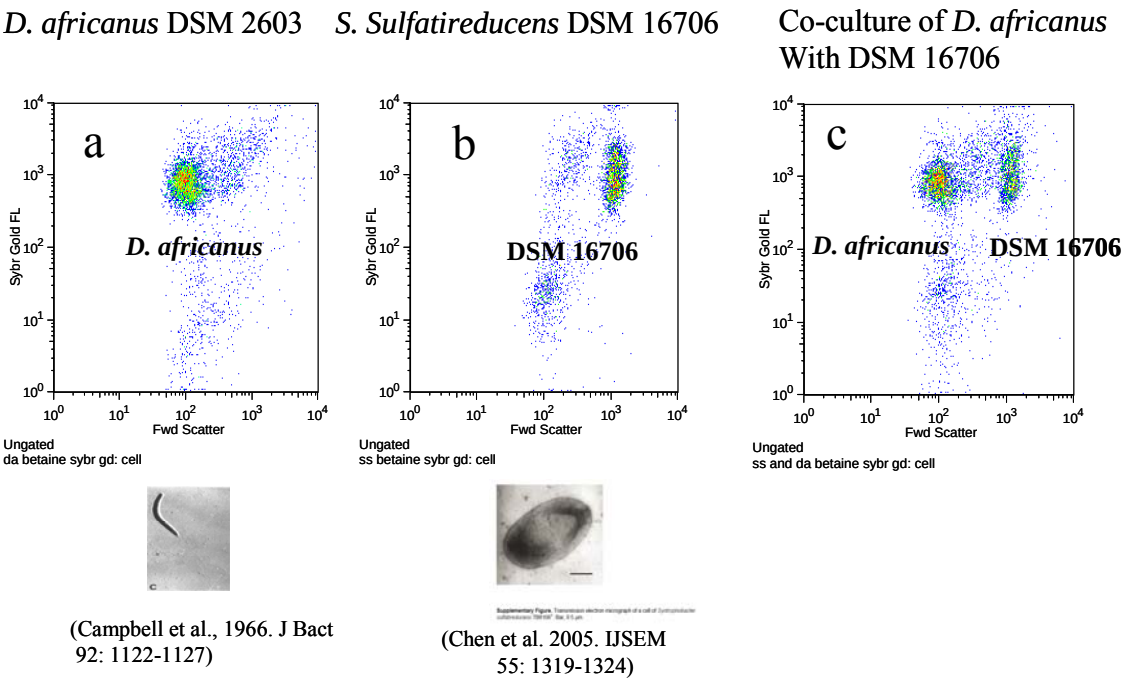
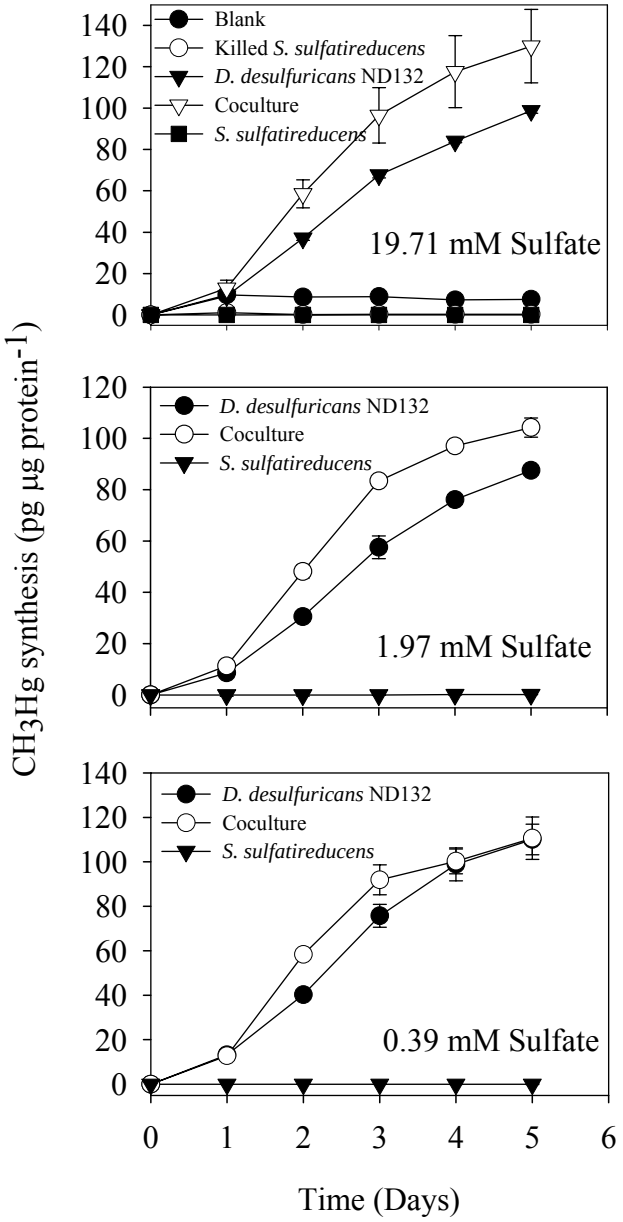
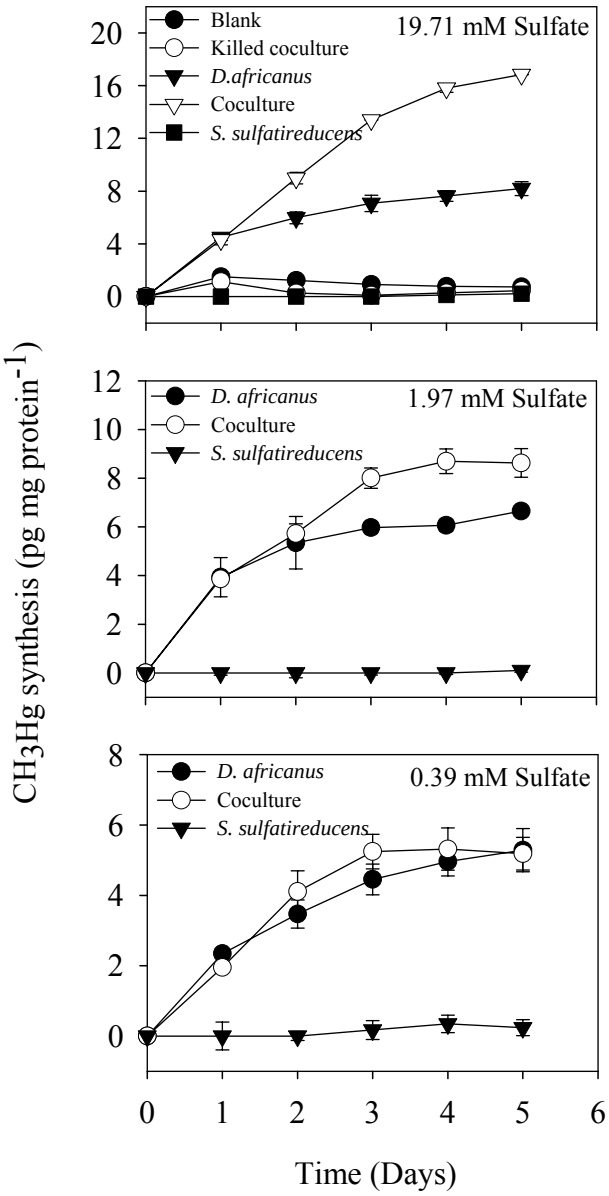


Fig. S3.2. Light scatter distributions of pure strains (a and b) and co-cultures (c) in the associations of *Syntrophobacter sulfatireducens* DSM 16706 with *Desulfovibrio africanus* DSM 2603 obtained with a BD Influx Mariner 209s Flow Cytometer and High Speed Cell sorter (FCSS) after SYBR Gold staining of cellular DNA.



Supplemental Fig. S3.3. CH_3Hg synthesis (pg of initial protein) in single strains and cocultures of *S. sulfatireducens* and *D. desulfuricans* ND 132 in sulfate-manipulated propionate media.



Supplemental Fig. S3.4. CH_3Hg synthesis (pg per initial protein) in single strains and cocultures of *S. sulfatireducens* and *D. africanus* in sulfate-manipulated propionate media.

CHAPTER 4

POTENTIAL FOR MICROBIAL METHYLMERCURY PRODUCTION AND DEGRADATION AND MOLECULAR CHARACTERIZATION OF ACTIVE MICROBIAL COMMUNITIES IN SEDIMENTS FROM THE SOUTH RIVER, VA

Abstract

The South River, VA, is an ecosystem with high levels of total mercury (THg) and methylmercury (MeHg) in water, sediments, and fish as a result of industrial Hg pollution. The roles of microbial mercury (Hg) methylation and methylmercury (MeHg) degradation as the two competing processes that together control net MeHg production in the environment, were therefore investigated in the South River sediments. Potential Hg methylation rates in samples collected at nine sites along a 21-km river reach downstream from a historic Hg contamination source were low in late spring (1.0 to 2.5% per day), and significantly higher in late summer (2.5 to 10.2% per day). Demethylation of ^{14}C-MeHg was dominated by $^{14}CH_4$ production, indicating a reductive degradation pathway in spring, but switched to producing mostly $^{14}CO_2$, suggesting an oxidative pathway in the summer. Rate comparison within river habitats suggested that fine grain sediments originating from the erosion of river banks were possible locations where MeHg accumulated, and hot spots for both methylation and demethylation activities. Demethylation rates exhibited a significantly positive relationship with sediment MeHg concentration. Molecular characterization based on 16S rRNA gene clone libraries obtained with RNA extracts of South River sediments showed that at least three groups of sulfate reducing

bacteria (SRB) and one group of *Geobacter*-like bacteria with a close affiliation with known Hg methylators, were active in three South River sediments. Evidence including strong positive correlation of sediment MeHg with porewater sulfate and occurrence of diverse SRB methylators in sediments suggested the involvement of SRB in Hg methylation. Low concentration of acid volatile sulfide (AVS) and high ratios of Fe(II):Fe(III) reported in all South River habitat types implicated iron reducing bacteria (IRB) as another group of principle methylators of Hg. Amendment experiments showing significant increase of methylation rates by addition of sulfate and obvious inhibition by molybdate further confirmed that SRB were a group of active Hg methylators in benthic South River habitats. Addition of low levels of ferric oxyhydroxide significantly stimulated methylation rates, indicating a role for IRB in MeHg synthesis. Overall, our studies are the first to show that coexisting SRB and IRB populations in sediments may both contribute to Hg methylation, possibly by temporally and spatially separated processes, in a MeHg-contaminated freshwater river ecosystem.

Introduction

Mercury (Hg) methylation converts inorganic Hg into methylmercury (MeHg) and increases Hg toxicity due to the trophic transfer of MeHg, a potent neurotoxicant, in aquatic food chains. Methylmercury synthesis in natural environments is dominantly a biologically-mediated process (Berman & Bartha, 1986) and is modulated by various chemical factors including levels of sulfate (Gilmour et al., 1992), nitrate (Todorova et al., 2009), sulfide (Benoit et al., 2001), organic matter content (Mitchell et al., 2008), total Hg (Hammerschmidt & Fitzgerald, 2004), and ferrous iron (Mehrotra & Sedlak, 2005). Even though Hg methylation in the environment has been studied for over 30 years, a few key issues regarding MeHg production are still not resolved. For example, potential rates of MeHg degradation as the competing process to methylation, have rarely been integrated with potential methylation rates assessing the dynamics of MeHg production. Likewise, the lack of knowledge regarding the genes which encode enzymes involved in microbial methylation hinders application of metagenomic approaches that could make a direct link of biotic Hg methylation and methylating microbes in the environments where MeHg is produced. To date, metagenomic approaches to this question have used 16S rRNA genes or those genes that specify sulfate reduction. Such studies are still useful by identifying potential methylators, information that provides insight into methylation in natural environments (Acha et al., 2005; Winch et al., 2009; Yu et al., 2010; Elias et al., 2011) and supports bioremediation efforts (Todorova et al., 2009).

The South River and South Fork Shenandoah River, VA, are scenic recreational streams. From 1929 to 1950, an industrial facility in Waynesboro, VA, used mercuric sulfate as a catalyst to produce acetate fiber and released thousands of kilograms of

Hg waste in the plant and into the surrounding landscape including the South River (Carter, 1977). River bank erosion and flooding eventually carried inorganic Hg into the river (Rhoades et al., 2009) leading to a continuing contamination of fish for decades, with methylmercury (MeHg) at levels that exceed the EPA criterion of 0.3 $\mu g\ g^{-1}$ by a factor of 4 to 13 (VADEQ, 2008). This contamination has also resulted in the trophic transfer of Hg from aquatic food chains to terrestrial birds and other animals in adjacent terrestrial ecosystems (Cristol et al., 2008). River sediments may be a major sink of Hg, and likely sites where microbial Hg methylation and methylmercury (MeHg) demethylation activities occur. The steady-state balance between Hg methylation and MeHg demethylation determines net accumulation of MeHg in aquatic ecosystems. Microbial methylation and demethylation, and the processes that control their dynamics and relationships to biogeochemical conditions have not been examined in the South River.

Sulfate reducing bacteria (SRB) have long been considered as the principal Hg methylating group in estuarine and freshwater ecosystems (Compeau & Bartha, 1985; Gilmour et al., 1992; Choi et al., 1994). However, more recently iron reducing bacteria (IRB) as a strain isolated from lake sediment and as pure cultures were shown to methylate Hg (Barkay & Wagner-Dobler, 2005; Fleming et al., 2006; Kerin et al., 2006). Sulfate and iron reducing bacteria are thus considered as two most important guilds of Hg(II) methylating microbes (Barkay & Wagner-Döbler, 2005; Fleming et al., 2006; Kerin et al., 2006). Nevertheless, the ecological significance of IRB as major Hg methylators in other environmental habitats such as in highly Hg-contaminated riverine ecosystems and the relative importance of SRB and IRB for *in-situ* MeHg production have been little reported. Molecular characterization of Hg methylating communities and examination of dominating microbial metabolic

pathways leading to Hg methylation as revealed by amendments with stimulators and inhibitors are two approaches important for understanding the complicated processes of methylation and demethylation. The objectives of this study were first to estimate the relative rates of Hg methylation and MeHg degradation in South River sediments; second, to identify possible habitats within the ecosystem where MeHg is likely to be produced; third, to relate methylation and demethylation rate potentials with microbial community structure; lastly, to test hypotheses regarding pathways of methylation and demethylation in the South River ecosystems.

Materials and Methods

Site description and Sampling Methods. The South River, located in northern Virginia, is a sinuous and steep gravel-bed river with a typical channel width of ~40 m and cohesive bank sediments (mostly silt and clay with some sand and gravel) (Pizzuto & O'Neal, 2009). It is one of the two main tributaries of the South Fork of the Shenandoah River. The riparian zone is characterized by abundant forest, pasture, or agricultural land uses. The ten study sites were along a 21-km expanse of the South River downstream from the contamination source, a previous textile (acetate fiber) manufacturing facility in Waynesboro, VA, referred to as the origin or 0 km (see Fig. 4.1). Sampling locations included five distinct habitat types: floodplain wetland (located at 2.6 and 13.8 km from Waynesboro, referred to hereafter as Relative River Distance-RRD 2.6 and RRD 13.8), bed sediment pool (RRD 4.8 and RRD 14.0), embedded pool (RRD 7.4 and RRD 11.9), island or mill race side channel pool (RRD 8.4 and RRD 15.9), and river pool edges with fine grained sediment deposit (RRD 10.0 and RRD 20.6). Fine-grained sediment (FGS) deposits (e.g., in RRD 10.0 and

20.6) in the South River refer to specific in-channel deposits of mud and sand caused by reduced water velocity due to bank obstructions. This fraction stored 17% to 43% of the annual mass equivalent of the suspended sediment load and was closely related to the transport of heavy metals in the river (Skalak & Pizzuto, 2010).

Surface sediment samples (0 to 7 cm depth) were collected from the streambed downstream of the Hg contamination source in Waynesboro, using a 5-cm polycarbonate core (3.3 mm wall thickness) in May (15.1- 16.5 °C in sediments) and August (19.7-20.6 °C in sediments) 2008. Surface sediment generally represents the layers where high Hg methylation and demethylation frequently occur in benthic habitats (Marvin-DiPasquale & Oremland, 1998; Hines et al., 2000; Hines et al., 2006). For sites with a course-grained streambed texture, where a coring method was not effective to take samples, a plastic hand-operated bilge pump was used to collect the fine-grained sediment into a clean 20-L polyethylene bucket, and the slurry samples were then taken after settling down for 20 min. Samples from site RRD 2.6 were only collected for chemical analysis. Samples collected for MeHg analysis were immediately placed on dry ice, while those for all other chemical analyses were kept in ice in the field. Samples for measuring potential methylation and demethylation rates were filled fully in sterile mason jars, shipped on ice to Rutgers University, and stored at 4°C prior to experiments. Samples for DNA/RNA extraction were shipped on dry ice and stored at -80° in the lab prior to processing. An additional set of samples was collected in May 2010 from RRD 10.0, RRD 14.0, and RRD 20.6 where high potential methylation (M) or demethylation (D) rates, or M/D ratios were detected in 2008 samples, for potential M and D measurements in the presence of metabolic stimulators and inhibitors.

Analytical Methods. Porewater in sediment samples was extracted by centrifugation (SLA-1500 rotor in the Sorvall RC-5B plus centrifuge) at 10, 000 rpm for 30 min at 4 °C. Aliquots of porewater were filtered by 0.22 μm membranes, and analyzed for nitrate and sulfate using an ICS-1000 ion chromatography system (Dionex, CA) equipped with an AS-40 autosampler and an Ionpac AS9-HC analytical column (4 mm × 250 mm). The effluent used for ion chromatography analysis was a 9 mM Na_2CO_3 solution. Porewater pH was measured by an Accumet 915 pH Meter (Fisher Sci.).

For whole sediment samples, organic matter content was analyzed by the loss-on-ignition (LOI) method (ASTM, 2007). Total solids and acid volatile sulfide (AVS) in sediment were measured following the procedures described by EPA Method 160.3 (USEPA, 1971) and 821/R-91-100 (USEPA, 1991), respectively. After processing the samples under strictly anaerobic conditions, Fe(II) and microbially reducible Fe(III) in the whole sediment and porewater were analyzed spectrophotometrically (Spectronic 20 Genesys, Spectronic Instruments, UK) by the ferrozine assay at 562 nm (Lovley & Phillips, 1987a). THg concentrations in sediment samples were analyzed by cold-vapor atomic fluorescence spectroscopy (CVAFS) detection following oxidation and volatilization by addition of $SnCl_2$ (Bloom & Fitzgerald, 1988). MeHg in wet sediment was separated by a solvent (methylene chloride) extraction procedure, and measured following aqueous ethylation with sodium tetraethylborate, purging and trapping, adsorption and desorption, separation by gas chromatography at 100 °C, reduction by a pyrolytic column, and detection by CVAFS (Bloom et al., 1997). Quality control experiments with reference sediment material showed an average of 103% recovery rates for the THg and of 90-95% for the MeHg analyses.

Mercury Methylation and Amendment Experiments. Sediments were completely homogenized in an anaerobic chamber (Coy Laboratories, MI) and processed under strictly anoxic conditions. Portions of sediment (3 ml) were dispensed by a cut-off plastic syringe into a 13 ml serum vials which were then sealed with Teflon-lined butyl rubber stoppers. Aliquot of N_2-deaerated site water (3 ml) was injected into each vial to create slurry incubations. After pre-incubation for 24 hour, 100 µl of ~0.1 µCi of $^{203}HgCl_2$ (Eckert & Ziegler Isotope Products, CA) in 0.1 N HCl, representing ~15 µg Hg(II) L^{-1} slurry, was injected into each vial and vortex homogenized. 100µl of 0.1 N NaOH was immediately injected into the same vial to neutralize the acid added. The radiotracer approach allowed quick measurement of potential methylation rates of sediments within hours at an ambient total Hg concentration (Gilmour & Riedel, 1995). Spiked Hg levels in this study were 110 to 1000 fold lower than ambient Hg concentrations, considering that the dry/wet ratio of the sediment was ~ 0.4, and thus ^{203}Hg was added at trace levels. After incubation for ~2 d in the dark at ambient temperature (~22 °C), 2.0 ml of 3 N HCl was injected into each vial to terminate microbial activities and vials were stored at -20 °C until analysis. The duration of incubation (~two days) used for the methylation experiments was judged based on preliminary tests and similar studies by other researchers (Hines et al., 2006; Drott et al., 2008). Preliminary experiments with two days incubation showed that this incubation period was adequate to distinguish rates from blank's means plus three standard deviations. Procedural blanks as killed controls for evaluating abiotic Hg methylation were processed as described for live samples except that acid (2.0 ml of 3 N HCl) was added prior to the injection of $^{203}HgCl_2$. This approach yielded nearly identical blank results as compared to procedural blanks that were obtained by using γ radiation (Yu et al., 2010).

