2010

Functional Identification of Microorganisms that Transform Mercury in Marine Sediments

Lisa Romas
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FUNCTIONAL IDENTIFICATION OF MICROORGANISMS THAT TRANSFORM
MERCURY IN MARINE SEDIMENTS

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

By

LISA M. ROMAS
B.S., St. Lawrence University, 2008

2010
Wright State University
I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Lisa Romas ENTITLED, Functional identification of microorganisms that transform mercury in marine sediments, BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Romas, Lisa M.  M.S., Department of Earth & Environmental Sciences, Wright State University, 2010. Functional identification of microorganisms that transform mercury in marine sediments.

Monomethylmercury (MMHg) is the toxic form of mercury (Hg) that biomagnifies in food webs, and human exposure to MMHg occurs predominantly via consumption of fish. The primary source of MMHg to the marine environment is thought to be in situ sedimentary production by benthic microorganisms, namely sulfate-reducing bacteria (SRB). I collected sediments from the continental shelf (stations 2 and 6) and slope (station 9) of the NW Atlantic Ocean, and amended them with various inhibitor and promoter solutions to target specific functional groups capable of Hg transformations. I also added stable enriched Hg isotopes (i.e., $^{200}$Hg(II) and CH$_3$$^{199}$Hg$^+$) to quantify gross Hg methylation and gross MMHg demethylation, respectively, which were detected with inductively couple plasma mass spectrometry (ICPMS). Hg isotope results suggest that biotic methylation accounted for at least 60% of gross Hg methylation and 0–40% of gross MMHg demethylation. Methanogens probably did not have a major role in MMHg production or demethylation at any of the stations. Iron-reducers (FeRB) were not primary Hg methylators, but iron-reduction (via a Fenton-like reaction) or Fe(III) limitation appear to have influenced MMHg demethylation. Nitrogen cyclers possibly were important in Hg methylation and MMHg demethylation may have been limited by nitrate. SRB were likely important producers of MMHg in nearly all of the sediments. Results of this study support previous research that SRB are important Hg methylators in sulfate-rich, marine environments. This study also highlights the potential importance of iron and nitrogen cycling in MMHg demethylation in marine sediments.
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ACKNOWLEDGMENTS

This study was supported by the U.S. National Science Foundation (OCE-0752116). I thank the R/V Endeavor captain and crew for assistance at sea, the USGS Water Resources Center, Dr. Chad Hammerschmidt, Dr. Geraldine Nogaro, Katlin Bowman, Allan Hutchins, Melissa Tabatchnick, Jaclyn Klaus, and Robbie Weller for assistance with sample collection, performing experiments, and laboratory analyses.
DEDICATION

I dedicate this thesis to my family and friends for all of their love and support. Thank you, Mom and Dad, for all that you have sacrificed to help me obtain my education. Thank you, also, for making frequent, long-distance trips to visit. Thanks to my brother, for always encouraging me to pursue my idealizations and keeping me in touch with global issues and events. I could not have achieved half of what I did without the support of my fiancé and future husband. Thank you for being a great listener and friend during my most difficult times. Thank you to old friends and ones that I met at W.S.U. for making things fun. Lastly, thank you to my advisor for sharing your knowledge about mercury, and for allowing me to partake in two oceanographic cruises and a conference. Because of your hard work, I had access to resources and opportunities for experiences that I never imagined having.
I. INTRODUCTION

Health Risks and Exposure. Mercury (Hg) is a pervasive global contaminant that poses serious health risks to humans (Gilbert and Grant-Webster, 1995; Friedmann et al., 1996; Grandjean et al., 1997; Amorim et al. 2000; Harada, 2005; Onishenko et al., 2007) and wildlife (Scheuhammer et al., 2007). Humans are exposed to Hg primarily as monomethylmercury (MMHg; Fitzgerald and Clarkson, 1991), which is the form of Hg that accumulates readily in organisms and biomagnifies in food webs (Wiener et al., 2003). Oceanic fish are a particularly important route of human exposure, because the majority of fish consumed by U.S. citizens are of marine origin (EPA, 2002; Sunderland, 2007). The ubiquitous distribution, high toxicity, and elevated levels of MMHg in fish have resulted in consumption advisories in 48 of the United States and a large portion of their coastal waters (U.S. EPA, 2010).