After incubation, newly synthesized $Me^{203}Hg$ was separated from the unreacted $^{203}HgCl_2$ in sediment slurries by a toluene extraction method which was modified from previous protocols (Guimaraes et al., 1995; Marvin-DiPasquale et al., 2003; Hines et al., 2006). Acidified slurries were poured and rinsed into 50 ml glass centrifuge tubes with 5 ml saturated KCl (in 0.6 M H_2SO_4), mixed with 0.5 ml saturated $CuSO_4$, and vortexed. The suspensions were heated at 70-75 °C for 30 min. Five ml of toluene was then added, vortexed for 1 min and tubes centrifuged by a GLC-2 general laboratory centrifuge (Sorvall Instruments, Miami, FL) to separate the organic toluene phase from the aqueous phase at 2,500 rpm for 5 min at room temperature. The top toluene phase was transferred to second centrifuge tube which was capped quickly to avoid toluene evaporation. Another 5 ml of toluene was added to the tube with slurry following a second round extraction (vortex and centrifuge). The toluene extracts were then combined and dehydrated with anhydrous Na_2SO_4 (ca. 0.5 g). 5 ml of the toluene extract was mixed with a scintillation fluid (Scintisafe, Fisher Sci.) and $CH_3{}^{203}Hg$ was quantified by scintillation counting. Potential Hg methylation rates (% day^{-1}) were calculated after substracting results of the abiotic controls from results of triplicate samples per site or treatment. To evaluate recovery rates of MeHg by the toluene extraction method, sediment samples from all nine sites were amended with 2 ml of 3N HCl to terminate microbial activities, spiked with ^{14}C-MeHgCl (specific activity, 60 mCi/mmol; radiochemical purity, 95.2%; Amersham Corp., Buckinghamshire, England), and subjected to the same extraction protocol. Recovery rates ranged from 76 to 95%, which were within the range of those reported in river sediments impacted by gold mining and salt marsh sediments (Guimaraes et al., 1995; Marvin-DiPasquale et al., 2003).

Molybdate (Na_2MoO_4) as an inhibitor and sulfate (Na_2SO_4) as a stimulator of sulfate reduction were amended at two spiked concentrations of 400 and 1,000 μM, respectively. These amendment levels were similar to the addition concentrations of molybdate in Clear Lake sediments described by Fleming et al. (2006). Fe (III) in form of $Fe(OH)_3$ were added to slurry samples (total 6 ml) at amended concentrations of 0.801, 1.604, and 3.210 mg g^{-1} dry weight (dwt) sediment for Site RRD 10.0, 2.538, 5.057, and 10.113 mg g^{-1} dwt for RRD 14.0, and 1.437, 2.870, and 5.738 mg g^{-1}dwt for RRD 20.6, respectively. Fresh ferric oxyhydroxide was synthesized according to the methods described by Cornell and Schwertmann (2003). Briefly, ferric oxyhydroxide was synthesized by titrating a $FeCl_3$ solution with NaOH to pH 7 in an anaerobic glove box. Precipitates were washed with deoxygenated distilled deionized water until the supernatant exhibited a constant pH. Aliquots of the suspension were filtered and dried, and dry weight was determined for calculating the concentration of ferrihydrite under nitrogen gas (Wiatrowski et al., 2009).

Methylmercury Demethylation and Amendment Experiments. Sediment slurry microcosms were prepared and incubated at ambient temperature (~22 °C) in the dark for 24 hrs prior to spiking of MeHg as described above for Hg methylation assays. To determine demethylation potentials, 100 μl of ~0.1 μCi of ^{14}C-MeHgCl (specific activity described previously) were spiked into 6 ml of slurry in a 13 ml serum vial. Before spiking, the stock ^{14}C-MeHgCl solution was extracted for purification with methylene chloride by the method of Schaefer et al. (2004). Each treatment was assayed in triplicate and two killed controls, which were prepared by adding 2 ml of 3 N NaOH to the slurry prior to spiking. The amended ^{14}C-MeHg (31 μg L^{-1} slurry) was comparable or lower than the *in-situ* sediment MeHg

concentrations, which ranged from 8.2 to 124.0 μg kg^{-1} wet sediment from RRD 4.8 to 20.6 (RRD 2.6 not included, Table 4.1).

The gaseous products ($^{14}CO_2$ and $^{14}CH_4$) from ^{14}C-MeHgCl degradation were flushed and trapped by the $^{14}CH_4$-combustion/$^{14}CO_2$-trapping technique modified from Hines et al. (2006) and Marvin-DiPasquale and Oremland (1998), which was originally developed by Ramlal et al. (1986). After incubating the slurry microcosms statically for 40 to 48 h, demethylation activities were terminated by adding 2 ml of 3 N NaOH, a treatment that also pre-trapped $^{14}CO_2$ in the aqueous phase as carbonate. $^{14}CH_4$ was then flushed by air (20 ml min^{-1} for 30 min.), combusted into CO_2 by passing through a CuO filled quartz tube in a tube furnace (Carbolite, Derbyshire, England) that was heated at 850 °C. The product $^{14}CO_2$ was then trapped by a mixed solution of 2-phenylethylamine (3 ml), methanol (3ml), and Scinti Verse cocktail (6 ml, Fisher Sci.). The remaining slurry was acidified by the addition of 1 ml 6 N HCl, incubated overnight at room temperature to release the trapped $^{14}CO_2$, which was then purged by N_2 and trapped as described above. Rates observed from killed controls were generally low and subtracted from those of live samples. Results for potential rates are expressed as % $Me^{203}Hg(II)$ demethylated per day. The $^{14}CO_2$ trapping efficiency was determined by spiking sediments with ^{14}C-$NaHCO_3$ (specific activity, 30-60 mCi $mmol^{-1}$; concentration, ~100 mCi ml^{-1}; ICN Biomedicals, Inc., Irvine, CA) to which 1 ml 6N HCl in anaerobic serum bottles was immediately added. Recovery rates were from 92 to 108%.

Metabolic stimulators and inhibitors were added to slurry incubations to investigate which microbial guilds were involved in demethylation. Slurries of samples from sites RRD 10.0 and 20.6, collected in May 2010, were amended with either 0.4 mM of sodium sulfate or 0.4 mM sodium molybdate to stimulate or inhibit

sulfate reduction, respectively, with 0.5 mM bromoethane sulfonic acid [BES] to inhibit methanogenesis, and with ferric oxyhydroxide (Fe[III]) to stimulate iron reduction. Fe(III) was added to the slurry incubation at spiked $Fe(OH)_3$ concentrations of 0.801 and 1.604 mg g^{-1} dwt sediment for site RRD 10.0, and 1.437 and 2.870 mg g^{-1}dwt for RRD 20.6. Samples were incubated and demethylation rates were measured as described above.

M/D ratio was calculated by dividing the potential methylation rate by the potential demethylation rate obtained for a same sample. Results of methylation and demethylation 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 (HSD) (one-way) or Tukey-Kramer (two-way) test using SAS software (SAS Institute, Cary, NC).

RNA extraction, cDNA synthesis, and PCR amplification of 16S rRNA genes. Molecular characterizations of microbial communities were performed with sediment samples from site RRD 10.0, RRD 14.0, and RRD 20.6. RNA and DNA of sediment samples were extracted using a modification of the Hurt method (Hurt et al., 2001). Briefly, nucleic acids from natural samples were first recovered by a modified bead-beating step (Hurt et al., 2001), and RNA and DNA in extracts were separated by Qiagen® RNA/DNA Mini Kit (Qiagen, CA) according to the manufacturer's protocol. Total RNA was then purified using a RNeasy kit (Qiagen, CA). DNA in extracted RNA preparation was removed with RQ1 RNase-free DNase. RNA was reverse-transcribed to cDNA by the Superscript III First-Strand Synthesis System (Invitrogen, CA).

16S rRNA genes for clone libraries representing active bacteria in sediments were amplified by using a bacterial universal primer set of 27f and 910r (Lane, 1991). PCR

amplifications of cDNA were performed in a GeneAmp PCR System 9700 (Applied Biosystems, CA). Reactions (25 or 50 µl each) included 0.4 µM PCR primers, $MgCl_2$ at 1.5 mM (final concentration), 1× PCR buffer provided by the polymerase manufacturer, 0.2 nM of each deoxynucleoside triphosphate, 0.25 mg of bovine serum albumin ml^{-1}, 50 to 250 ng of cDNA, and 0.025 U of *Taq* polymerase (Denville, NJ). PCR conditions were an initial 5-min hot start at 95 °C, 35 cycles of 94 °C for 10 sec, annealing at 55 °C for 30 sec, and extension at 72 °C for 1 min and 30 sec, concluding with a final extension at 72 °C for 12 min.

Clone library construction and sequencing of 16S rRNA genes. Clone libraries of 16S rRNA genes were constructed using purified PCR products which were ligated into pGEM®-T Easy vectors (Promega, WI) and transformed into *Escherichia coli* DH 10B cells according to the manufacturer's instructions. Recombinant plasmid DNA for sequencing from randomly selected white colonies were purified using a Concert™ 96 Plasmid Purification System (Invitrogen, CA). The presence and size of inserts in plasmid DNA extracts were verified by EcoRI restriction digests (New England BioLabs, MA) followed by gel electrophoresis. 16S rRNA gene inserts were sequenced using primer 27r by Genewiz. Inc. (Piscataway, NJ).

Phylogenetic analyses. DNA sequences of clone libraries were edited by using MEGA (http://www.megasoftware.net/), and the sequence similarity of clone 16S rRNA genes to those in all databases was compared using BlastN (http://www.ncbi.nih.gov/). Edited sequences were 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 16S rRNA gene sequences from the South River are xxx to xxx, and xxx to xxx, respectively.

Results

Sediment characteristics. Porewater pH in surface sediment samples from the 10 sites was nearly neutral (6.2 to 7.4) (Table 4.1). Nitrate concentrations in porewater from all sites were low (from 14.7 to 18.6 μM) in 2008, and slightly higher concentrations were observed in May 2010. Sulfate in porewater, with ranges from 25.4 to 2558.2 μM (Table 4.1), fluctuated obviously seasonally in sites RRD 7.4, 10.0, 13.8, 14.0 and 20.6, with the highest (2558.2 μM) and lowest (25.4) values in sites RRD 13.8 (August 2008) on RRD 2.6 (May 2008), respectively. Analysis on sediments collected in May 2010 from the three sites (RRD 10.0, 14.0 and 20.6) showed that sulfate concentrations in the sediment porewater were in the range from 102.2 to 288.2 μM.

Total solids in whole sediment samples ranged from 18.3% to 63.5% (Table 4.1). Sediment samples from all 10 sites were sandy and silty and contained organic matter that, by appearance and texture, originated from woody debris. Most samples were composed of sand (33 to 88%), silt (10 to 63%), and clay (1.9 to 6.1%) (Flanders et al., 2010). The highest organic content was 54.9% at site RRD 15.9. For most sampling sites, however, organic content represented 8.2 to 18.5% of the total sediment weight. AVS was detected at higher levels in August than in May 2008 (data not shown), with the highest concentration at site RRD 15.9 in both samplings. The highest level of Fe(II) and Fe(III) measured by the ferrozine assay was both found at site RRD 2.6, with a range of 9.4-9.6 and 7.3-7.4 mg g^{-1} dwt sediment, respectively.

For the remaining sampling sites which were selected for assay of methylation and demethylation rates, samples of RRD 14.0 had the highest Fe(III) values (2.6 to 2.9 mg g^{-1} dwt sediment), both in May 2008 and May 2010 samples. Fe(II) levels in the May 2010 samples from the three sites were comparable, while microbially reducible Fe(III) content was site-specific with large variation, ranging from 0.2 to 2.8 mg g^{-1} dry wt (Table 4.1). Concentrations of Fe(II) and Fe(III) in porewater were low; below the detection limit of 0.01 μM (data not shown).

There was no clear trend in sediment THg concentrations downstream from the historic Hg contamination source in Waynesboro. Five of the 10 river sites contained comparable levels of total Hg at around 20 μg g^{-1} wet weight of sediment, with the highest THg concentration (56.6 μg g^{-1}) found at site RRD 8.4, a mill race channel sediment located in the middle section of the studied river expanse (Table 4.1). The fine-grained sediment (FGS) deposit from sites RRD 10.0 and 20.6 had the highest MeHg concentrations (123.0 and 124.0 ng g^{-1} wet sediment weight, respectively) in May 2008 samples. The trend of MeHg concentrations downstream from the industrial Hg source was also not obvious. However, sediment MeHg levels in samples taken in May 2008 for most sites were significantly higher than those from the August 2008 samples (data not shown).

Assay of Hg methylation and demethylation potentials. Preliminary time course experiments of methylation and demethylation conducted with sediments collected in May 2008 revealed that rates increased linearly in two days of incubation from all nines sites and were obviously different from the rates in the killed controls. Thus the incubation period of around two days was selected for all later methylation and demethylation experiments.

Two-Way ANOVA tests between sites and between sampling seasons demonstrated that seasonal changes of Hg methylation potentials between May and August 2008 were highly significant ($p < 0.0001$). Differences among sites for each sampling season were also highly significant ($p < 0.0001$). For samples collected in May 2008, potential methylation rates ranged from 1.0 to 2.5% per day (Fig. 4.2). Sites including RRD 4.8, 7.4, 10.0 and 14.0 had comparable methylation rates, which were significantly higher than the rates from the remaining sites ($p < 0.05$) (Fig. 4.2). Significantly higher potential methylation rates in the August samples were shown compared with the rates from the May samples, and ranged from 2.5 to 10.2 % per day. The methylation rate in the RRD 20.6 sample (10.2 % day^{-1}) was significantly higher than those of all other samples ($p < 0.0001$), while rates in RRD 10.0 (7.1 % day^{-1}) and 14.0 (5.1 % day^{-1}) as intermediate rates also showed significant difference from those of the remaining samples (Fig. 4.2).

Reductive degradation of ^{14}C-MeHg, i.e., $^{14}CH_4$ production, dominated in May 2008 samples, with the only significantly higher rate (3.92 % day^{-1}) at RRD 4.8 ($p < 0.01$) (Fig. 4.3). In contrast, oxidative demethylation, production of $^{14}CO_2$, dominated in August 2008 samples. The production of $^{14}CO_2$ in August samples was significantly higher than in May samples (two-way ANOVA; $p < 0.0001$), and showed a roughly increasing trend with distance from the Hg source, reaching the highest rate (3.50 % day^{-1}) of CO_2 production at site RRD 20.6 in the August samples. In May 2008, potential demethylation rates (combining $^{14}CH_4$ with $^{14}CO_2$) varied from 0.0 to 3.9%-day and were highest at site RRD 4.8 (3.9 % day^{-1}) in May 2008. In August 2008, potential rates ranged from 0.06 to 4.44% day^{-1} and were highest at site RRD 20.6. Site RRD 4.8 or 20.6 in its respective sampling time was the sole samples

with significantly different rates from other samples ($p < 0.05$), suggesting that activities were season and site specific (Fig. 4.3).

Methylation to demethylation ratios (M/D), which reflect the potential of net MeHg production, were higher than 1.6 for May 2008 samples (with the exception of RRD 15.9, M/D= 0.62), and higher than 3.2 for August 2008 samples (Fig. 4.4), suggesting a high potential for MeHg production especially in August 2008. The highest M/D ratio (~ 70) was observed at RRD 14.0 in May 2008 and RRD 10.0 in August 2008 and was mainly due to low potential demethylation rates in these two samples (Fig. 4.4). The relatively low M/D ratio (3.2) in RRD 20.6 in August 2008 resulted from the simultaneous occurrence of high potential rates for both methylation and demethylation at this same site.

When the habitat types within the South River are considered, the highest methylation potentials were observed in fine-grained sediment deposits collected along the river pool edge (RRD 10.0 and 20.6), with lower potentials shown in mill race side channel pools (RRD 8.4 and 15.9) and floodplain wetlands (RRD 13.8; Fig. 4.2). Sediments from RRD 20.6 (August sampling) had a high potential for oxidative demethylation, while the May samples from RRD 4.8 had a high potential for reductive demethylation (Fig. 4.3). Mixed results of demethylation rates were observed in sites that were characterized as embedded river pools (RRD 7.4 and 11.9) with low flow velocities. Samples form RRD 7.4 had a high methylation potential in May but not in August 2008, and those from RRD 11.9 had low methylation potentials at both samplings times (Fig. 4.2). Sediments from sites RRD 4.8 and 14.0, characterized by moderately higher concentrations of THg, also showed substantial methylation potentials in May 2008.

Factors correlating with sediment MeHg, Hg methylation and demethylation activities. The relationships among parameters known to significantly affect net MeHg accumulation patterns were examined in order to better understand the mechanisms of methylation and demethylation activities. For sites where potential methylation and demethylation rates were assayed, sediment MeHg concentrations were significantly positively correlated both with porewater sulfate (r^2 = 0.72, p = 0.0002; Fig. 4.5a) and with sediment THg concentrations (r^2 = 0.64, p = 0.003; Fig. 4.5b). However, neither AVS nor Fe(II)/Fe(III) ratios were strongly correlated with sediment MeHg (r^2 = 0.005, p = 0.91, and r^2 = 0.11, p = 0.95, respectively; data not shown). The relationships of potential methylation rates with porewater sulfate, sediment AVS, and Fe(II)/Fe(III) ratios, respectively, were insignificant (r^2 = 0.003, 0.06, and 0.11, respectively; data not shown).

Potential demethylation rates exhibited a significantly positive correlation with sediment MeHg (r^2 = 0.75, p = 0.0001; Fig. 4.5c). No obvious relationships of potential demethylation rates with sediment THg (r^2 = 0.23), porewater sulfate concentrations (r^2 = 0.05), or Fe(II)/Fe(III) ratios (r^2 = 0.03), respectively, were observed. M/D ratios, a measure of the potential for MeHg production, were significantly positively correlated with sediment THg (r^2 = 0.60, p = 0.001; Fig. 4.5d), but had a significantly negative correlation with sediment MeHg (r^2 = 0.61, p = 0.002; data not shown).

Effects of sulfate, molybdate, and amorphous ferric oxyhydroxide on Hg methylation. Potential rates of methylation and demethylation were determined in the presence of metabolic stimulators and inhibitors in order to examine which microbial guilds contributed to MeHg synthesis in the three most active sites RRD10.0, RRD14, and RRD20.6. Analysis on sediment samples collected in May

2010 from the three sites showed that sulfate concentrations in the sediment porewater were in the range from 102.2 to 288.2 μM, and the average content of microbially reducible Fe (III) ranged from 0.839 to 2.643 mg/g dry weight (Table 4.1). Sodium molybdate as an inhibitor and sodium sulfate as a stimulator for SRB were then amended at spiked concentrations of 400 and 1,000 μM (not including the background concentration), 2 and 5 fold of the average background sulfate level (i.e., 200 μM), respectively. Based on the measured background levels of microbially reducible Fe (III) in May 2010 (Table 4.1), Fe (III) in form of $Fe(OH)_3$ were added to slurry samples at spiked concentrations which were ½, 1, 2 times of the background reducible Fe(III) level for each site (at an average content of 0.598, 0.848, and 0.964 mg g^{-1} for site RRD 10.0, 14.0, and 20.6, respectively) by using a freshly-made $Fe(OH)_3$ slurry stock (20.05 g L^{-1}). The corresponding amendment concentrations of $Fe(OH)_3$ in the slurry incubation (total 6 ml) were 0.801, 1.604, and 3.210 mg g^{-1} dry weight (dwt) sediment for Site RRD 10.0, 2.538, 5.057, and 10.113 mg g^{-1} dwt for RRD 14.0, and 1.437, 2.870, and 5.738 mg g^{-1}dwt for RRD 20.6, respectively.

Sulfate addition, at concentrations which were 2 and 5 times higher than ambient pore water sulfate levels, significantly stimulated Hg methylation in all sediments samples (Fig. 4.6). Amendment of 0.4 mM and 1.0 mM sulfate increased potential methylation rates by 1.6 ($p = 0.002$) and 1.9 ($p = 0.0002$) fold for RRD 10.0, 1.7 ($p < 0.0001$) and 2.6 ($p < 0.0001$) fold for RRD 14.0, and 2.5 ($p < 0.0001$) and 2.6 ($p < 0.0001$) fold for RRD 20.6, respectively. Only in RRD14 sediment, increasing sulfate concentrations to 1.0 mM resulted in significantly higher potential methylation rates as compared with the effect of the 0.4 mM additions ($p < 0.0001$). Thus, in sediments of RRD 10.0 and RRD20.6, amendments with 0.4 mM sulfate were sufficient to

stimulate methylation while sulfate addition with 1.0 mM probably reached the saturation of the needs for the methylating SRB.

Molybdate addition at 0.4 and 1.0 mM which were 2 and 5 fold of ambient sulfate levels, respectively, significantly inhibited methylation rates by 27.8% ($p = 0.003$) and 27.2% ($p = 0.003$) for RRD10.0, and by 26.4 ($p = 0.001$) and 24.6% ($p = 0.002$) for RRD14 (Fig. 4.6). For site RRD 20.6, however, molybdate addition had no significant effects ($p > 0.368$). There was also no significant difference for molybdate addition between 0.4 mM and 1.0 mM in all sediment samples tested. The results indicated that at most, molybdate addition inhibited only 28% of the potential methylation rates and that further addition of molybdate did not result in a more efficient inhibition of methylation.

Amorphous ferric oxyhydroxide was added, calculated as Fe(III), at levels which were ½, 1, and 2 fold of ambient microbially-reducible Fe(III). The stimulation of potential methylation was conspicuous at ½ fold level for RRD 10.0 and 14.0 (Fig. 4.6). For site RRD 10.0, addition of ferric oxyhydroxide at 1/2 and 1 fold as Fe(III) (corresponding spiked concentrations of $Fe(OH)_3$ were 0.801 and 1.604 mg g^{-1} dwt sediment, respectively) increased potential methylation rates by 47.6% ($p = 0.002$) and 10.3% ($p = 0.607$), respectively, while amendments at 2 fold as Fe(III) (at spiked $Fe(OH)_3$ of 3.210 mg g^{-1} dwt sediment), significantly decreased potential rates by 61.2% ($p = 0.0003$). For site RRD 14.0, amendment of Fe(III) at ½, 1, and 2 fold of background levels (corresponding to 2.538, 5.057, and 10.113 mg $Fe(OH)_3$ g^{-1} dwt sediment) significantly increased methylation rates by 97.9% ($p = 0.0005$), 87.9% ($p = 0.001$), and 56.5% ($p = 0.015$), respectively. With RRD 20.6, amendment of 1.437 mg $Fe(OH)_3$ g^{-1} dwt sediment (1/2 fold as Fe(III)) did not significantly increased the methylation rates (by 12.5%, $p = 0.453$), while at 2.870 (1 fold) and 5.738 (2 fold) mg

$Fe(OH)_3$ g^{-1} dwt sediment potential methylation rates were significantly reduced by 52.4% ($p = 0.0009$) and 100% ($p < 0.0001$).

Effects of sulfate, molybdate, amorphous ferric oxyhydroxide, and bromoethane sulfonic [BES] acid on potential Hg demethylation rates. The degradation of ^{14}C-MeHg in sediment incubation was followed by formation of the two gaseous carbonaceous products including $^{14}CH_4$ and $^{14}CO_2$. For native samples from RRD 10.0 and 20.6 taken in May 2010, formation of $^{14}CH_4$ accounted for 75.5 and 65.3% of the total gaseous carbon product of MeHg degradation, respectively (Fig. 4.7), consistent with the May 2008 results showing reductive demethylation as the dominating pathway (Fig. 4.3).

In RRD 10.0 sediment incubation, overall, total gaseous carbon products by degradation of ^{14}C-MeHg were significantly stimulated by molybdate (0.4 mM), Fe(III) addition at 1 fold of the ambient level (1.604 mg $Fe(OH)_3$ g^{-1} dwt sediment), and BES (0.5 mM) by 61.8% ($p = 0.002$), 43.2% ($p = 0.031$), and 144.5% ($p < 0.0001$), respectively (Fig. 4.7a). For CH_4 production, amendments with ferric oxyhydroxide (1/2 and 1 fold as Fe(III)) significantly inhibited CH_4 production by 55.5% and 64.9% as compared to the unamended control, respectively (Fig. 4.7b). Addition of 0.4 mM molybdate, ½ and 1 fold as Fe(III), and 0.5 mM BES greatly enhanced production of $^{14}CO_2$ evolution in RRD 10.0 sediment incubations by 4.0, 3.1, 4.8, and 7.1 fold, respectively (Fig. 4.7c), while addition of 0.4 mM sulfate had no effect on CO_2 generation ($p = 0.989$).

For site RRD 20.6, amendments of 0.4 mM sulfate, 0.4 mM molybdate, and 0.5 mM BES to slurry samples significantly reduced total gaseous production in comparison with the native samples by 15.5% ($p=0.02$), 20.2% ($p=0.005$), 23.3% ($p=0.002$), respectively (Fig. 4.7a). Fe(III) addition at ½ and 1 fold caused even

higher reduction by 58.6% ($p<0.0001$) and 74.5% ($p<0.0001$), respectively. Spiking 0.4 mM sulfate to site RRD 20.6 sediment highly inhibited formation of CH_4 as reductive demethylation by 38.6% ($p = 0.007$) and significantly increased CO_2 as oxidative demethyltion (by 28.0 %; $p < 0.0001$) (Fig. 4.7b and c). Addition of 0.4 mM molybdate inhibited the formation of $^{14}CH_4$ and $^{14}CO_2$ by 17.1% ($p = 0.393$) and 26.1% ($p < 0.0001$), respectively. These results indicated the involvement of SRB in oxidative demethylation when sulfate reducing conditions were favorable. Additions of $Fe(OH)_3$ by 1/2 and 1 fold above ambient microbial reducible Fe(III) concentration caused a pronounced reduction of $^{14}CH_4$ by 68.8 and 83.0%, and that of $^{14}CO_2$ by 39.4 and 58.6% (Fig. 4.7b and c), suggesting that these amendments suppressed demethylation. Production of $^{14}CH_4$, but not of $^{14}CO_2$, from ^{14}C-MeHg was significantly inhibited by BES addition (33.0%; $p <0.002$), suggesting the involvement of methanogens in reductive demethylation.

Molecular characterization of sediment communities. The composition of the active microbial communities in the South River sediments was determined by examining the dominant 16S rRNA sequences in RNA extracts of sediment samples. Previous experiments described in this study showed that site RRD 10.0 had high methylation potential in both May and August 2008, that RRD 14.0 exhibited the highest M/D ratio and relatively high methylation potential in May 2008, and that RRD 20.6 was a site with the highest potential of methylation and demethylation in August 2008. Sediments from RRD 10.0 (May and August 2008 samples), RRD 14.0 (May 2008), and RRD 20.6 (August 2008) were thus selected for the community analysis (Fig. 4.8). A total of 386 clones, 96 and 98 clones representing each sample, were generated. By working with RNA, rather than DNA, extracts allowed the recovery and identification of microbial taxa which were active, rather than those

which were present at the site at the time of sampling. Sequences similar to all classes of the *Proteobacteria* (alpha-, belta-, gamma-, and delta-) dominated the active communities in the four sediments (Fig. 4.8), accounting for 60.4% (May) and 52.1% (August) in RRD 10.0, 54.2% in RRD 14.0 (May), and 56.2% in RRD 20.8 (August) for respective clone library. The remaining taxa included those commonly found in riverine and other freshwater sediments, including *Planctomycetes*, *Firmicutes*, *Bacteroidetes*, Cyanobacteria, *Acidobacteria*, and Green non sulfur bacteria (Fig. 4.8 and Tables C-1 to C-4 in Appendix C).

Clones representing the *Deltaproteobacteria,* the class to which most Hg-methylating sulfate and iron reducing bacteria belong (Ranchou-Peyruse et al., 2009), were present in all four libraries representing 3.1, 9.4, 10.4 and 11.2 % of the clones from RRD 10.0 (May), RRD 10.0 (August), RRD 14.0 (May) and RRD 20.6 (August), respectively. Sequence similarities of *Deltaproteobacteria*-affiliated clones showed that 27.6% of the clones were most similar (> 93 % similarity) to sulfate and iron reducers with documented methylation activities (Table S4.1). Such clones were most abundant in the clone library representing the active community in site RRD 20.6 (Fig. 4.8), where the highest potential methylation rate was observed in August 2008 (Fig. 4.2). A higher representation of *Deltaproteobacteria* was obtained for site RRD 10.0 in the August as compared to the May 2008 libraries, corresponding to the 2.9 fold higher potential methylation rates in the August sample (Fig. 4.2). Thus, the abundance of active bacteria affiliated with taxa known to methylate Hg was related to the potential methylation rates of sediment incubations, suggesting that active methylators were a dominant component of the microbial communities in the South River sediments. This relationship was further examined by relating potential methylation rates with the abundance of SRB-, IRB-, and *Deltaproteobacteria*-like

clones in each of the clone libraries (Fig. 4.9). A significant correlation ($r^2 = 0.93$, $p = 0.04$) was only observed for potential methylation rates and SRB-like sequences, supporting a role for SRB in Hg methylation.

The phylogenetic analysis of cloned 16S rRNA genes with affiliation to iron and sulfate reducing bacteria from *Deltaproteobacteria* showed that, at least three groups of active SRB and one group of *Geobacter*-like microbes formed from South River sediments strongly supported clusters with known Hg methylators (Fig. 4.10). First, at the top of the phylogenetic tree, two clones from RRD 14.0 (May.) were clustered with *Desulfococcus multivorans*, a strong Hg methylator (Ekstrom et al., 2003). Four clones including two (RRD10.0Aug-48 and RRD20.6Aug-97) which were 95-98% similar to a uncultured *Desulfobacteraceae* bacterium (Table S4.1), were weakly clustered with the known methylators *Desulfobacter* sp. BG8 and *Desulfobacterium* sp. BG33 (King et al., 2000) (Fig. 4.10). The second SRB group including RRD10.0Aug-25 and RRD20.6Aug-82 showed 91-96% similarity to uncultured *Desulfobulbaceae* bactereium (Table S4.1), and clustered at 100% bootstrap values with *Desulfobulbus propionicus* 1pr3, another strong Hg methylator (Ekstrom et al., 2003). The third SRB group of clones at the bottom of the tree was from RRD 10.0 and 20.6, loosely grouped with *Desulfovibrio desulfuricans* ND 132 which is also a known strain for Hg methylation (Jay et al., 2002), and with *Desulfovibrio africanus*, a weak methylator (Ekstrom et al., 2003). In the group of *Geobacter*-like bacteria, two clones (RRD10.0May-56 and RRD20.6Aug-24) were closely clustered with *Geobacter* sp. CLFeRB, one Hg methylator of IRB isolated from a freshwater lake sediment (Fleming et al., 2006), and another two clones (RRD10.0May-28 and RRD14.0May-76) also tightly grouped with the whole *Geobacter* cluster which contained another two strong methylators, *Geobacter metallireducens* and *Geobacter*

sulfurreducens PCA (Kerin et al., 2006). All the four clones were 93-96% similar to uncultured *Geobacter* strain or *Geobacter psychrophilus* stain P35 (Table S4.1).

Discussion

Hg contamination and the microbial potential for methylmercury production in the South River. A three years' study of Hg and MeHg in soil, surface water and sediment identified that the major non-point sources of inorganic Hg to the South River, transported by particle-bound forms downstream, were within the first 14 km downstream from the historic point source in Waynesboro (Flanders et al., 2010). Our study included this reach by focusing on the first 20.6 km downstream from Waynesboro. Total Hg content in South River sediments was comparable to those in the highly contaminated sediments (Fort Churchill, 15.8-44.3 μg Hg g^{-1}) in the Carson River, Nevada (Oremland et al., 1995), those from impoundment sediments in the Soča River, Slovenia (5-20 μg Hg g^{-1}) (Hines et al., 2006), and much higher than those in sediments from Minamata Bay, Japan (2.2 – 6.4 μg Hg dwt g^{-1}) (Tomiyasu et al., 2006). In the South River, the highest MeHg concentrations were detected in sites with fine-grained sediment deposits such as RRD 10.0 and 20.6 ranging from 123.0 to 124.0 ng g^{-1} and they were much higher than those reported for Minamata Bay (5.1 to 7.3 ng dwt g^{-1}) (Tomiyasu et al., 2006) and those from the Soča River (around 5 ng g^{-1}) (Hines et al., 2006).

Potential methylation rates in South River sediments (1.1 to 10.2 day^{-1}) were significantly higher than those observed in other mercury impacted environments. For example, potential methylation rates in Adirondack wetland soils and sediments were 0.1 to 1.2 % day^{-1} (Yu et al., 2010) and those reported for the Idrica River were

0.5 to 1.5 % day^{-1} (Hines et al., 2000). However, South River rates were similar to those in Valdeazogues River sediments in the Almadén mining region, Spain (0.38 to 13.0 % day^{-1}), the world's largest Hg mining region (Gray et al., 2004). The potential demethylation rates in this study, however, were generally lower than those reported in the Idrica River (6 to 8 % day^{-1}) (Hines et al., 2000) and Almadén sediments (0.04 - 17 % day^{-1}) (Gray et al., 2004).

In conclusion, our results suggest that the potentials for Hg methylation in the South River were at the higher range of those reported for other riverine systems while potentials for demethylation were at the lower range reported by others. Consequently, the potential for net MeHg production, as indicated by the M/D ratios (Fig. 4.4), was high especially for samples collected in August. The findings of higher MeHg production rates in August than in May were unexpected because previous reports showed higher MeHg concentrations in water and sediments in spring as compared to summer (Flanders et al., 2010). Higher M/D ratios in August than in May 2008 could be due to higher organic content detected by % total volatile solids in warmer seasons (Flanders et al., 2010) and higher temperatures, creating more favorable conditions for microbial metabolism in the summer. Korthals and Winfrey (1987) also observed that the methylation potentials in surficial lake sediments increased from spring to late summer and decreased in the fall. Fine-grained sediment deposits (RRD 10.0 and 20.6) collected along the river pool edge had the highest rates of potential methylation and demethylation (Fig. 4.2 and 4.3). The occurrence of high potentials for both methylation and demethylation in the same site was also observed in other sediment types (Pak & Bartha, 1998b; Hines et al., 2000). These observations suggest that fine-grained sediments originating in eroded

river banks were hot spots for both methylation and demethylation activities and habitats within the South River ecosystem where MeHg is produced.

Temporal patterns were also observed for demethylation pathways with reductive demethylation dominating in spring and oxidative demethylation dominated in summer. While reductive demethylation may lead to the conversion of MeHg to elemental Hg(0) and its transport into the gaseous phase, oxidative demethylation with the likely production of Hg(II) may result in a futile cycle of methylation and demethylation (Schaefer et al., 2004). Since both methanogens and SRB could be involved in oxidative degradation of MeHg, Marvin-DiPasquale and Oremland (1998) hypothesized that the $^{14}CO_2/^{14}CH_4$ ratios from oxidative degradation by methanogens were near or below 0.3, while the gaseous product from the demethylation by SRB was only CO_2. Therefore, detection of CH_4 does not necessarily mean the production of elemental Hg.

The relationships of geochemical factors with potential methylation, demethylation rates, and MeHg accumulation in South River sediemnt. The strong positive correlation of sediment MeHg with porewater sulfate (Fig. 4.5a) suggested that SRB could be involved in Hg methylation in South River sediments. A positive correlation was observed between sediment THg and MeHg concentrations in sediments (Fig. 4.5b), which demonstrated that increased MeHg was strongly related to higher THg in sediments. Drott et al. (2008) also reported a positive correlation between THg and MeHg in sediments from Hg-contaminated environments, and proposed that total Hg is one factor contributing to the MeHg synthesis.

The strong correlation between demethylation rates and sediment MeHg concentrations (Fig. 4.5c) is consistent with the known relationship between reaction rate and substrate concentration. Similar correlation was shown in Hg-contaminated

river sediments in Belgium showing increased degradation rates in response to increased concentrations of sediment MeHg (Billen et al., 1974). Likewise, the positive relationship between M/D ratios and sediment THg (Fig. 4.5d) may be explained by THg representing the likely substrate for methylation being one factor controlling Hg methylation.

The link of Hg methylation potentials to putative methylators in South River sediments. Low concentrations of AVS and high Fe(II):Fe(III) ratios were found for all South River habitat types (Tables 1), indicating that iron rather than sulfate reduction was the terminal oxidation process in anoxic sediments. This conclusion would suggest that iron reducers were the principle methylators of Hg in South River sediments. Results of 16S rRNA cloning and sequencing, however, partially contradicted this suggestion by showing that both iron and sulfate reducers were active in the sediments.

Phylogenic analysis showed that clones with high similarity to the 16S rRNA genes of strong Hg methylators were distributed across three sediment sites (RRD 10.0, 14.0 and 20.6; Fig. 4.10 and Table S4.1) where high potential methylation rates were observed (Fig. 4.2), suggesting that SRB actively methylated Hg in these locations. This conclusion is consistent with the significant positive correlations between (i) sediment MeHg and pore water sulfate concentrations (Fig. 4.5a), and (ii) potential methylation rates and percentage of SRB-like clones in all 16S rRNA clones representing the sediment communities (Fig. 4.9). However, sequences most similar to those of iron reducers, *Geobacter*-like and *Shewanella*-like strains (data not shown), were also present in all clone libraries (Fig. 4.10). *Geobacter* spp. are important Hg methylators (Fleming et al., 2006; Kerin et al., 2006), although *Shewanella* spp. might not be involved in Hg methylation (Kerin et al., 2006).

However, the abundance of *Geobacter*-like sequences in the clone libraries was not correlated with potential methylation rates (Fig. 4.9). Nevertheless, the results pointed out that both iron and sulfate reducers were potential methylators in the South River sediments.

Average pore water sulfate concentrations in South River sediments, ranging from 29.6 to 227.1 μM (excluding the extremely high value at RRD13.8), were similar to typical sulfate levels (<0.2 mM) in freshwater ecosystems (Gilmour & Henry, 1991; Warner et al., 2003). In the amendment experiments, molybdate was used as an inhibitor of SRB since molybdate acts as a competitive inhibitor in dissimilatory sulfate reduction (Peck, 1959), and could efficiently inhibit Hg methylation both in sediments under sulfate reducing conditions (Compeau & Bartha, 1985) and in pure cultures under both fermentative and sulfate reducing conditions (Pak & Bartha, 1998a). Spiked concentrations of molybdate as an inhibitor and sulfate as a stimulator in this study were chosen at 2 and 5 fold of the *in-situ* pore water sulfate levels following Fleming et al. (2006), although an equimolar level of molybdate (or sulfate) to ambient sulfate was previously employed (Oremland & Capone, 1988). Research by others indicated that, molybdate up to 20 mM did not inhibit reduction of iron or manganese (Burdige & Nealson, 1985; King & Garey, 1999), and methylation by IRB is probably "molybdate-independent" (Fleming et al., 2006). Thus clear differentiation between metabolism of SRB and IRB is possible by molybdate and sulfate amendments. Since there are no specific inhibitors for IRB (Gorby & Lovley, 1991; Woznica et al., 2003), it is relatively difficult to directly discriminate the contribution of IRB from MeHg synthesis in sediments. However, as the purpose of stimulating iron reduction, additions of $Fe(OH)_3$ to freshwater river sediments inhibited sulfate reduction by 86 to 100% (Lovley & Phillips, 1987b) and reduced

methane production by 50-90% (Lovley & Phillips, 1986, 1987b). Therefore, addition of $Fe(OH)_3$ should stimulate iron reduction and may also inhibit sulfate reduction and methanogenesis by directing available reducing equivalents from these processes toward iron reduction. In this study, amendment of Fe(III) in the form of $Fe(OH)_3$ was based on the content of measured ambient microbially reducible ferric ion (Lovley & Phillips, 1987a) instead of the total Fe content; an approach justified based on the low bioavailability of solid iron phases in sediments and the assumption that freshly added ferrihydroxide is highly available for Fe(III) reduction.

Sulfate addition significantly increased potential methylation rates by 1.6 to 2.6 fold, and molybdate addition significantly inhibited methylation by 24.6 to 27.8%, clearly implicating SRB actively involved in synthesis of MeHg in the South River sediments. However, more than 72% of the potential methylation rates could not be further inhibited, indicating that SRB only partially contributed to MeHg production and that other methylating microbial guilds, e.g., IRB or other unknown organisms, contributed to this process.