Sources. Hg in the environment is derived from natural and anthropogenic sources. It is estimated that about one-third of environmental Hg is from natural sources, which include volcanism and weathering of natural mineral deposits (Fitzgerald and Lamborg, 2003), while the majority is from human activities. Anthropogenic sources, principally fossil-fuel combustion, have increased atmospheric emissions of Hg by about 3× since the Industrial Revolution (Mason et al., 1994; Fitzgerald et al., 1998; Pacyna et al., 2006; Fitzgerald et al., 2007). This estimate is supported by Hg residues in multiple environmental mediums from across the globe, including seabird feathers (Monteiro and
Furness, 1997), lake sediment cores (Swain et al., 1992; Kamman and Engstrom, 2001; Lamborg et al., 2002), peat bog cores (Biester et al., 2002), air over the Atlantic Ocean (Slemr and Langer, 1992), and ice cores (Schuster et al., 2002).

**Biogeochemical Cycling of Hg: Microbial MMHg Production.** Hg has a complex biogeochemical cycle that includes a variety of transformations in the atmosphere and surface waters (Figure 1). Due to its relatively high volatility (Schroeder and Munthe, 1998) and predominance as elemental Hg ($\text{Hg}^0$) in air, Hg has a long residence time in the troposphere ($\sim$ 1 y; Slemr et al., 1985; Lamborg et al., 2002) in comparison to other metals. As a result, $\text{Hg}^0$ is distributed globally. It is removed principally from the atmosphere by oxidation to divalent ionic Hg (Hg(II)), which is scavenged and deposited to the earth’s surface in wet and dry deposition (Fitzgerald and Lamborg, 2003). Hg(II) is reduced biologically and photochemically in surface waters (Amyot et al., 1994; 1997a; 1997b; Costa and Liss, 1999), resulting in supersaturation of $\text{Hg}^0$, net evasion, and rapid cycling at the sea-water interface (Mason et al., 1998; Gardfeldt et al., 2003). Hg(II) is scavenged rapidly from the water column. While Hg(II) can be methylated in the atmosphere (Hammerschmidt et al., 2007) and surface ocean (Mason et al., 1998), microbial methylation of Hg(II) in hypoxic and anoxic regions (Benoit et al., 2003), namely sediments (Watras et al., 1994; Balcom et al., 2004), is hypothesized to be one of the principal sources of MMHg in the marine environment (Hammerschmidt et al., 2004; Hammerschmidt and Fitzgerald, 2008).
Biogeochemical conditions affect Hg speciation, microbial composition, and microbial activity, which, in turn, affect net microbial production of MMHg (King et al., 1999; King et al., 2000; Benoit et al., 2003; Hammerschmidt and Fitzgerald, 2004; Fitzgerald et al., 2007). On a global scale, Hg(II) loadings to sediments appear to be a primary control on MMHg production in marine sediments (Figure 2; Fitzgerald et al., 2007). The average flux of net MMHg (i.e., net production) from sediments has been measured to be about 8% of inorganic Hg(II) loadings (Hammerschmidt et al., 2006; Fitzgerald et al., 2007).
The chemical speciation of Hg(II) can affect its availability for microbial methylation. The main ligands competing for complexation of Hg(II) in aquatic environments are sulfide and organic matter (Benoit et al., 2001; Lamborg et al., 2004; Fitzgerald et al., 2007; Hammerschmidt et al., 2008; Skyllberg, 2008). Benoit et al. (1999, 2001) have demonstrated that microbial MMHg production is affected largely by sulfide, which controls the speciation and associated bioavailability of Hg(II). In low-sulfide pore fluids, Hg(II) is predicted to be present predominantly as the HgS\(^0\) ion pair. Because it is small and uncharged, HgS\(^0\) is thought to diffuse passively through bacterial cell membranes (Benoit et al., 1999, 2001) where it is methylated intracellularly and inadvertently by unknown mechanisms (Ekstrom et al., 2003). Sulfide concentrations
greater than about $10^{-5}$ M often result in reduced *in situ* MMHg production (Hammerschmidt et al., 2004, 2008). This may result from either a change in speciation from HgS$^0$ to HgHS$^-$, rendering it less bioavailable (Fitzgerald et al., 2007), or by inhibiting Hg methylating organisms, particularly sulfate-reducing bacteria (SRB; Gilmour and Henry, 1991). Complexation by organic matter, particularly in the solid phase, also can inhibit Hg methylation by rendering Hg(II) less bioavailable (Hammerschmidt and Fitzgerald, 2004; Hammerschmidt et al., 2008).