Potential methylation rates were stimulated by the addition of $Fe(OH)_3$ at ½ fold over ambient Fe(III) levels on site RRD 10.0 and at all three amendment levels on site RRD 14.0, suggesting an active role for IRB in methylation. Addition at 2 fold Fe(III) to RRD 10.0 and 1 and 2 fold to RRD 20.6 inhibited methylation possibly due to the inhibition of sulfate reduction by added Fe(III) as a stronger oxidizer and a reduced bioavailability of Hg^{2+} caused by scavenging of added $Fe(OH)_3$ (Jackson, 1989; (Mastrine et al., 1999). To date the few studies that examined the effect of stimulating iron reduction on Hg methylation in environmental incubations reported contradicting results (Mitchell & Gilmour, 2008). Warner et al. (2003) reported that methylation was inhibited in riverine sediments that were oxidized to produce Fe(III)), while

Jackson (1989) reported that the addition of iron oxide promoted Hg methylation in lake sediments. Gilmour et al. (1998) indicated that spiking Everglades sediments with soluble iron citrate did not result in any significant effect on Hg methylation. Overall, our results indicated that low level of $Fe(OH)_3$ addition significantly stimulated microbial Hg methylation in most of South River sediments. With $Fe(OH)_3$ amendment at a higher level, influence on Hg methylation rates was site-specific possibly because ambient reducible Fe (III) and the composition of microbial communities varied.

The capacity of IRB to utilize solid phase Fe(III) and absorbed Hg(II) may extend the depth of Hg methylation in sediments. Methylation by sulfate reducers is likely restricted to the upper layers of sediment due to its dependence on soluble electron acceptors and Hg (Fleming et al., 2006). This difference in niches where methylation takes place would enable methylation by both SRB and IRB in the same sediment samples. Our results support this hypothesis by showing stimulation of methylation when sediments were amended to enhance either sulfate or iron reduction and by the co-occurrence of 16S rRNA sequences in the community RNA pool that were most similar to those of known SRB and IRB. We therefore conclude that both IRB and SRB contribute to MeHg production in South River sediments.

Demethylation pathways and related microbial metabolic activities. Two pathways, distinguished by their gaseous carbonaceous products, have been proposed for microbial MeHg degradation. Oxidative demethylation is a co-metabolism process that converts the methyl group in MeHg to CO_2 with or without formation of a small amount of CH_4 (Oremland et al., 1991; Marvin-Dipasquale & Oremland, 1998). Pure cultures of SRB and methanogens were shown to produce CH_4 with $^{14}CO_2$ (Oremland et al., 1991) when incubated with MeHg. Reductive demethylation is mediated by Hg

resistant with broad spectrum *mer* operons, in which organomercury lyase (MerB) encoded by *merB* genes cleaves the C-Hg bond to form elemental Hg and CH_4 as the sole carbon product (Schottel, 1978; Barkay et al., 2003).

In the South River sediments, the dominant pathway for demethylation varied between May and August. In May, most ^{14}C-MeHg was degraded to $^{14}CH_4$, implicating reductive degradation (Fig. 4.3), while $^{14}CO_2$ production dominated in August suggestive of oxidative demethylation. Some previous studies have hypothesized that oxidative demethylation is the leading pathway in anaerobic habitats with low levels of Hg contamination, whereas reductive demethylation dominates in aerobic environments and where high Hg concentrations are present (Oremland et al., 1991; Marvin-DiPasquale et al., 2000; Barkay et al., 2003). Clearly, the results reported here for the South River sediments do not support this hypothesis. Hines et al. (2006) also noted a temporal change in demethylation pathways in coastal marine sediments collected at the Gulf of Trieste. Degradation that was solely oxidative in two surface sediment samples (the upper 1.5 cm) collected in August switched to partial reductive degradation (20-40% as $^{14}CH_4$) in samples collected in March, suggesting that the relative redox potential, rather than the concentration of Hg in surface sediment, was controlling which pathway of demethylation was dominating. A study by Oremland et al. (1995) also showed that there was no apparent relationships between the degree of Hg contamination and the occurrence of oxidative MeHg degradation. It is possible that, in surface sediments of the South River, reduction potential tends to be higher in the colder months (e.g., May) than in warmer months (e.g., August) due to higher solubility and less consumption of O_2, thus possibly switching the MeHg degradation to reductive pathway (Hines et al., 2006). A shift of microbial community structure could also explain the change in the

demethylation pathway (Marvin-DiPasquale et al., 2000). Such a change was noted for active members in the RRD10.0 community in which sequences most similar to those of the Gammaproteobacteria, among which *mer* operons are common (Barkay et al., 2010). Number of this group declined in the August relative to the May community, while those most similar to Deltaproteobacteria, among which *mer* is rare, increased in the August sample (Fig. 4.8).

Amendments differently influenced MeHg degradation in sediments from site RRD 10.0 and 20.6 (Fig. 4.7a). In RRD20.6 samples from May 2008 and 2010, the reductive pathway dominated in unamended sediments (Fig. 4.3 and 4.6b) while sulfate significantly stimulated $^{14}CO_2$ production and strongly inhibited $^{14}CH_4$ formation, and molybdate significantly reduced evolution of $^{14}CO_2$, implicating SRB involving in oxidative demethylation. Similar trends were reported for the Carson River sediments with high Hg contamination (Oremland et al., 1995). Formation of $^{14}CH_4$ was greatly inhibited by BES (33.0%; Fig. 4.7b), which was also an effect found in the Carson River sediment (Oremland et al., 1995). This result suggested an involvement of methanogens in reductive demethylation. Amendments with $Fe(OH)_3$ significantly decreased production of both reductive and oxidative demethylation in RRD 20.6 sediment incubations, indicating that iron reducing conditions impacted demethylation rates probably by inhibiting demethylating SRB and methanogens, Thus, IRB were not likely to degrade MeHg in RRD20.6. While little information is available on the role of IRB in demethylation, the addition of nitrate, whose redox potential (+420 mV) is highly positive as is that of ferric iron (+760 mV), partially inhibited MeHg degradation in Flordia Everglades peat sediments, suggesting that denitrifyers, while not directly degrade MeHg, may influence this process by inhibiting SRB and methanogens (Marvin-DiPasquale & Oremland, 1998).

For site RRD 10.6, amendment of sediment incubations with ferric oxyhydroxide enhanced oxidative demethylation (Fig. 4.7c), suggesting that IRB might be involved in this process. In this site, sulfate addition did not influence either reductive or oxidative demethylation. Amendment of molybdate and BES conspicuously stimulated oxidative demethylation by 4 and 7 fold, respectively, suggesting that other demethylators besides SRB and methanogens were stimulated for MeHg degradation because the inhibition on these two might provide more substrate or electron donors for other potential demethylators.

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Table 4.1. Physical and chemical characteristics of sediments from the South River, VA in 2008 and 2010[*]

Study site (RRD, km)	Porewater			Sediment						
	pH	Nitrate (μM)	Sulfate (μM)	Total Solids (%)	Organic content, LOI (%) [†]	AVS (μmol g^{-1})	Fe (II) [‡] (mg g^{-1})	Fe (III) [‡] (mg g^{-1})	Total Hg (μg g^{-1}) [§]	MeHg (ng g^{-1}) [§]
					May and August 2008					
2.6	6.8-7.0	14.8-18.6	25.4-36.5	36.0-48.7	13.8-14.2	1.6-1.8	9.4-9.6	7.3-7.4	3.5-8.0	4.9-5.7
4.8	6.9-7.3	14.7-15.1	111.2-226.8	34.4-39.8	10.3-10.3	1.7-2.7	1.4-1.5	1.4-1.5	20.3-26.5	32.6-58.3
7.4	6.9-7.4	14.8-17.7	46.9-174.6	29.0-39.9	8.2-11.5	1.9-2.1	2.8-2.9	1.1-1.2	18.4-27.6	56.6-87.7
8.4	6.7-7.0	15.7-16.5	44.8-69.2	34.1-61.8	5.3-7.6	1.0-1.2	1.6-1.7	0.8-0.9	31.4-56.6	23.5-62.6
10.0	6.9-7.0	15.2-16.5	62.3-182.2	21.9-63.5	15.2-15.4	2.4-6.1	3.3-3.4	1.0-1.3	6.8-21.1	17.9-123.0
11.9	6.6-7.2	14.7-14.8	113.0-166.9	30.0-35.1	12.0-12.3	2.1-2.6	2.2-2.3	1.7-1.8	19.8-24.1	37.3-97.8
13.8	6.6-6.9	14.7-14.9	60.3-2558.2	30.7-49.5	10.8-11.6	2.3-4.9	2.6-2.7	1.5-1.6	14.2-19.7	23.6-103.0

14.0	6.9-7.3	14.7-15.3	130.3-406.9	25.9-40.6	11.4-12.4	2.4-2.9	1.1-1.1	2.8-2.9	20.8-21.2	37.5-47.4
15.9	6.8-7.0	14.7-14.9	27.8-79.6	22.9-39.5	18.5-54.9	4.6-13.6	4.4-4.6	0.6-0.6	4.3-8.9	8.2-49.0
20.6	6.9-7.0	14.8-15.1	79.0-176.6	18.3-48.0	0.25-12.9	1.3-3.7	3.7-3.7	0.8-0.9	10.5-28.6	24.1-124.0
					May 2010					
10.0	6.2-6.3	36.3-37.6	102.2-153.5	-	-	-	2.1-2.1	0.4-1.3	-	-
14.0	6.3-6.4	38.8-51.6	154.4-229.9	-	-	-	1.9-2.0	2.6-2.7	-	-
20.6	6.2-6.3	169.0-19	204.2-288.2	-	-	-	1.9-2.3	0.2-2.8	-	-
		9.1								

* Values represent the range of concentrations detected from all samples collected in May (n=2) and August 2008 (n=2), or in May 2010 (n=3).

† The content range was only from the samples collected in May 2008.

‡ The content values on dry weight sediment basis were only from the samples collected in May 2008.

§ Unit of values was based on wet sediment weight (g^{-1}).

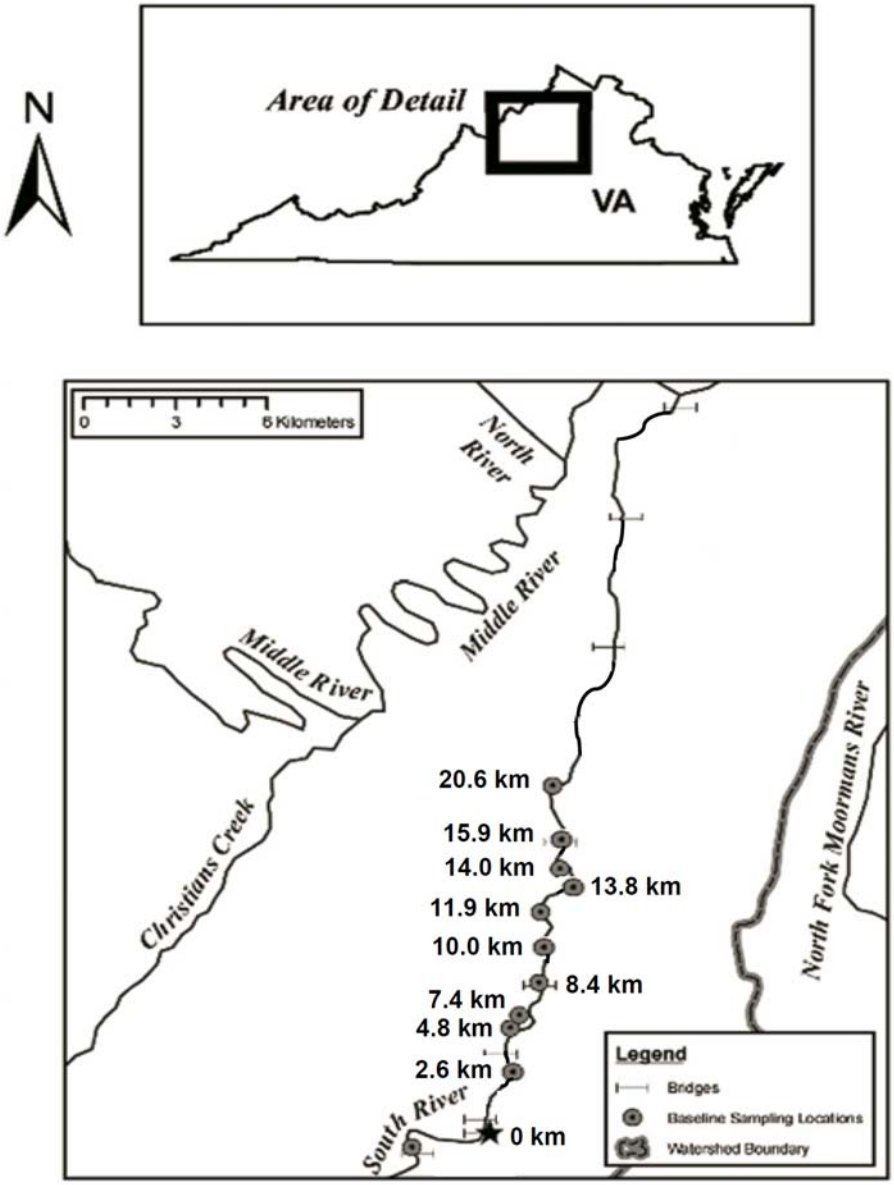

Fig. 4.1. Location of study area and sampling sites. Lower panel shows the downstream distance from the historic point source of mercury in Waynesboro, VA (★) along the river centerline, in km.

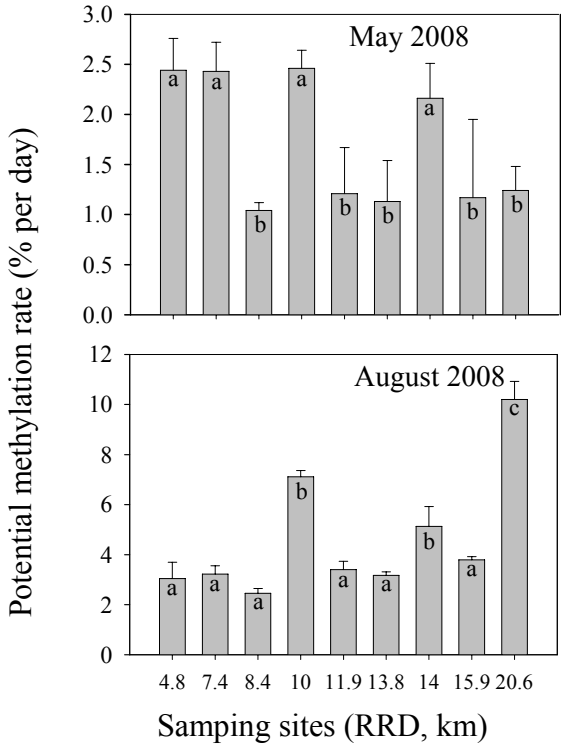

Fig. 4.2. Potential methylation rates by slurry incubations for nine sampling sites in the South River, VA. RRD - Relative River Distance (RRD, km) downstream from the Hg contamination source in Waynesboro. Different letters inside the bars indicate significant differences (ANOVA, $p<0.05$).

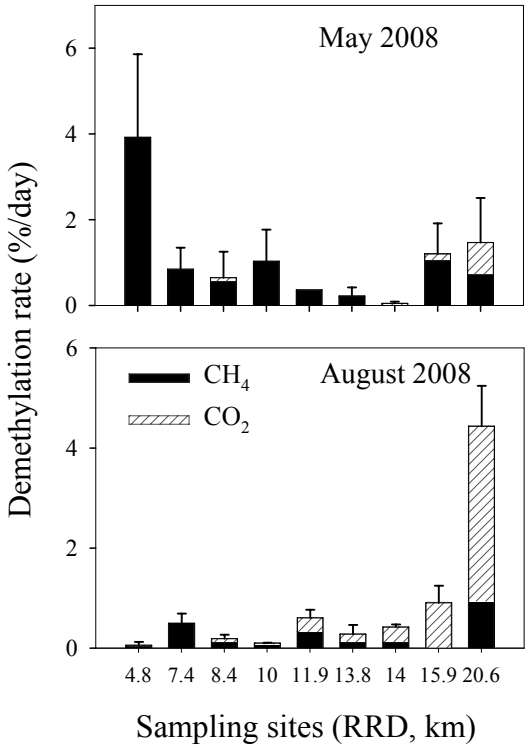

Fig. 4.3. Potential demethylation rates by slurry incubations for nine sampling sites in the South River, VA. RRD - Relative River Distance (RRD, km) downstream from the Hg contamination source in Waynesboro.

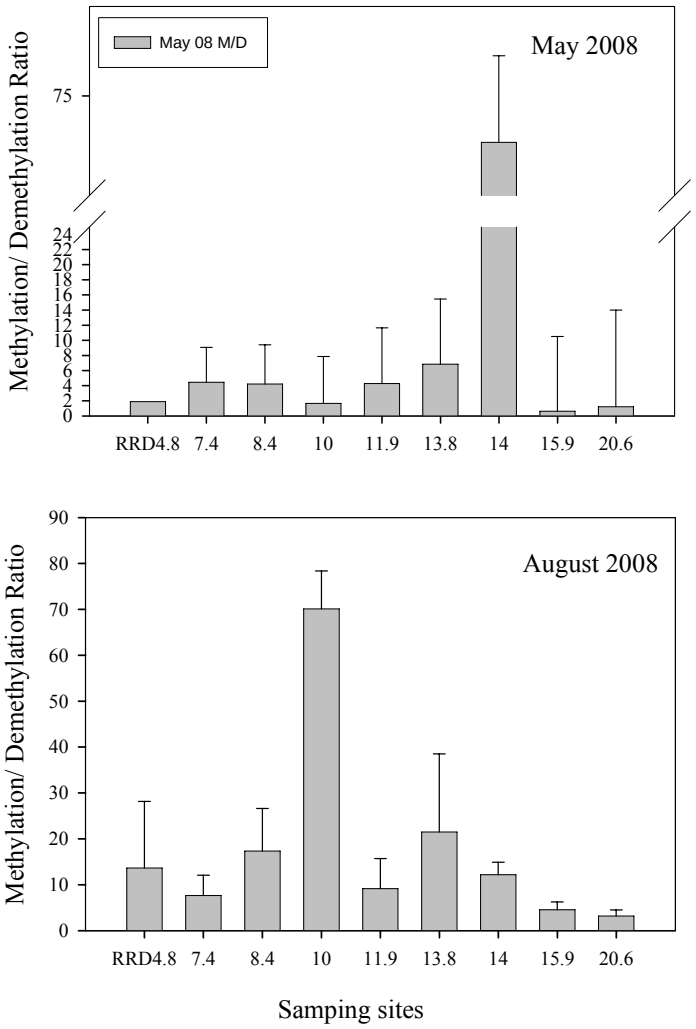

Fig. 4.4. Potential Methylation/ Demethylation ratios for sediment samples collected in May and August 2008 from nine different sites in the South River, VA.

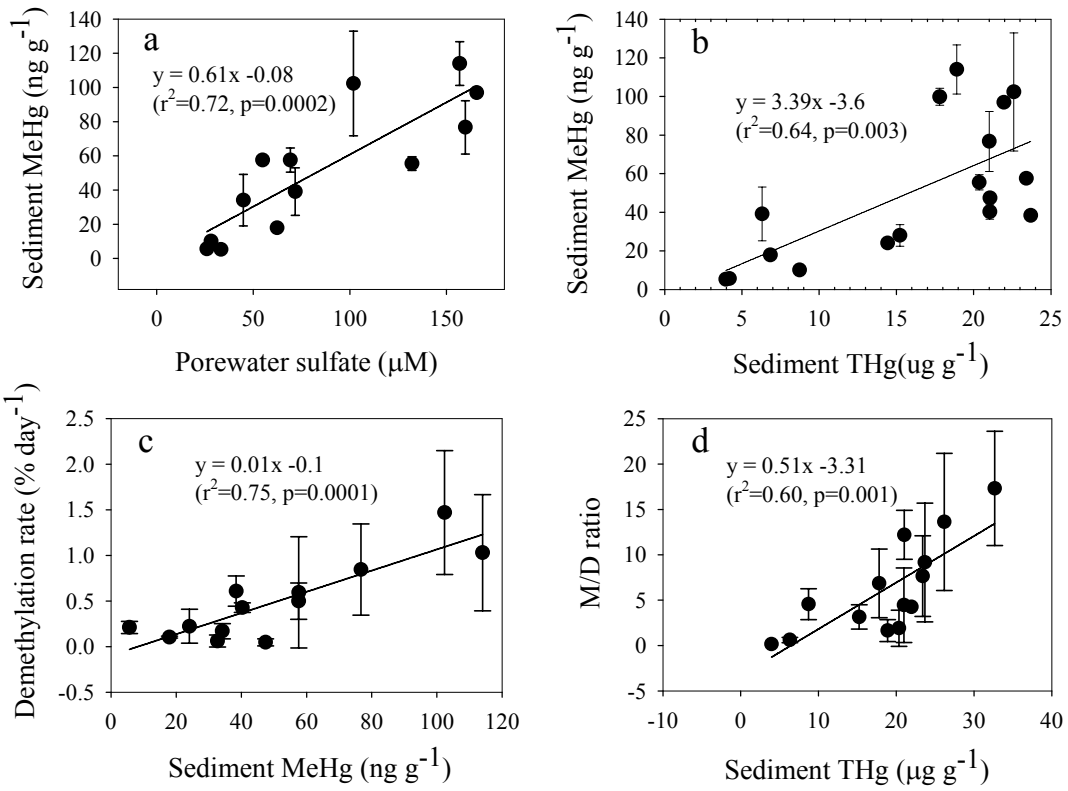

Fig. 4.5. Regression analysis of a) porewater sulfate versus sediment MeHg concentrations (n = 13), b) sediment THg concentrations versus sediment MeHg concentrations (n = 16), c) sediment MeHg concentrations versus potential demethylation rates (n = 13), d) sediment THg concentrations versus ratios of potential methylation over potential demethylation rates (M/D) (n=14).

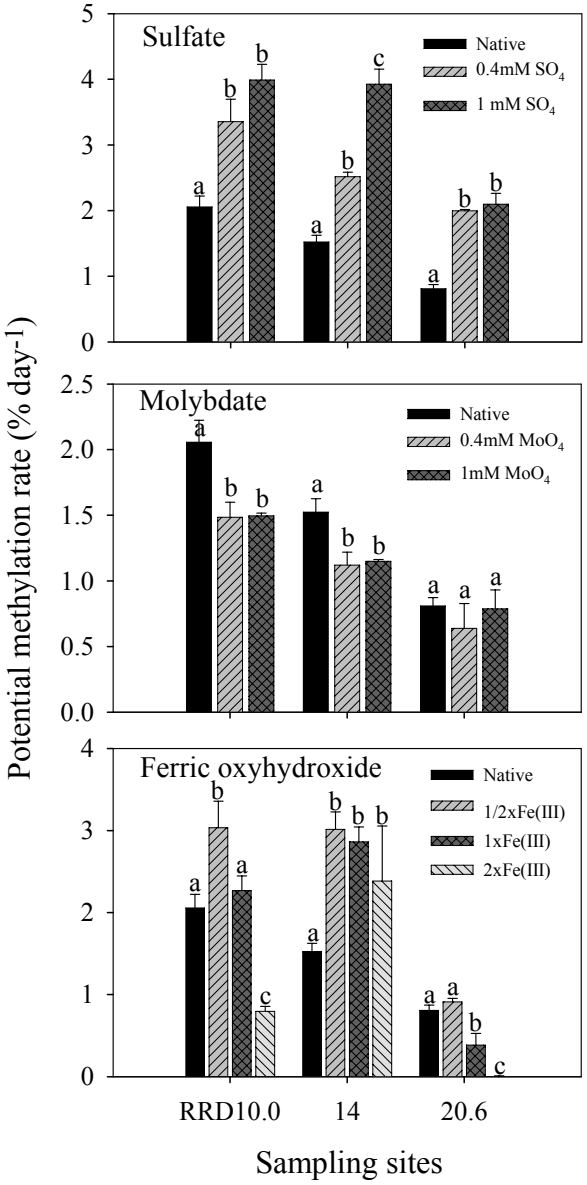

Fig. 4.6. The effects of metabolic stimulators and inhibitors on potential methylation rates. Fe(III) in form of $Fe(OH)_3$ were added at 1/2, 1 and 2 fold of the measured *in-situ* microbially reducible Fe(III). The final amended $Fe(OH)_3$ levels in the slurry were 0.801, 1.604, and 3.210 mg g^{-1} dwt sediment for RRD 10.0, 2.538, 5.057, and 10.113 mg g^{-1}dwt for RRD 14.0, and 1.437, 2.870, and 5.738 mg g^{-1} dwt for RRD 20.6, respectively. Different letters above bars indicate significant difference (ANOVA).

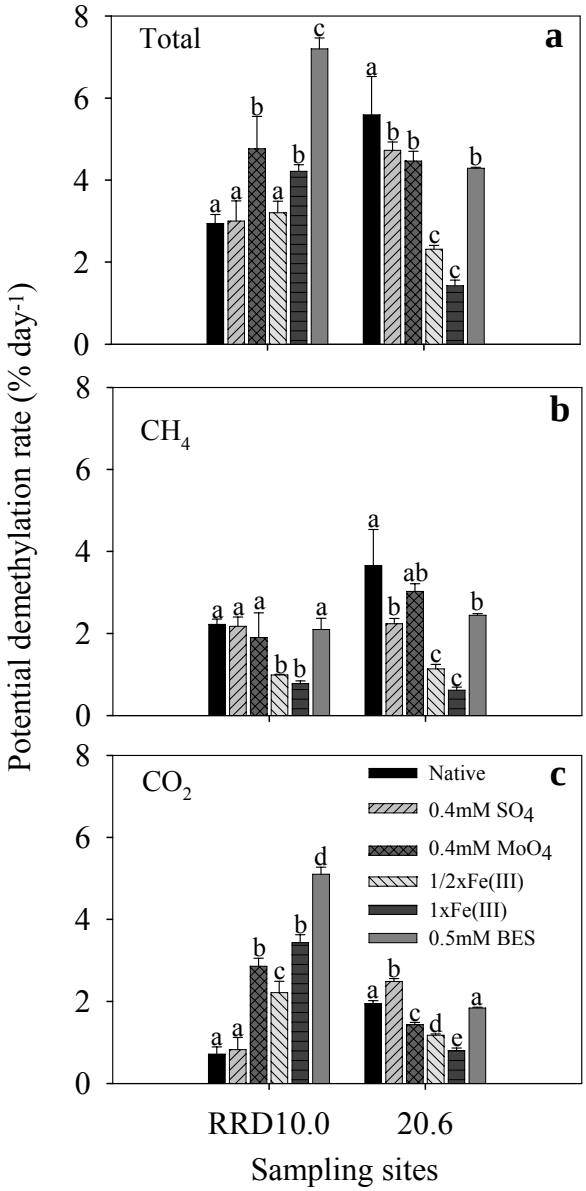

Fig. 4.7. The effects of metabolic stimulators and inhibitors on potential demethylation rates (total CH_4 and CO_2, a) and the carbonaceous gaseous products of demethylation of CH_4 (b), CO_2 (c) in site RRD10.0 and 20.6. Amendments included sulfate, molybdate, amorphous ferric oxyhydroxide ($Fe(OH)_3$), and bromoethane

sulfonic acid (BES). Final amended $Fe(OH)_3$ levels in the slurry incubations were 0.801 and 1.604 mg g^{-1}dwt sediment for RRD 10.0, and 1.437 and 2.870 mg g^{-1} dwt sediment for RRD 20.6, respectively. Different letters above bars indicated significant difference (ANOVA).

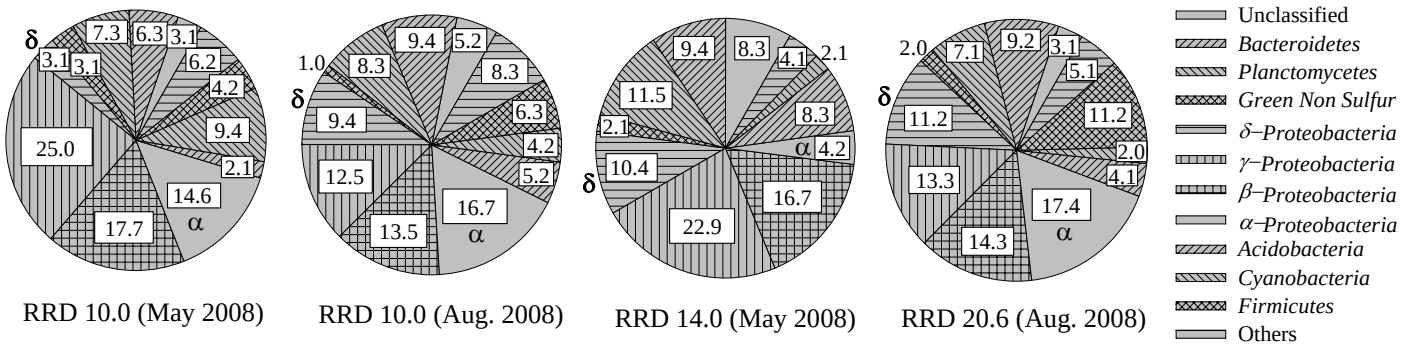

Fig. 4.8. Active bacterial community composition of Site RRD10.0, RRD14.0 and RRD20.6. Numbers indicate percentages of clones belonging to each taxon. Others included minor groups of *Flavobacteria*, *Fibrobacteres*, *Spirochaetes*, *Nitrospirae*, *Verrucomicrobia*, *Actinobacteria*, *Chlorobi*, *Fusobacteria*, and *Lentisphaerae*.

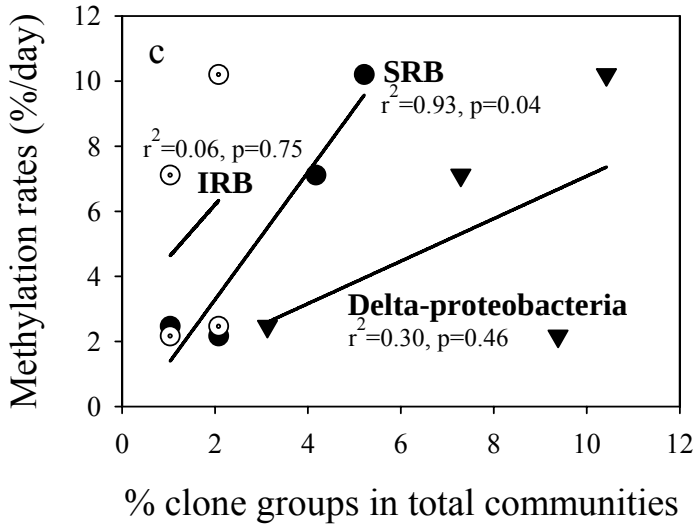

Fig. 4.9. The relationship between potential methylation rates and % of clones with 16S rRNA sequence similar to those of SRB, IRB, and all *Deltaproteobacteria* in total clones sequenced for each clone library.

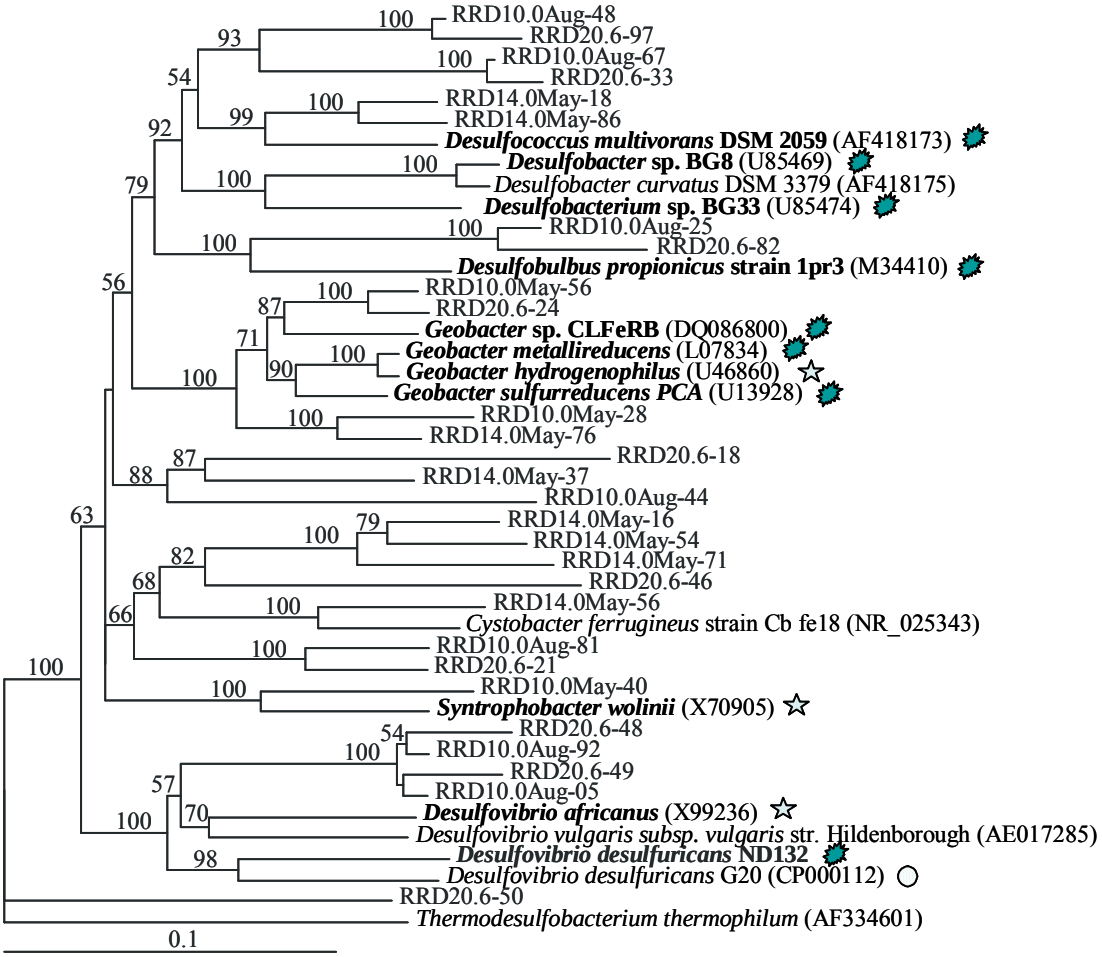

Fig. 4.10. A phylogenetic tree of 16S rRNA clone sequences affiliated with Delta-proteobacteria from cDNA extracts in the South River sediments and their affiliation with known iron and sulfate reducing bacteria. Symbols following reference species names indicate strong Hg methylators (✹), weak methylators (☆), and non-methylators (○). Clone designation denotes the sampling site (the initial three letters and three numbers), sampling time (May or Aug.), and clone number (last two digits). The 16S rRNA gene of *Thermodesulfobacterium thermophilum*, was used as an outgroup.

Supplemental Table S4.1 A list of clones with 16S rRNA sequences with affiliation with the Deltaproteobacteria in the South River sediments*

Clones	Most closely related sequence (Accession number)	Similarity (%)	Clones	Most closely related sequence (Accession number)	Similarity (%)
RRD 10.0 May 2008			**RRD 10.0 Aug 2008**		
10.0-28	Uncultured *Geobacter* sp. clone D12_30 (EU266834)	95	**10.0-5**	*Desulfovibrio putealis*(AY574979)	96
10.0-40	Uncultured *Syntrophobacter* sp. clone B02 (EU888826)	94	**10.0-25**	Uncultured Desulfobulbaceae bacterium clone TDNP_USbc97_108_1_6 (FJ516880)	96
10.0-56	Uncultured *Geobacter* sp. clone LH-27 (AB265850)	96	**10.0-44**	Uncultured *Geobacter* sp. clone GASP-WA2S1_B10 (EF072550)	86
			10.0-48	Uncultured Desulfobacteraceae clone TDNP_USbc97_107_1_5 (FJ516879)	98
			10.0-67	Uncultured Delta proteobacterium clone Z114MB93 (FJ485081)	93
			10.0-81	Uncultured Delta proteobacterium clone GASP-MA4S1_C10 (EF663952)	95
			10.0-92	*Desulfovibrio* sp. BSY-C (AB303306)	94
RRD 14.0 May 2008			**RRD 20.6 Aug 2008**		
14.0-11	Uncultured Desulfuromonadales bacterium clone AMBH11 (AM935776)	85	**20.6-18**	Iron-reducing bacterium enrichment culture clone FEA_2_D7 (FJ802334)	98
14.0-16	Uncultured Delta proteobacterium clone AKYH836 (AY921872)	91	**20.6-21**	Uncultured Delta proteobacterium clone TDNP_Wbc97_103_1_10 (FJ516992)	96
14.0-18	Uncultured Desulfobacteraceae bacterium clone TDNP_USbc97_21_4_87 (FJ516927)	95	**20.6-24**	*Geobacter psychrophilus* stain P35 (AY653549)	95
14.0-37	Uncultured Delta proteobacterium clone HF70_10I02 (EU361323)	91	**20.6-33**	Uncultured delta proteobacterium clone Z27M49B (FJ485583)	91
14.0-54	Uncultured Delta proteobacterium clone sw-xj259 (GQ302548)	96	**20.6-46**	Uncultured delta proteobacterium clone GASP-KA1W3_A11 (EU297366)	91
14.0-56	Uncultured *Cystobacter* sp. clone 4.08 (GQ183416) (Myxococcales; Cystobacterineae;Cystobacteraceae)	99	**20.6-48**	*Desulfovibrio putealis* (AY574979)	92
			20.6-49	*Desulfovibrio putealis* (AY574979)	93
			20.6-50	*Desulfovibrio fairfieldensis* (U42221)	84
			20.6-82	Uncultured Desulfobulbaceae bacterium clone TDNP_USbc97_108_1_6 (FJ516880)	91
			20.6-97	Uncultured Desulfobacteraceae	96

14.0-71	Uncultured Delta proteobacterium clone sw-xj259 (GQ302548)	87		bacterium clone TDNP_USbc97_107_1_5 (FJ516879)	
14.0-76	Uncultured *Geobacter* sp. clone D12_30 (EU266834)	93			
14.0-86	Uncultured Desulfobacteraceae bacterium clone TDNP_USbc97_21_4_87	95			

*The most closely related sequence is the name of the data base-deposited sequence with the highest similarity score as obtained by blastn searches and similarity (%) indicates how closely similar our cloned SR sequence is to that sequence.

CHAPTER 5

CONCLUSION

The major purpose of this study was to elucidate the Hg methylation mechanisms by linking potential methylation rates with characterized microbes which may be involved in methylation processes. Freshwater bodies such as lakes are much smaller and more enclosed water bodies than the open ocean, and therefore are more sensitive to atmospheric deposition of Hg and MeHg contamination as a result of microbial methylation. This is especially true for most isolated lakes, for example, in the Adirondack region (Driscoll et al., 2007). As a highly evolved forest peatland lake in the Adirondack Mountains, Sunday Lake has been exposed to Hg solely via atmospheric deposition and has low concentrations of total Hg detected in the wetland. MeHg levels in fish, however, are significantly elevated. After investigation of THg and MeHg concentrations, and determining potential methylation rates in various habitats, floating *Sphagnum* moss mats near the lake water front were identified as the most important site for total Hg and MeHg accumulation and for microbial methylation. By incubation with specific metabolic inhibitors and stimulators, SRB were confirmed as the principal Hg methylators in the *Sphagnum* mats.

Studies were then focused on the molecular characterization on samples from the floating *Sphagnum* moss mats. Although preliminary sequencing attempt of 16S rRNA genes by cloning with a universal primer set (total 144 clones) failed to detect either SRB or IRB in soil samples taken from a riparian site of a creek that drained into the lake (Data not shown), sequencing of *dsrB* genes in the floating moss mats demonstrated that

the SRB community was quite diverse. The community as characterized by *dsrB* was dominated by novel SRB groups, *Desulfobulbus elongatus*-like bacteria, and *Syntrophobacter*-like bacteria (Fig. 2.3). When 16S rRNA genes of extracted DNA from the same moss mats were characterized by nested PCR and DGGE, *Syntrophobacter*-like bacteria represented 58% of sequenced DGGE bands. The difference of SRB community composition profiles characterized by clone libraries of *dsrB* genes and by 16S rRNA genes obtained with PCR-DGGE, was most likely due to differences in the targeted sequences that were dictated by the employed PCR primers. However, both approaches showed a dominance of *Syntrophobacter* spp. in the *Sphagnum* moss mats. This conclusion was supported by MPN enrichment results in which propionate-oxidizing SRB (e.g., *Syntrophobacter* spp.) accounted for almost a 1/3 of the SRB counts, and by the detection of 16S rRNA genes with a *Syntrophobacter*-specific 16S rRNA primer set in the MPN enrichments. A previous study by Loy et al. (2004) reported the frequent detection of the family "*Syntrophobacteraceae*" in a similar peatland covered by mosses in Germany. PCR-DGGE analysis of *dsrB* from my MPN enrichments clearly exhibited the dominance of *Desulfovibrio* spp. in the *Sphagnum* mat. Additionally, PCR detection of *mcr* genes from extracted cDNA from the mats showed the presence and activity of methanogens in the moss mats. Together, these findings led me to hypothesize that syntrophy suggested by the presence of *Syntrophobacter* spp., a group of classic syntrophs associated methanogen (Boone & Bryant, 1980), was likely active in the *Sphagnum* mosses and probably played an important role in carbon recycling and Hg methylation.