The surrounding biogeochemical conditions dictate the microbial community composition, behavior, and activity which, in turn, affect net MMHg production. Variations in seasonal temperature have been shown to influence the rate of bacterial MMHg production (Winfrey and Rudd, 1990; Benoit et al., 2001; Hammerschmidt and Fitzgerald, 2004). Benoit et al. (2001) demonstrated that the bulk of MMHg production by SRB occurs during log-phase growth, while Harmon et al. (2007) noticed a lag in Hg methylation rates during a lag in bacterial growth. Bioturbation also enhances MMHg production (Hammerschmidt et al., 2004; Benoit et al., 2009). Furthermore, the specific location of intracellular Hg methylation reactions and cell physiology (e.g., size, membrane composition) may differ among and within microbial functional groups, which may explain, in part, differences in MMHg production (Benoit et al., 2003).

While the relative influence of various biogeochemical factors on MMHg production has been investigated for various aquatic systems, the primary microbial functional groups responsible for Hg transformations, and the mechanisms employed, remain unclear for a variety of natural aquatic sediments. Research on this topic would
promote our understanding of the Hg biogeochemical cycle and help improve environmental cycling models.

**Hg Methylating Organisms.** It is evident that microbes are the chief methylators of Hg in aquatic sediments, and that environmental conditions control spatial and temporal variations in microbial consortia as well as their relative contribution to MMHg production. Most studies have implicated SRB as the principal methylators of Hg in aquatic environments (Compeau and Bartha, 1985; Gilmour et al., 1992; King et al., 1999; King et al., 2000; Benoit et al., 2001). With regard to Hg methylation by SRB, there are several discrepancies in the peer-reviewed literature related to the methylating agents involved and metabolic pathways used in bacterial MMHg production (Ekstrom and Morel, 2008). While particular agents and pathways have been implicated, researchers have not identified a universal agent or pathway involved in bacterial MMHg production. Furthermore, recent studies with environmentally realistic levels of added molybdate, which is a metabolic inhibitor of sulfate reduction (Wilson and Bandurski, 1958), suggest that some strains of iron-reducing bacteria (FeRB) may be responsible for a significant portion of MMHg production in sediments. It also is possible that other functional groups of microorganisms may be important producers of MMHg under certain environmental conditions. Because net MMHg production is a function of both gross Hg methylation and gross MMHg demethylation, it is pertinent to apply many of the same questions regarding the primary group of Hg methylating microorganisms to microbial demethylators of MMHg.
SRB as Principal Hg Methylators—Compeau and Bartha’s (1985) seminal study established the paradigm of SRB as the principal methylators of Hg in natural sediments. The authors found a 95% decrease in MMHg production when anoxic, low-salinity estuarine sediments were treated with 20 mM molybdate. Molybdate (MoO$_4^{2-}$) was used to inhibit SRB metabolism, because it is known to compete with SO$_4^{2-}$ in the SRB electron transport system (Wilson and Bandurski, 1958). A subsequent study by Gilmour et al. (1992) reaffirmed that SRB are important MMHg producers in lacustrine sediments by adding SO$_4^{2-}$ (≤ 200 µM) to promote and MoO$_4^{2-}$ (20 mM) to inhibit SRB respiration. King and colleagues (1999, 2000) found a correlation between sulfate reduction rates and Hg methylation rates in pure cultures of SRB and salt marsh sediments.