This hypothesis was tested with pure strains including three *Syntrophobacter* spp., a methanogen, *Methanospirillum hungatei*, and two H_2-producing and/or utilizing *Desulfovibrio desulfuricans* ND132 and *Desulfovibrio africanus*, known Hg methylating SRB. *Syntrophobacter wolinii* and its association with *M. hungatei* had low Hg methylating activities. Association of *Desulfovibrio* spp. with *M. hungatei* in sulfate-free lactate medium stimulated MeHg synthesis by 2.1-19.4 fold relative to the monocultures of *Desulfovibrio* spp., suggesting that this syntrophic pathway could contribute significantly to MeHg synthesis in sulfate-deplete environments.

"Classic" syntrophy generally refers to the association of a methanogen with *Syntrophobacter* sp. (Boone & Bryant, 1980) or the association of a methanogen with *Desulfovibrio* sp. (Bryant et al., 1977). The association of *Syntrophobacter sulfatireducens* with *Desulfovibrio* spp. which stimulated Hg methylation in a propionate sulfate medium was accidentally discovered in a preliminary Hg methylation experiment in which a sulfate-free propionate medium for coculture was inoculated with an unwashed *Desulfovibrio* pre-grown culture by the carryover of sulfate. The stimulation of Hg methylation by the syntrophy of *S. sulfatireducens* with *D. desulfuricans* ND132 or *D. africanus* DSM 2603 was observed in propionate medium amended with 0.39 to 19.71 mM sulfate. In co-culture medium with 3.94 mM of sulfate, the *Syntrophobacter-Desulfovibrio* coculture significantly stimulated Hg methylation, by 1.3 to 2.0 fold, as compared to methylation by monocultures of ND132 or DSM 2603. Flow cytometry analysis showed that growth of *Desulfovibrio* spp. within the coculture increased 3.3 to 4.3 times in five days, while monoculture of *Desulfovibrio* spp. failed to grow. It seems that the stimulation of Hg methylation by the syntrophy was mostly due to the activities

of the Hg methylating *Desulfovibrio* spp., that were stimulated by the syntrophic association. All the evidence insinuates that this syntrophy could be important for MeHg production in natural environments when propionate and sulfate are available to Hg methylating *Desulfovibrio* spp. To further elucidate the mechanism of this syntrophy, future studies on the metabolic pathways of propionate utiliziation by the cocultures, electron transfer pathways and activities of membrane associated hydrogenases and dehydrogenases, and gene regulation relating to these processes in the two partners of the coculture are warranted. To summarize, syntrophy could stimulate MeHg synthesis by either co-growth of *Desulfovibrio* spp. with methanogens in sulfate-free environments, or by co-growth of *Desulfovibrio* with *Syntrophobacter* spp. in sulfate-limited environments where sources of energy and carbon are limiting.

The South River in VA downstream of Waynesboro has been contaminated by industrial Hg source from an old DuPont facility for decades. Long term adaptation to high concentrations of THg and MeHg in benthic sediments might have shifted the microbial community structure, and likely increased the diversity and abundance of Hg resistant bacteria. Therefore, highly contaminated areas such as the South River may have different characteristics regarding microbial Hg methylation and demethylation in comparison with relatively pristine ecosystems, such as Sunday Lake. When the methylation rates in the two sites are compared, it is obvious that rates in South River sediments (1.0-10.2% day^{-1}) were significantly higher than those from the floating moss mats in Sunday Lake (up to 0.4 % day^{-1}). By sequencing cDNA, this study showed the coexistence of active Hg methylators of SRB and IRB in South River sediments.

Metabolic inhibition and stimulation experiments further indicated that both sulfate reduction and iron reduction activities were coupling with Hg methylation.

These findings may support future management of the Hg pollution in the South River. For instance, iron amendments as a strategy to inhibit Hg methylation of SRB should be used with caution because increased Fe(III) concentrations by either direct addition or by the oxidation of Fe(II) when added to sediments could greatly stimulate MeHg synthesis by IRB.

REFERENCES

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Bryant MP, Campbell LL, Reddy CA & Crabill MR (1977) Growth of *desulfovibrio* in lactate or ethanol media low in sulfate in association with H_2-utilizing methanogenic bacteria. *Appl Environ Microbiol* **33**: 1162-1169.

Driscoll CT, Han YJ, Chen CY, Evers DC, Lambert KF, Holsen TM, Kamman NC & Munson RK (2007) Mercury contamination in forest and freshwater ecosystems in the Northeastern United States. *Bioscience* **57**: 17-28.

Loy A, Kusel K, Lehner A, Drake HL & Wagner M (2004) Microarray and functional gene analyses of sulfate-reducing prokaryotes in low-sulfate, acidic fens reveal cooccurrence of recognized genera and novel lineages. *Appl Environ Microbiol* **70**: 6998-7009.

APPENDIX A

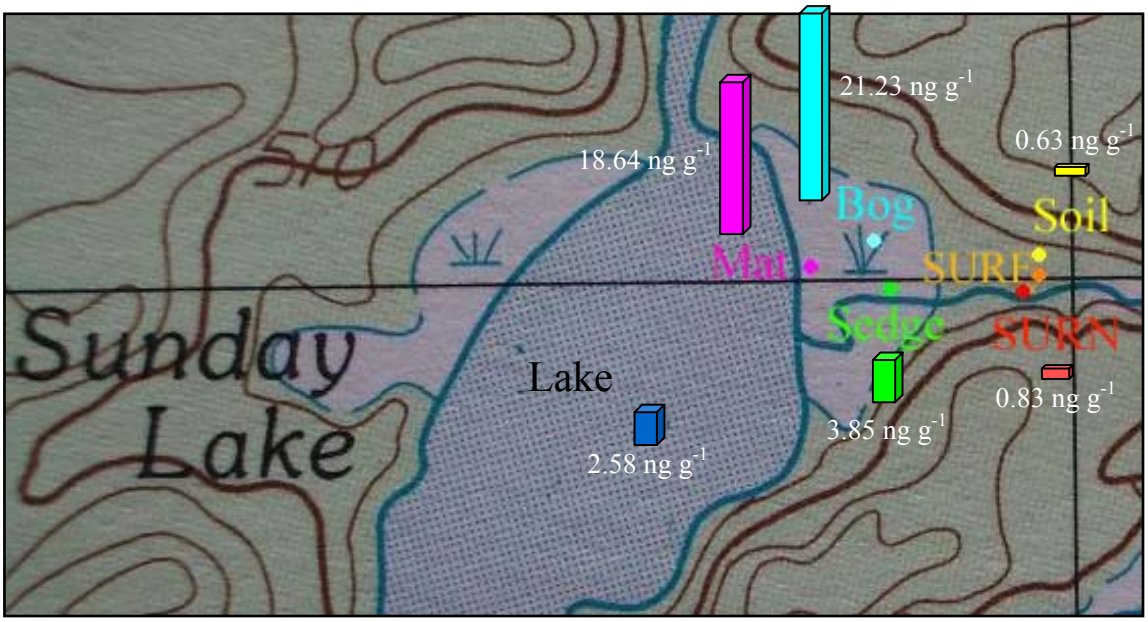

Fig. A-1. Sampling sites in Sunday Lake, Adirondack Mountains, New York. **Soil:** Upland soil; **SURF:** Sunday Lake Riparian (site) Far (from water); **SURN:** Sunday Lake Riparian (site) Near (water); **Sedge:** Sedge proximal to lake; **Mat:** Floating moss mat near lake waterfront; **Bog:** moss bog to lake.

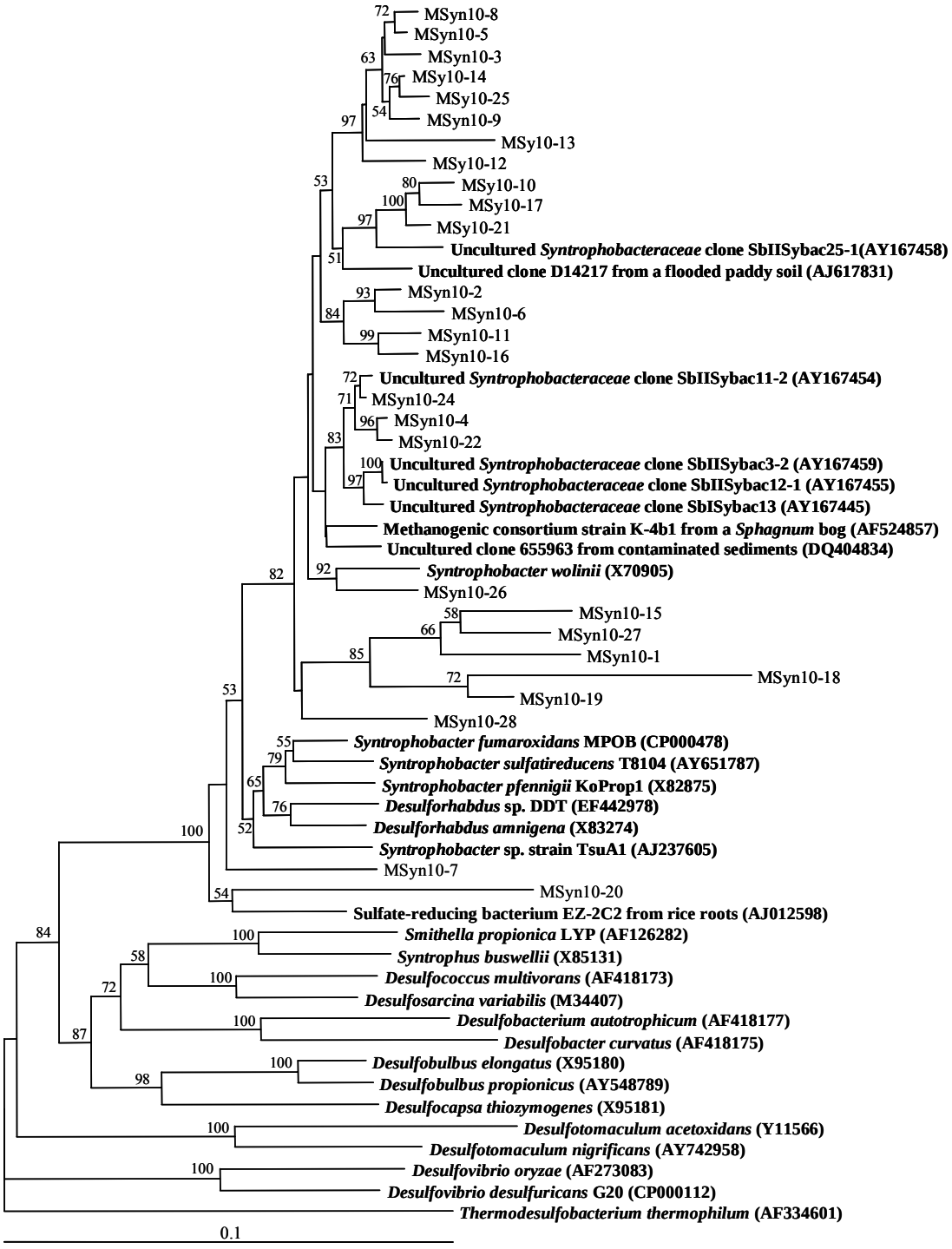

Fig.A-2. Neighbor-joining tree of *Syntrophobacteraceae*-like clone 16S rRNA gene sequences from the enriched MAT communities (10^{-1} dilution cultures) with propionate-sulfate medium, Sunday Lake. Bootstrap values are shown at branch points (%). The scale bar indicated 10% sequence difference.

APPENDIX B

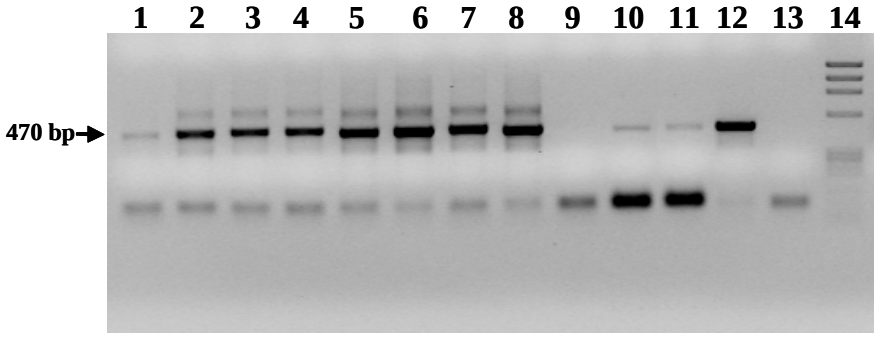

Fig. B-1. Methyl coenzyme-M reductase (*mcrA*) genes in the native samples of Sunday Lake wetland. Lanes marked as: 1. SURN-Top; 2. SURN-Bottom; 3. SURF-Top; 4. SURF-Bottom; 5. BOG-Top; 6. BOG-Bottom; 7. Sedge; 8. MAT; 9. Upland soil; 10. MAT-cDNA; 11. Cheesequake (saltmarsh mat); 12. *Methanospirillum hungatei* (positive control); 13. Blank; 14. Size marker – phiX 174 Hae III.

APPENDIX C

Table C-1. A list of clones with 16S rRNA sequences indicating their affiliation with sequences in databases for the clone library of RRD 10.0, the South River in May 2008

Clones	Most similar organism (accession number)	Similarity (%)	Clones	Most similar organism (accession number)	Similarity (%)
10.0-1	Uncultured Beta proteobacterium clone DFAW-011 (AY823958)	99	**10.0-50**	Uncultured Cyanobacterium clone GASP-WA2W1_H06 (EF072789)	97
10.0-2	Beta proteobacterium HF007 (AB376664)	97	**10.0-51**	*Dechloromonas* sp. EMB 269 (DQ413167)(Betaproteobacteria; Rhodocyclales; Rhodocyclaceae)	97
10.0-3	Uncultured Gamma proteobacterium clone SM1E05 (AF445683)	90	**10.0-52**	Uncultured Bacteroidetes bacterium clone AKYH767 (AY921784)	97
10.0-4	Uncultured Betaproteobacteria bacterium clone QEDN10DF12 (CU926928)	97	**10.0-53**	Uncultured Rickettsiales bacterium clone EV221H2111601SAH71 (DQ223223) (Alphaproteobacteria; Rickettsiales)	99
10.0-5	Uncultured Acidobacteria bacterium clone QEDQ2BG09 (CU923031)	98			
10.0-6	Uncultured Alphaproteobacteria bacterium clone QEDV2BG10 (CU919468)	98	**10.0-54**	Uncultured Gamma proteobacterium clone AMEG9 (AM935343)	93
10.0-7	Uncultured Chloroflexi bacterium clone TDNP_Wbc97_223_1_102 (FJ517056) (Green non-sulfur bacteria)	93	**10.0-55**	Uncultured Cyanobacterium clone AS-39-6 (FJ866619)	91
			10.0-56	Uncultured *Geobacter* sp. clone LH-27 (AB265850)	96
10.0-8	Uncultured *Mesorhizobium* sp. clone AMLH9 (AM934992) (Alphaproteobacteria; Rhizobiales; Phyllobacteriaceae)	91	**10.0-57**	Uncultured Planctomycetes bacterium clone QEDN7DC12 (CU926205)	98
			10.0-58	Uncultured Bacteroidetes bacterium clone Skagen107 (DQ640726)	98
10.0-9	*Chroococcidiopsis* sp. PCC 6712 strain PCC 6712 (AJ344557) (Cyanobacteria; Pleurocapsales)	91	**10.0-59**	Uncultured Chloroflexi bacterium clone CARS17 (FJ902424) (Green non-sulfur bacteria)	97
10.0-10	Uncultured Gamma proteobacterium clone UH-59 (AB265953)	98	**10.0-60**	Uncultured Beta proteobacteria bacterium clone QEDS2DC04 (CU921407)	98
10.0-11	Uncultured *Flavobacterium* sp. clone JG37-AG-150 (AJ519404) (Bacteroidetes; Flavobacteria; Flavobacteriales;Flavobacteriaceae)	98	**10.0-61**	Uncultured Alphaproteobacteria bacterium clone QEDN3AG01 (CU926948)	98
10.0-12	Uncultured Planctomycetes bacterium clone QEDV2CD12 (CU919129)	97	**10.0-62**	Uncultured Gamma proteobacterium clone JG37-AG-94 (AJ518786)	93
10.0-13	Uncultured Gamma proteobacterium clone NABOS_SSPbact4 (EU544828)	91	**10.0-63**	*Methylomonas* sp. LW21 (AF150800) (Gammaproteobacteria; Methylococcales; Methylococcaceae)	97
10.0-14	Uncultured Hyphomicrobiaceae bacterium clone Amb_16S_929 (EF018645) (Alphaproteobacteria; Rhizobiales)	97	**10.0-64**	*Methylobacter tundripaludum* type strain SV96T (AJ414655)	97
10.0-15	Uncultured Chloroflexi bacterium clone AKYG651 (AY921737) (Green non-sulfur bacteria)	97	**10.0-65**	Uncultured Firmicutes bacterium clone RB_13f (EF123527) (Phylum)	92
			10.0-66	Uncultured type I methanotroph clone site1-39 (EF101324) (Gammaproteobacteria; Methylococcales; Methylococcaceae)	99
10.0-16	Uncultured Alphaproteobacteria bacterium clone QEDV3CG10 (CU919640)	97			
10.0-17	Uncultured Planctomycetaceae bacterium clone P7 (FJ788655)	91	**10.0-67**	Uncultured Planctomycetes bacterium clone QEDN7DC12 (CU926205)	97
10.0-18	Uncultured Rhodobacteraceae bacterium clone TDNP_Bbc97_260_1_68	97	**10.0-68**	Uncultured *Acidobacterium* sp. clone Dolo_26 (AB257649)	97

	(FJ516785) (Alphaproteobacteria; Rhodobacterales)			(Acidobacteria; Acidobacteriales; Acidobacteriaceae)	
10.0-19	Uncultured Burkholderiales bacterium clone GC12m-3-32 (EU641055) (Betaproteobacteria)	98	**10.0-69**	Uncultured Beta proteobacterium clone Elb252. (AJ421937)	98
10.0-20	Uncultured Firmicutes (Phylum) bacterium clone GASP-KB1S1_F03 (EU297503)	86	**10.0-70**	Uncultured Betaproteobacteria bacterium clone QEDS2DC04 (CU921407)	99
10.0-21	*Methylobacter tundripaludum* type strain SV96T (AJ414655) (Gammaproteobacteria; Methylococcales; Methylococcaceae)	97	**10.0-71**	*Methylobacter tundripaludum* type strain SV96T (AJ414655)	95
10.0-22	Uncultured Betaproteobacteria bacterium clone QEDR1BH03 (CU922158)	97	**10.0-72**	Uncultured bacterium clone GASP-KC2S3_B11 (EU299776)	93
10.0-23	Uncultured Gamma proteobacterium clone UH-59 (AB265953)	90	**10.0-73**	Uncultured sulfur-oxidizing symbiont bacterium clone AMCA4 (Gamma proteobacteria) (AM935643)	94
10.0-24	Uncultured Clostridia bacterium clone NS2_31G12 (EU722117) (Firmicutes)	91	**10.0-74**	Uncultured Rhodobacteraceae bacterium clone GC12m-3-30 (EU641039) (Alpha-proteobacteria)	98
10.0-25	Alpha proteobacterium SK200a-9 (AB231946)	95	**10.0-75**	*Rhodobacter* sp. MOLA 438 (AM990705)(Alpha proteobacteria; Rhodobacterales; Rhodobacteraceae)	86
10.0-26	Uncultured Moraxellaceae bacterium clone MS-K43 (FJ949177) (Gammaproteobacteria; Pseudomonadales)	92	**10.0-76**	Uncultured Clostridiales bacterium clone RsW02-066 (AB198539) (Firmicutes; Clostridia)	94
			10.0-77	Uncultured *Methylobacter* pAMC419 (AF150775)	94
10.0-27	*Methylobacter tundripaludum* type strain SV96T (AJ414655)	92	**10.0-78**	Uncultured Verrucomicrobia bacterium clone MVP-78 (DQ676384) (Phylum)	98
10.0-28	Uncultured *Geobacter* sp. clone D12_30 (EU266834) (Deltaproteobacteria; Desulfuromonadales;Geobacteraceae)	95	**10.0-79**	Uncultured *Methylobacter* pAMC419 (AF150775)	95
10.0-29	Uncultured Spirochaetes bacterium clone QEDN2BE08 (CU925939) (Phylum)	95	**10.0-80**	Uncultured Alpha proteobacterium clone JG34-KF-245 (AJ532704)	99
10.0-30	Uncultured *Pseudomonas* sp. clone 2.28 (GQ183242) (Gammaproteobacteria; Pseudomonadales;Pseudomonadaceae)	95	**10.0-81**	Uncultured Bacteroidetes bacterium clone CM3D12 (AM936269)	96
10.0-31	Uncultured bacterium clone LaC15H77 in river sediment (EF667745)	94	**10.0-82**	Uncultured Cyanobacterium clone AM-20-36 (FJ866617)	99
10.0-32	Uncultured Betaproteobacteria bacterium clone QEDN10DF01 (CU925363)	97	**10.0-83**	Uncultured Beta proteobacterium clone MVP-58 (DQ676380)	97
10.0-33	Uncultured Gammaproteobacteria bacterium clone QEDN4CH07 (CU924906)	94	**10.0-84**	Uncultured bacterium clone KS-360 (EU809687)	98
			10.0-85	Uncultured Gamma proteobacterium clone AKYG1795 (AY921681)	96
10.0-34	Uncultured Gammaproteobacterium clone GC12m-1-64 (EU640647)	99	**10.0-86**	Uncultured Bacteroidetes bacterium clone TDNP_USbc97_208_1_82 (FJ516920)	94
10.0-35	Uncultured Beta proteobacterium clone AS6 (EU283348)	97	**10.0-87**	*Pseudomonas* sp. SMT-9 strain SMT-9 (AM689953)	98
10.0-36	Uncultured Cyanobacterium clone SepB-17 (EF032663)	96	**10.0-88**	Uncultured Planctomycete clone Amb_16S_966 (EF018677)	97
10.0-37	*Methylobacter tundripaludum* type strain SV96T (AJ414655)	98	**10.0-89**	Uncultured Bacteroidetes bacterium clone CM3D12	80
10.0-38	*Nitrospira* sp. clone o14 (AJ224044) (Nitrospirae; Nitrospirales; Nitrospiraceae)	98	**10.0-90**	Uncultured Cyanobacterium clone AS-39-6 (FJ866619)	92
10.0-39	Uncultured Beta proteobacterium clone B12-27_GoMY (AB476705)	94	**10.0-91**	Uncultured Planctomycetacia bacterium clone GASP-MA3S3_G03 (EF663623)	91
10.0-40	Uncultured *Syntrophobacter* sp. clone	94			

	B02 (EU888826) (Deltaproteobacteria)		**10.0-92**	Uncultured Planctomycetacia bacterium clone GASP-WC1S1_B07 (EF074378)	93
10.0-41	Uncultured Betaproteobacterium clone RBE2CI-23 (EF111143)	96			
10.0-42	Uncultured Cyanobacterium clone AM-20-36 (FJ866617)	99	**10.0-93**	Uncultured Burkholderiales bacterium clone GC12m-2-91 (EU641712) (Betaproteobacteria)	98
10.0-43	Uncultured Cyanobacterium clone AS-39-6 (FJ866619)	92	**10.0-94**	*Methylocystis* sp. H9a strain H9a (AJ458490) (Alphaproteobacteria; Rhizobiales; Methylocystaceae)	99
10.0-44	Uncultured *Methylobacter* sp. clone GASP-0KA-565-E11 (EU043582)	98			
10.0-45	Uncultured Alphaproteobacteria bacterium clone QEDN6DA01 (CU927456)	98	**10.0-95**	Uncultured Alpha proteobacterium clone HB125 (EF648106)	95
			10.0-96	Uncultured Cyanobacterium clone SepB-17 (EF032663)	96
10.0-46	*Methylobacter tundripaludum* type strain SV96T (AJ414655)	98			
10.0-47	Uncultured Betaproteobacterium clone 61-05-22c423 (DQ316837)	95			
10.0-48	Uncultured Comamonadaceae bacterium clone GC12m-3-24(EU641089) (Betaproteobacteria; Burkholderiale)	86			
10.0-49	Uncultured *Geobacter* sp. clone: LH-23 (AB265846)	95			

Table C-2. A list of clones with 16S rRNA sequences indicating their affiliation with sequences in databases for the clone library of RRD 14.0, the South River, VA in May 2008

Clones	Most similar organism (accession number)	Similarity (%)	Clones	Most similar organism (accession number)	Similarity (%)
14.0-1	Uncultured Planctomycetacia bacterium clone GASP-MB3W1_H05 (EF665797) (Planctomycetes)	92	**14.0-50**	Uncultured Planctomycete clone B2 (AY266448) (Planctomycetacia; Planctomycetales)	92
14.0-2	Uncultured *Methylobacter* sp. clone GASP-0KA-565-E11 (EU043582) (Gammaproteobacteria; Methylococcales;Methylococcaceae)	94	**14.0-51**	Uncultured Alphaproteobacteria bacterium clone QEDS1DF06 (CU921259)	92
			14.0-52	Uncultured Gamma proteobacterium clone 63 EDB3 (AM882582)	90
14.0-3	Uncultured Comamonadaceae bacterium clone GC6m-3-56 (EU641904) (Betaproteobacteria; Burkholderiales;Comamonadaceae)	98	**14.0-53**	Uncultured Planctomycetaceae bacterium clone A06-12D (FJ542876) (Planctomycetes; Planctomycetacia; Planctomycetales; Planctomycetaceae)	94
14.0-4	Uncultured Bacteroidetes bacterium clone IRD18F07 (AY947957)	97	**14.0-54**	Uncultured Delta proteobacterium clone sw-xj259 (GQ302548)	96
14.0-5	Uncultured Oceanospirillales bacterium clone TDNP_USbc97_159_1_28 (FJ516900) (Gammaproteobacteria)	99	**14.0-55**	Uncultured Burkholderiales bacterium clone GC12m-4-53 (EU641130) (Betaproteobacteria; Burkholderiales)	98
			14.0-56	Uncultured *Cystobacter* sp. clone 4.08 (GQ183416) (Deltaproteobacteria; Myxococcales; Cystobacterineae; Cystobacteraceae)	99
14.0-6	Uncultured Beta proteobacterium clone Elb252 (AJ421937)	98			
14.0-7	Uncultured Polyangiaceae bacterium clone GASP-MA2S1_D02 (EF662854) (Deltaproteobacteria; Myxococcales; Sorangiineae)	96	**14.0-57**	Uncultured Acidobacteria bacterium clone lhad15 (DQ648914)	98
			14.0-58	Uncultured Gamma proteobacterium clone RBE2CI-96 (EF111182)	99
14.0-8	*Methylobacter tundripaludum* strain SV96T (AJ414655) (Gammaproteobacteria; Methylococcales; Methylococcaceae)	98	**14.0-59**	Uncultured *Alkanindiges* sp. clone REV_P1PII_1E (FJ933440) (Gammaproteobacteria; Pseudomonadales; Moraxellaceae)	95
14.0-9	*Methylomonas* sp. LW21 (AF150800) (Gammaproteobacteria; Methylococcales;Methylococcaceae)	98	**14.0-60**	Uncultured Bacteroidetes bacterium clone AS35 (EU283365)	96
			14.0-61	Uncultured type I methanotroph clone site1-5 (EF101317) (Gammaproteobacteria; Methylococcales; Methylococcaceae)	95
14.0-10	Uncultured Unclassified bacterium clone QEDN8DG02 (CU924929)	96			
14.0-11	Uncultured Desulfuromonadales bacterium clone AMBH11 (AM935776) (Deltaproteobacteria; Desulfuromonadales)	85	**14.0-62**	Uncultured Beta proteobacterium clone Elb252 (AJ421937)	98
			14.0-63	Uncultured *Petrimonas* sp. clone CL5.H48 (FM176310)(Bacteroidetes; Bacteroidia; Bacteroidales; Porphyromonadaceae)	90
14.0-12	*Methylobacter tundripaludum* strain SV96T (AJ414655) (Gammaproteobacteria; Methylococcales; Methylococcaceae)	98	**14.0-64**	Uncultured Acidobacteria bacterium clone QEDQ2BG09 (CU923031)	89
			14.0-65	Uncultured Burkholderiales bacterium clone Plot4-D11 (EU449562) (Betaproteobacteria)	96
14.0-13	Uncultured Fibrobacteres bacterium clone LiUU-9-330 (AY509521) (Phylum)	87	**14.0-66**	Uncultured Planctomycete clone GASP-MA3W1_B03 (EF663660) (Planctomycetes; Planctomycetacia; Planctomycetales)	92
14.0-14	Uncultured Betaproteobacteria bacterium clone QEDR1CC01 (CU922458)	98			

14.0-15	*Methylibium* sp. YIM 61602 (FJ615290) (Betaproteobacteria; Burkholderiales)	91	**14.0-67**	Uncultured Bacteroidetes bacterium clone QEDQ2AB06 (CU923649)	91
14.0-16	Uncultured Delta proteobacterium clone AKYH836 (AY921872)	91	**14.0-68**	Uncultured Bacteroidetes bacterium clone IRD18G07 (AY947969)	97
14.0-17	Uncultured methanotrophic proteobacterium clone Littoralsite2-Type1-7 (EF587747) (Gammaproteobacteria)	95	**14.0-69**	Uncultured Beta proteobacterium clone R2E (EU499556)	97
14.0-18	Uncultured Desulfobacteraceae bacterium clone TDNP_USbc97_21_4_87 (FJ516927) (Deltaproteobacteria; Desulfobacterales; Desulfobacteraceae)	95	**14.0-70**	Uncultured Planctomycete clone DEL34 (AJ616275) (Planctomycetes; Planctomycetacia; Planctomycetales)	97
14.0-19	Uncultured Betaproteobacteria bacterium clone QEDS3DG02 (CU921251)	97	**14.0-71**	Uncultured Delta proteobacterium clone sw-xj259 (GQ302548) (Deltaproteobacteria)	87
14.0-20	*Crenothrix polyspora* clone 6 (DQ295898) (Gammaproteobacteria; Methylococcales; Crenotrichaceae	95	**14.0-72**	Unidentified Cytophagales/green sulfur bacterium OPB56 clone MFC-EB28 (AJ630296)	96
14.0-21	Uncultured Sphingomonadaceae bacterium clone 113-Cadma (AB478689) (Alphaproteobacteria; Sphingomonadales; Sphingomonadaceae)	98	**14.0-73**	*Methylobacter tundripaludum* strain SV96T (AJ414655) (Gammaproteobacteria; Methylococcales; Methylococcaceae)	94
14.0-22	Uncultured Gamma proteobacterium clone VERDEA80 (FJ902650)	98	**14.0-74**	Uncultured *Methylobacter* pAMC419 (AF150775) (Gammaproteobacteria; Methylococcales; Methylococcaceae)	98
14.0-23	Uncultured Beta proteobacterium clone TDNP_USbc97_13_7_13 (FJ516892)	97	**14.0-75**	Uncultured Bacteroidetes bacterium clone AMKD8 (AM935033)	90
14.0-24	Uncultured Chloroflexi bacterium gene clone: B11-Capima (AB479051) (Green non-sulfur bacteria)	89	**14.0-76**	Uncultured *Geobacter* sp. clone D12_30 (EU266834) (Deltaproteobacteria; Desulfuromonadales; Geobacteraceae)	93
14.0-25	Uncultured Planctomycete clone Z273MB72 (FJ484707) (Planctomycetes; Planctomycetacia; Planctomycetales)	93	**14.0-77**	Uncultured Planctomycete clone DEL30 (AJ616272) (Planctomycetes; Planctomycetacia; Planctomycetales)	95
14.0-26	Uncultured Planctomycete clone AKYG1739 (AY921845) (Planctomycetes; Planctomycetacia; Planctomycetales)	88	**14.0-78**	Uncultured Gamma proteobacterium clone GC12m-1-64 (EU640647)	99
14.0-27	Uncultured Cytophagales bacterium clone TDNP_Wbc97_203_1_91 (FJ517047) (Bacteroidetes; Sphingobacteria; Sphingobacteriales)	97	**14.0-79**	Beta proteobacterium RG-4 (AY561571)	96
14.0-28	Uncultured bacterium clone nbw1179g04c1 (GQ079436)	98	**14.0-80**	Uncultured Acidobacterium sp. clone Z195MBM55 (FJ485444) (Acidobacteria; Acidobacteriales; Acidobacteriaceae)	94
14.0-29	Uncultured *Pseudomonas* sp. clone 2.28 (GQ183242) (Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae)	99	**14.0-81**	Uncultured Gamma proteobacterium clone GC12m-4-21 in nearshore bacterioplankton communities of Lake Michigan	99
			14.0-82	Uncultured Beta proteobacterium (AB276369)	97
			14.0-83	Uncultured Cyanobacterium clone A206 (DQ181671)	98
			14.0-84	*Flavobacterium* sp. BF86 strain BF86 (AM934679) (Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae)	96
			14.0-85	Uncultured Alpha proteobacterium clone IRD18A04 (AY947895)	90
			14.0-86	Uncultured Desulfobacteraceae	95

14.0-30	Uncultured Acidobacteria bacterium clone AMKF9 (AM935051)	98		bacterium clone TDNP_USbc97_21_4_87 (FJ516927) (Deltaproteobacteria; Desulfobacterales)	
14.0-31	Uncultured Gamma proteobacterium clone GC12m-4-21(EU641167)	94			
14.0-32	Uncultured Gamma proteobacterium clone GC12m-4-21(EU641167)	99	**14.0-87**	Uncultured *Methylobacter* sp. clone GASP-0KA-565-E11 (EU043582)(Gammaproteobacteria)	97
14.0-33	Uncultured Flexibacteraceae bacterium clone LiUU-9-122 (AY509312) (Bacteroidetes; Sphingobacteria; Sphingobacteriales)	89	**14.0-88**	Uncultured Acidobacteriaceae bacterium clone AMGG11 (AM935426)	94
			14.0-89	Uncultured Actinobacterium clone GASP-MB3S2_B07 (EF665649) (Actinobacteria)	98
14.0-34	Uncultured Beta proteobacterium clone PIB-31 (AM849442)	99			
14.0-35	Uncultured Betaproteobacteria bacterium clone QEDQ1AC03 (CU922963)	91	**14.0-90**	Uncultured Planctomycetaceae bacterium clone AMPF6 (AM935123)	95
			14.0-91	*Phormidium autumnale* Arct-Ph5 (DQ493873) (Cyanobacteria; Oscillatoriales)	98
14.0-36	Uncultured Beta proteobacterium clone DFAW-034(AY823978)	96			
14.0-37	Uncultured Delta proteobacterium clone HF70_10I02 (EU361323)	91	**14.0-92**	Uncultured Gamma proteobacterium clone M0018_032 (EF071136)	93
14.0-38	Uncultured Planctomycetes bacterium clone QEDN7DC12 (CU926205)	98	**14.0-93**	Uncultured *Acidobacterium* sp. clone sw-xj128 (GQ302578)	94
			14.0-94	*Lysobacter brunescens* (AB161360) (Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae)	98
14.0-39	Uncultured microorganism clone A0 (EU841050)	99			
14.0-40	Uncultured Chlorobi bacterium clone QEDR2DA04 (CU922236)	88	**14.0-95**	Uncultured Acidobacteria bacterium clone QEDQ2BG09 (CU923031)	97
14.0-41	Uncultured Planctomycetes bacterium clone QEDN7DC12 (CU926205)	88	**14.0-96**	Uncultured bacterium clone Roi_L1-E8-T7 (FN296920)	95
14.0-42	Uncultured bacterium clone AMKG2 (AM935056)	98			
14.0-43	Uncultured Betaproteobacteria bacterium clone QEDS2DC04 (CU921407)	99			
14.0-44	Uncultured microorganism clone A0 (EU841050)	99			
14.0-45	Uncultured microorganism clone A0 (EU841050)	99			
14.0-46	Uncultured bacterium clone rRNA_oxic_4 (AM949476)	99			
14.0-47	*Lentisphaera araneosa* strain HTCC2160 (AY390429) (Lentisphaerae; Lentisphaerales; Lentisphaeraceae)	84			
14.0-48	Uncultured Acidobacteria bacterium clone i5 (DQ453805)	95			
14.0-49	*Roseococcus suduntuyensis* strain SHET (EU012448) (Alphaproteobacteria; Rhodospirillales; Acetobacteraceae)	94			

Table C-3. A list of clones with 16S rRNA sequences indicating their affiliation with sequences in databases for the clone library of RRD 10.0, the South River, VA in August 2008

Clones	Most similar organism (accession number)	Similarity (%)	Clones	Most similar organism (accession number)	Similarity (%)
10.0-1	Uncultured Beta proteobacterium clone 1233 (EF109927)	93	**10.0-50**	Uncultured Beta proteobacterium clone Elb252 (AJ421937)	98
10.0-2	Uncultured Cytophagales bacterium clone TDNP_USbc97_199_1_51 (FJ516918) (Bacteroidetes; Sphingobacteria;Sphingobacteriales)	91	**10.0-51**	Uncultured Methylobacter sp. clone GASP-0KA-565-E11 (EU043582)	98
			10.0-52	*Rhizobium daejeonense* strain L22 (DQ089696) (Alphaproteobacteria; Rhizobiales; Rhizobiaceae)	98
10.0-3	Uncultured Bacteroidetes bacterium clone A840 (EU283476)	97	**10.0-53**	Uncultured Planctomycetes bacterium clone QEDN7AD10 (CU925538)	98
10.0-4	Uncultured Gamma proteobacterium clone E4-00yk7 (EU810927)	99	**10.0-54**	Uncultured Cyanobacterium clone TAF-A34 (AY038729)	96
10.0-5	*Desulfovibrio putealis* (AY574979)	96	**10.0-55**	Uncultured Acidobacteria bacterium clone 7025P4B47 (EF562076)	97
10.0-6	Uncultured Verrucomicrobia bacterium clone g3 (EU979012) (Phylum)	95	**10.0-56**	Uncultured Actinobacterium clone TS-45-11 (GQ406194)	98
10.0-7	Uncultured Bacteroidetes bacterium clone Skagenf93 (DQ640676)	95	**10.0-57**	Uncultured Beta proteobacterium clone Elb252	99
10.0-8	Uncultured Beta proteobacterium clone 187 (AB252908)	98	**10.0-58**	Uncultured Gamma proteobacterium clone GC12m-4-21 (EU641167)	99
10.0-9	*Bacillus* sp. TSWCSN14 (AM888183) (Firmicutes; Bacillales; Bacillaceae)	94	**10.0-59**	Uncultured Cyanobacterium clone CD204B01 (DQ200411)	93
			10.0-60	Uncultured Oceanospirillales bacterium clone GC12m-2-84 (EU641745) (Gammaproteobacteria)	97
10.0-10	*Clostridium* sp. enrichment culture clone MB3_7 (AM933653) Firmicutes; Clostridia; Clostridiales; Clostridiaceae)	96	**10.0-61**	Uncultured Flavobacterium sp. clone XZTSH72 (EU703375) (Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae)	96
10.0-11	Uncultured Planctomycetes bacterium clone QEDN7DC12 (CU926205)	97			
10.0-12	Uncultured Chloroflexi bacterium isolate OTU17/APA (AM902609) (Phylum)	97	**10.0-62**	Uncultured bacterium clone LaC15H92 (EF667798)	98
			10.0-63	*Clostridium* sp. enrichment culture clone MB3_7 (AM933653) (Firmicutes; Clostridia; Clostridiales; Clostridiaceae)	98
10.0-13	Uncultured Cyanobacterium clone AM-20-36 (FJ866617)	91			
10.0-14	Uncultured Verrucomicrobia bacterium clone g3 (EU979012) (Phylum)	94	**10.0-64**	Uncultured *Hyphomicrobium* sp. clone GASP-WA2W3_B04 (EF072981) (Alphaproteobacteria; Rhizobiales; Hyphomicrobiaceae)	98
10.0-15	Uncultured Planctomycete clone MVP-54 (DQ676352)	95			
10.0-16	Uncultured Beta proteobacterium clone Elb252 (AJ421937)	98	**10.0-65**	Uncultured Clostridiaceae bacterium clone HrhB49 (AM159266)	99
10.0-17	Uncultured *Acidobacterium* sp. clone Z4MB66 (FJ484865) (Acidobacteria; Acidobacteriales; Acidobacteriaceae)	95	**10.0-66**	Uncultured bacterium clone 1013-28-CG28 (AY532573)	99
			10.0-67	Uncultured Delta proteobacterium clone Z114MB93 (FJ485081)	93
10.0-18	Uncultured Betaproteobacteria bacterium clone QEDR1AF04 (CU922442)	98	**10.0-68**	Uncultured Xanthomonadaceae bacterium clone GC12m-3-93 (EU641060)(Gammaproteobacteria; Xanthomonadales)	98
10.0-19	Uncultured Bacteroidetes bacterium clone 033T7 (DQ110029)	95			
10.0-20	Uncultured Alphaproteobacteria bacterium clone QEDR2AG08	99	**10.0-69**	*Chondromyces apiculatus* strain BICC 8620 (DQ491072)	94