Methylating Agent and Metabolic Pathways Involved—It has been proposed that cysteine, an amino acid, enhances Hg methylation by facilitating uptake of Hg$^{2+}$ and promoting enzymatic formation of MMHg, possibly via the methionine biosynthesis pathway (Landner, 1971; Schaefer and Morel, 2009; Sparling, 2009). Methylcobalamin, the methyl derivative of vitamin B$_{12}$, also is suspected to play a key role in Hg methylation (Jensen and Jernelov, 1969; Wood et al., 1968; Choi and Bartha, 1993; Ekstrom and Morel, 2008). Although Hg methylation involving methylcobalamin may be a non-enzymatic, abiotic process (Chemaly, 2002), studies have suggested that MMHg production by a strain of SRB is catalyzed enzymatically (Choi and Bartha, 1993; Choi et al., 1994a, 1994b). Choi et al. (1994a, 1994b) determined that Hg methylation was 40× greater when catalyzed by an enzyme than without one. They described that methylcobalamin is a co-enzyme in the transfer of a methyl carbanion group from methyltetrahydrofolate to Hg(II) (Choi et al., 1994a; Choi et al., 1994b). These studies
associated MMHg production by a pure culture of SRB, *Desulfovibrio desulfuricans*, with the acetyl-coenzyme A (CoA) metabolic pathway (Choi et al., 1994a, 1994b).

Recent research has concluded that neither vitamin B$_{12}$ nor the acetyl-CoA pathway are requisite for bacterial Hg methylation (Ekstrom et al., 2003; Ekstrom and Morel, 2008). MMHg is produced by pure cultures of SRB that utilize a variety of metabolic pathways, including: (1) complete oxidizers of carbon that use the acetyl-CoA pathway, (2) incomplete oxidizers that do not use this pathway, (3) bacteria that use the citric acid cycle, (4) bacteria that use a B$_{12}$-containing methyltransferase, and (5) bacteria that are independent of a B$_{12}$-containing methyltransferase (Ekstrom et al., 2003; Ekstrom and Morel, 2008). It is evident that vitamin B$_{12}$ and/or the acetyl-CoA pathway are often involved in bacterial MMHg production, but other metabolic or anabolic pathways may be associated with Hg methylation.

**SRB Inhibition with Molybdate: Are FeRB Important Hg Methylators?**—As noted, recent studies have suggested that FeRB may be important methylators of Hg in aquatic sediments. 16s ribosomal RNA (rRNA) sequencing has shown that SRB and FeRB may be related evolutionarily (Longergan et al., 1996); therefore, the relative contribution of FeRB to MMHg production and/or the metabolic pathway(s) utilized by FeRB during Hg methylation may be difficult to distinguish from SRB. Fleming et al. (2006) found a decoupling of sulfate reduction and Hg methylation in iron-rich, freshwater sediments of Clear Lake, CA. The authors ascertained that experimental additions of MoO$_4^{2-}$ at levels equimolar to *in situ* SO$_4^{2-}$ (~100 µM) inhibited SRB effectively in Clear Lake deposits, but had little impact on MMHg production. This
suggested that a functional group of microorganisms other than SRB was largely responsible for Hg methylation, which, after isolation tests, was found to be an iron-reducing bacterium, *Geobacter* sp. strain CLFeRB.

Fleming et al. (2006) noted that the levels of MoO$_4^{2-}$ used to inhibit SRB in previous studies may have led to the incorrect interpretation that SRB are principal methylators of Hg because MoO$_4^{2-}$ additions were excessive (Fleming et al., 2006). In particular, MoO$_4^{2-}$ used to inhibit SRB should be equimolar to ambient SO$_4^{2-}$ (Fleming et al., 2006). In past experiments with estuarine and marine sediments, it is plausible that SRB, as opposed to FeRB, are dominant Hg methylators, because MoO$_4^{2-}$ additions (typically 20 mM) were comparable to ambient SO$_4^{2-}$ (≤ 28 mM). However, ambient SO$_4^{2-}$ is at least 10× less in most freshwaters, suggesting that ≥ 20 mM amendments of MoO$_4^{2-}$ to freshwater deposits may inhibit many microbial processes, including iron-reduction, and lead to an incorrect interpretation of findings (Fleming et al., 2006).

Warner et al. (2004) added 2mM MoO$_4^{2-}$ and found suppressed Hg methylation rates under iron-reducing—rather than sulfate-reducing or methane-producing conditions—in slurries of wetland sediment. However, added MoO$_4^{2-}$ was an order-of-magnitude greater than the maximum ambient SO$_4^{2-}$ concentration of 685 µM. This may have lead to an incorrect interpretation, similar to many past inhibition studies.