	(CU922101)			(Deltaproteobacteria; Myxococcales;	
10.0-21	*Methylocystis* sp. strain Ch22	96		Sorangiineae; Polyangiaceae)	
	(AJ458487) (Alphaproteobacteria;		**10.0-70**	Uncultured Planctomycetales	93
	Rhizobiales; Methylocystaceae)			bacterium	
10.0-22	Uncultured *Fusobacterium* sp. clone	91		cloneTDNP_USbc97_271_1_109	
	HrhB94 in rice roots			(FJ516944)	
	(AM159309) (Fusobacteria;		**10.0-71**	Uncultured Gamma proteobacterium	99
	Fusobacteriales; Fusobacteriaceae)			clone GC12m-4-21 (EU641167)	
10.0-23	Uncultured Alpha proteobacterium	97	**10.0-72**	Uncultured Alpha proteobacterium	87
	clone GASP-WB1W3_E12			clone RBE1CI-148 (EF111101)	
	(EF073787)		**10.0-73**	Uncultured Actinobacterium clone:	95
10.0-24	Uncultured Gamma proteobacterium	97		UH-26 (AB265920) (Phylum)	
	clone GC12m-4-21 (EU641167)		**10.0-74**	Uncultured Alpha proteobacteria	99
10.0-25	Uncultured Desulfobulbaceae	96		bacterium clone QEDS2AF01	
	bacterium clone			(CU921840)	
	TDNP_USbc97_108_1_6		**10.0-75**	*Dechloromonas hortensis* strain MA-1	98
	(FJ516880) (Deltaproteobacteria;			(AY277621) (Betaproteobacteria;	
	Desulfobacterales;Desulfobulbaceae)			Rhodocyclales; Rhodocyclaceae)	
10.0-26	*Clostridium* sp. enrichment culture	98	**10.0-76**	Uncultured Myxococcales bacterium	93
	clone MB3_7 (AM933653)			clone Plot4-2G07 (EU449593) (Delta	
	(Firmicutes; Clostridia; Clostridiales;			proteobacteria)	
	Clostridiaceae)		**10.0-77**	Uncultured *Methylobacter* sp. clone	97
10.0-27	Uncultured Alphaproteobacteria	99		GASP-0KA-565-E11	
	bacterium clone QEDR2AG08			(EU043582) (Gamma proteobacteria;	
	(CU922101)			Methylococcales; Methylococcaceae)	
10.0-28	Uncultured Acidobacteria bacterium	98	**10.0-78**	Uncultured Planctomycete clone	96
	clone CM3F06 (AM936249)			GASP-WA2W1_A07 (EF072800)	
10.0-29	Uncultured Cyanobacterium clone	91	**10.0-79**	Uncultured Bacteroidetes bacterium	99
	AM-20-36 (FJ866617)			clone MEsu06b11D9 (FJ828466)	
10.0-30	Uncultured Gamma proteobacterium	99	**10.0-80**	Uncultured Betaproteobacteria	96
	clone GC12m-4-21 (EU641167)			bacterium clone QEDS3DG02	
10.0-31	Uncultured Rhodocyclales bacterium	97		(CU921251)	
	clone 7025P4B88 (EF562112) (		**10.0-81**	Uncultured Delta proteobacterium	95
	Betaproteobacteria)			clone GASP-MA4S1_C10	
10.0-32	Uncultured Alphaproteobacteria	98		(EF663952)	
	bacterium clone QEDR2AG08		**10.0-82**	Uncultured Alpha proteobacterium	97
	(CU922101)			clone GASP-WA1S1_A01	
10.0-33	Uncultured Beta proteobacterium	99		(EF071996)	
	clone 233 (AB252904)		**10.0-83**	Uncultured Planctomycetes bacterium	96
10.0-34	Uncultured Acidobacteria bacterium	98		clone QEDN7CE10 (CU927466)	
	clone SS_LKC29_UB26 (AM180885)		**10.0-84**	No significant similarity	
10.0-35	Uncultured Planctomycetaceae	94	**10.0-85**	Uncultured Acidobacteriaceae	94
	bacterium clone AMPF6 (AM935123)			bacterium clone CMME10	
10.0-36	*Roseomonas vinaceus* strain CPCC	94		(AM935953) (Acidobacteria)	
	100056 (EF368368)		**10.0-86**	Uncultured Bacteroidetes bacterium	93
	(Alphaproteobacteria;			clone CB_10 (EF562552)	
	Rhodospirillales; Acetobacteraceae)		**10.0-87**	Uncultured Beta proteobacterium gene	98
10.0-37	Uncultured Alphaproteobacteria	99		clone 187 (AB252908)	
	bacterium clone QEDN10CF09		**10.0-88**	Uncultured Bacteroidetes bacterium	92
	(CU926428)			clone Z82M19B (FJ484307)	
10.0-38	*Sandarakinorhabdus limnophila* strain	96	**10.0-89**	Uncultured Planctomycete clone	96
	so42 (AY902680) (			GASP-MB1S1_E08 (EF664523)	
	Alphaproteobacteria;		**10.0-90**	Uncultured *Actinobacterium* clone TS-	98
	Sphingomonadales;			45-11 (GQ406194) (Actinobacteria)	
	Sphingomonadaceae)		**10.0-91**	*Methylocystis* sp. KS8a strain KS8a	97
10.0-39	Uncultured Beta proteobacterium	91		(AJ458493) (Alphaproteobacteria;	

	clone 187 (AB252908)			Rhizobiales; Methylocystaceae)	
10.0-40	Uncultured Bacteroidetes bacterium clone QEDN11AB07 (CU927037)	93	**10.0-92**	*Desulfovibrio* sp. BSY-C (AB303306) (Deltaproteobacteria; Desulfovibrionales; Desulfovibrionaceae)	94
10.0-41	Alpha proteobacterium BAC247 (EU180521)	99			
10.0-42	Uncultured bacterium clone SRRT67 from the rhizosphere biofilm of Phragmites at Sosei River, Japan (AB240493)	99	**10.0-93**	Uncultured Alphaproteobacteria bacterium clone QEDN10CF09 (CU926428)	99
			10.0-94	Uncultured Clostridiales bacterium clone M10Ba34 (AY360624) (Firmicutes; Clostridia)	98
10.0-43	Uncultured Rhodocyclaceae bacterium clone Amb_16S_1641 (EF019065) (Betaproteobacteria; Rhodocyclales)	97	**10.0-95**	Uncultured Bacteroidetes bacterium clone B7A (FJ205242)	87
10.0-44	Uncultured *Geobacter* sp. clone GASP-WA2S1_B10 (EF072550)	86	**10.0-96**	Uncultured bacterium clone Kas165B (EF203202)	95
10.0-45	Uncultured Gamma proteobacterium clone LiUU-11-89 (AY509481)	93			
10.0-46	Uncultured Chlorobi bacterium clone QEDN2DF11(CU927353)	99			
10.0-47	*Methylobacter tundripaludum*, type strain SV96T (AJ414655)	89			
10.0-48	Uncultured Desulfobacteraceae bacterium clone TDNP_USbc97_107_1_5 (FJ516879) (Deltaproteobacteria; Desulfobacterales)	98			
10.0-49	Uncultured Gamma proteobacterium clone GC12m-1-64 (EU640647)	99			

Table C-4. A list of clones with 16S rRNA sequences indicating their affiliation with sequences in databases for the clone library of RRD 20.6, the South River, VA in Aug. 2008

Clones	Most similar organism (accession number)	Similarity (%)	Clones	Most similar organism (accession number)	Similarity (%)
20.6-1	Denitrifying bacterium enrichment culture clone (FJ802237)	94	**20.6-49**	*Desulfovibrio putealis* (AY574979)	93
20.6-2	*Clostridium* sp. enrichment culture clone (AM933653) (Firmicutes; Clostridia; Clostridiales; Clostridiaceae;)	98	**20.6-50**	*Desulfovibrio fairfieldensis* (U42221)	84
			20.6-51	Uncultured Bacteroidetes bacterium clone QEDS2AE06 (CU921862)	95
20.6-3	Uncultured *Acidobacterium* sp. clone Z4MB66 (FJ484865) (*Acidobacteria (class)*;	91	**20.6-52**	Uncultured Cyanobacterium clone AM-20-36 (FJ866617)	91
			20.6-53	Uncultured Alpha proteobacteria bacterium clone QEDN10CF09 (CU926428)	99
20.6-4	*Methylobacter tundripaludum*, type strain SV96T (AJ414655), a methane-oxidizing bacterium (*Gammaproteobacteria*; *Methylococcales;* *Methylococcaceae*)	98	**20.6-54**	*Methylobacter tundripaludum*, type strain SV96T (AJ414655)	93
			20.6-55	Uncultured Planctomycetacia bacterium clone QEDN7AD10 (CU925538)	97
20.6-5	Uncultured *Rubrivivax* sp. clone GASP-0KB-649-A10 (EU043625) (Beta-proteobacteria; Burkholderiales)	98	**20.6-56**	Uncultured Bacteroidetes bacterium clone LA1E13 (FJ916489)	95
			20.6-57	Uncultured Beta proteobacterium clone Elb252 (AJ421937)	99
20.6-6	Uncultured Bacteroidetes (Phylum) bacterium from clone QEDV1CE11 (CU919887)	95	**20.6-58**	Uncultured type I methanotroph clone site1-39 (EF101324)	97
			20.6-59	Uncultured Acidobacteriaceae bacterium clone CMME10 (AM935953)	94
20.6-7	Uncultured Fibrobacteres (Phylum) bacterium clone 2.23 (GQ183237)	92	**20.6-60**	Uncultured Alpha proteobacteria bacterium clone QEDN10CF09 (CU926428)	95
20.6-8	Uncultured *Leptothrix* sp. clone 3.18 (GQ183308) (Betaproteobacteria; Burkholderiales)	98	**20.6-61**	Uncultured *Myxococcales* bacterium clone SIMO-2054 (AY711420) (Deltaproteobacteria; Myxococcales)	93
20.6-9	*Clostridium* sp. enrichment culture clone MB3_7 (AM933653) (Firmicutes; Clostridia; Clostridiales; Clostridiaceae)	99	**20.6-62**	Uncultured Verrucomicrobia bacterium clone g3 (EU979012)	93
			20.6-63	Uncultured Planctomycetales bacterium clone 464T3 (DQ110111)	96
20.6-10	Uncultured Planctomycetacia bacterium clone GASP-WA2W3_A05 (EF072971) (Planctomycetes)	98	**20.6-64**	Uncultured Gamma proteobacterium clone GC12m-4-21 (EU641167)	98
20.6-11	Uncultured Beta proteobacteria bacterium clone QEDN3CD07 (CU926783)	89	**20.6-65**	*Sandarakinorhabdus limnophila* strain so42 (AY902680) (Alphaproteobacteria; Sphingomonadales;Sphingomonadaceae)	97
20.6-12	*Chloroflexi* bacterium ET9 (EU875530) (Green non-sulfur bacteria, phylum)	91	**20.6-66**	Uncultured Chlorobi bacterium clone QEDN2DF11 (CU927353) (Green sulfur bacteria)	96
20.6-13	*Acinetobacter* sp. B113 (EU883929) (Gamma Proteobacteria; Pseudomonadales; Moraxellaceae)	99	**20.6-67**	Uncultured *Methylobacter* sp. clone LD_MO_6 (EU124843)	98
			20.6-68	Uncultured Rhodobacteraceae bacterium clone GC12m-3-30 (EU641039) (Alphaproteobacteria; Rhodobacterales)	98
20.6-14	*Clostridium* sp. enrichment culture clone MB3_7 (AM933653)	93	**20.6-69**	Uncultured Bacteroidetes bacterium clone QEDN1CG04 (CU925202)	92
20.6-15	Uncultured Beta proteobacterium clone AS86 (EU283393)	96	**20.6-70**	Uncultured Planctomycete clone GASP-	96

20.6-16	*Clostridium* sp. enrichment culture clone MB3_7 (AM933653)	98		KC1S1_A04 (EU299051)	
20.6-17	Uncultured Acidobacteria bacterium clone SS_LKC29 UB26 (AM180885)	97	**20.6-71**	Uncultured Gamma proteobacterium clone CM38G7 (AM936493)	90
20.6-18	Iron-reducing bacterium enrichment culture clone FEA_2_D7 (FJ802334)	98	**20.6-72**	Uncultured Clostridiales bacterium clone M10Ba34 (AY360624)	95
20.6-19	Uncultured Bacteroidetes bacterium clone QEDN5CB12 (CU925900)	95	**20.6-73**	Uncultured Bacteroidetes bacterium clone Skagenf93 (DQ640676)	90
20.6-20	Uncultured Proteobacterium clone Amb_16S_804 (EF018543)	86	**20.6-74**	Uncultured eubacterium WCHB1-02 (AF050593) (Firmicutes)	97
20.6-21	Uncultured Delta Proteobacterium clone TDNP_Wbc97_103_1_10 (FJ516992)	96	**20.6-75**	Uncultured Alpha proteobacterium clone GASP-WB1W3_E12 (EF073787)	96
20.6-22	Uncultured Rhodobacteraceae bacterium clone LW18m-2-46 (EU642384) (Alphaproteobacteria; Rhodobacterales)	96	**20.6-76**	Uncultured Rhodocyclales bacterium clone 7025P4B88 (EF562112) (Beta proteobacteria; Rhodocyclales)	96
20.6-23	Uncultured Alpha Proteobacterium clone GASP-WA1S1_A01 (EF071996)	96	**20.6-77**	Uncultured Beta proteobacterium clone 187 (AB252908)	96
20.6-24	*Geobacter psychrophilus* stain P35 (AY653549)	95	**20.6-78**	*Methylobacter tundripaludum*, type strain SV96T (AJ414655)	92
20.6-25	Uncultured Methylobacter pAMC419 (AF150775)	94	**20.6-79**	*Dechloromonas* sp. A34 (EF632559) (Betaproteobacteria; Rhodocyclales; Rhodocyclaceae)	97
20.6-26	Uncultured Firmicute clone MVP-51(DQ676350)	97	**20.6-80**	*Clostridium* sp. enrichment culture clone (AM933653) (Firmicutes)	98
20.6-27	Uncultured Eubacterium sp. (AM422350)(Firmicutes; Clostridia; Clostridiales; Eubacteriaceae)	94	**20.6-81**	Uncultured Gamma proteobacterium clone GC12m-4-21 (EU641167)	97
20.6-28	Uncultured Alpha proteobacteria bacterium clone QEDR2AG08 (CU922101)	98	**20.6-82**	Uncultured Desulfobulbaceae bacterium clone TDNP_USbc97_108_1_6 (FJ516880) (Deltaproteobacteria; Desulfobacterales)	91
20.6-29	Uncultured Planctomycetaceae bacterium clone AMPF6 (AM935123)	95	**20.6-83**	Uncultured Beta proteobacterium clone 187 (AB252908)	96
20.6-30	Uncultured Rhodocyclaceae bacterium clone Amb_16S_1641 (EF019065) (Betaproteobacteria; Rhodocyclales)	94	**20.6-84**	*Bradyrhizobium* sp. Tv2a-2 (AF216780) (Alphaproteobacteria; Rhizobiales; Bradyrhizobiaceae)	99
20.6-31	Uncultured Gamma proteobacterium clone TRK11 (AY874095)	94	**20.6-85**	Uncultured Beta proteobacterium clone LF029 (EF417718)	90
20.6-32	*Cyanothece* sp. WH 8902 (AY620238) (Cyanobacteria; Chroococcales)	91	**20.6-86**	Uncultured Planctomycete clone DEL31 (AJ616273)	98
20.6-33	Uncultured delta proteobacterium clone Z27M49B (FJ485583) (Deltaproteobacteria)	91	**20.6-87**	Uncultured Gamma proteobacterium clone RBE2CI-108 (EF111188)	94
20.6-34	*Dechloromonas hortensis* strain MA-1 (AY277621) (Betaproteobacteria; Rhodocyclales; Rhodocyclaceae)	98	**20.6-88**	Uncultured Alphaproteobacteria bacterium clone QEDR2AG08 (CU922101)	97
20.6-35	Uncultured Planctomycetacia	95	**20.6-89**	Uncultured Bacteroidetes bacterium clone Asc1b-w-32 (FJ213816)	91
			20.6-90	Uncultured Actinobacterium clone AUVE_04D03 (EF651133)	92
			20.6-91	Uncultured *Bradyrhizobium* sp. clone AMMA12 (AM934793) (Alphaproteobacteria; Rhizobiales; Bradyrhizobiaceae)	99
			20.6-92	Hyphomicrobiaceae bacterium AP-33 (AY145566) (Alphaproteobacteria; Rhizobiales)	95
			20.6-93	Uncultured Alpha proteobacterium clone N-C-98 (AB201614)	90

	bacterium clone QEDN7CE10 (CU927466)		**20.6-94**	Uncultured Betaproteobacteria bacterium clone QEDN10DF01 (CU925363)	96
20.6-36	*Methylocystis* sp. strain KS7 (AJ458498) (Alphaproteobacteria; Rhizobiales; Methylocystaceae)	94	**20.6-95**	Uncultured Firmicutes bacterium clone AUVE_02A11 (EF650943)	91
20.6-37	Uncultured *Chloroflexi* bacterium clone A1836 (EU283570)	95	**20.6-96**	Uncultured Chlorobi bacterium clone A19 (GQ390385) (Phylum)	93
20.6-38	Uncultured Eubacterium clone U54-3 (DQ137931) (Firmicutes)	95	**20.6-97**	Uncultured Desulfobacteraceae bacterium clone TDNP_USbc97_107_1_5 (FJ516879) (Deltaproteobacteria; Desulfobacterales)	96
20.6-39	Uncultured Alphaproteobacteria bacterium clone QEDR2AG08 (CU922101)	94	**20.6-98**	Uncultured Bacteroidetes bacterium clone LiUU-9-137 (AY509313)	96
20.6-40	Uncultured *Acidobacteria* bacterium clone CM3F06 (AM936249)	97			
20.6-41	*Paracraurococcus* sp.strain ORS 1473 (AJ968702) (Alphaproteobacteria; Rhodospirillales; Acetobacteraceae)	94			
20.6-42	Uncultured Bacteroidetes (phylum) bacterium clone CYC_29 (EF562574)	95			
20.6-43	*Rhizobium daejeonense* strain L22 (DQ089696) (Alphaproteobacteria; Rhizobiales;Rhizobiaceae)	96			
20.6-44	Uncultured Oceanospirillales bacterium clone GC12m-2-84 (EU641745) from Lake Michigan (Gammaproteobacteria; Oceanospirillales)	96			
20.6-45	Uncultured Xanthomonadaceae bacterium clone GC12m-3-93 (EU641060) (Gammaproteobacteria; Xanthomonadales)	94			
20.6-46	Uncultured delta proteobacterium clone GASP-KA1W3_A11 (EU297366)	91			
20.6-47	Uncultured Beta proteobacterium clone Elb252 (AJ421937)	98			
20.6-48	*Desulfovibrio putealis* (AY574979)	92			