A laboratory investigation with pure cultures of FeRB, concurrent with the work of Fleming and colleagues (2006) further identified the potential role of FeRB in MMHg production. Kerin et al. (2006) determined that not all FeRB are capable of methylating Hg. For example, *Geobacter metallireducens* and *Geobacter sulfurreducens* methylate
Hg, while *Shewanella* spp. cannot. Moreover, pure cultures of FeRB capable of producing MMHg were found to utilize electron acceptors other than Fe(III), such as fumarate and nitrate (Kerin et al., 2006). This illustrates that active Fe(III) reduction is not necessary for MMHg production by FeRB (Kerin et al., 2006). Results of both Kerin and colleagues (2006) and Fleming et al. (2006) suggest that FeRB may be important producers of MMHg in iron-rich, low-SO$_4^{2-}$ sediments.

MMHg production may be inhibited with Fe(II). Fe(II) additions to anoxic wetland sediments reduced MMHg production (Mehotra et al., 2003; Mehrotra and Sedlak, 2005). While the authors suggested that the mechanism for this inhibition is due to precipitation of FeS and an associated decrease in HgS$^0$, they did not rule out the possibility that the Fe(II) amendment may have inhibited iron-reduction directly (and concomitant Hg methylation by FeRB). Hence, this study highlights the need to better understand not only which functional groups of microorganisms are methylating Hg, but also how the function of specific metabolic inhibitors may be altered environmentally.

*Other Microbial Functional Groups Involved in Gross MMHg Production*— Because FeRB are potentially important Hg methylators in aquatic sediments, the current paradigm that SRB are primary Hg methylators must be revisited. Further confounding this issue is the question of whether functional groups of bacteria other than SRB and FeRB may contribute substantially to *in situ* MMHg production. Early research with cell extracts showed that methanogens may be important producers of MMHg (Wood et al., 1968). Additions of nitrate and chloramphenicol (an inhibitor of the eubacteria domain) to periphyton cultures pointed to denitrifiers as important producers of MMHg in
freshwater marshes (Cleckner et al., 1999). The syntrophic association between SRB and methanogens also is thought to play a role in the Hg methylation process (Pak and Bartha, 1998a, 1998b).

**Net MMHg Production.** Net MMHg production is a function of both gross Hg methylation and gross MMHg demethylation, which are concomitant processes in natural sediments. There are knowledge gaps regarding the identity of bacterial functional groups that produce and demethylate MMHg in the environment. Oremland and coworkers (1991, 1995) illustrated that many bacteria capable of methylating Hg also are capable of demethylating MMHg. Oxidative demethylation, for example, is prominent in zones of denitrification, sulfate-reduction, and methanogenesis (Pak and Bartha, 1998b; Oremland et al., 1991, 1995), which are associated with Hg methylation, as described previously.

Mechanisms of MMHg demethylation are, however, better understood than those of Hg methylation. MMHg demethylation occurs via abiotic (e.g., photodegradation; Sellers et al., 1996) and biotic mechanisms, which include oxidative and reductive demethylation pathways. Oxidative demethylation occurs when the methyl group on MMHg is oxidized to CO₂ and is typically associated with aerobic and/or anaerobic respiration in sediments that are not severely contaminated with Hg (Oremland et al., 1991; Marvin-Dipasquale et al., 2000). Reductive demethylation, on the other hand, is thought to be a detoxification mechanism, because it occurs in highly contaminated deposits (Marvin-Dipasquale et al., 2000). The role of the *mer* operon in reductive demethylation has been studied thoroughly, and it is well-known that transcription of *mer*
genes is induced by high levels of Hg (Robinson and Tuovinen, 1984; Barkay et al., 1992; Nazaret et al., 1994; Barkay et al., 2003; Schaefer et al., 2004). The \textit{mer} B gene produces the organomercurial lyase enzyme, which cleaves the methyl group from MMHg and reduces it to CH$_4$, and the mercuric reductase enzyme, resulting from the \textit{mer} A gene, reduces Hg(II) to Hg$^0$, which can volatilize (Robinson and Tuovinen, 1984; Drott et al., 2008).

**Hypotheses.** As derived or inferred from prior studies, I tested the following hypotheses related to bacterial transformation of Hg in continental shelf and slope sediments of the northwest Atlantic Ocean: 1) SRB are the principal functional group of Hg methylating organisms in sediments; and 2) SRB also are the major group of MMHg demethylating bacteria. The sampling sites included three stations in order of increasing distance from shore (Figure 3)—Station 2 (43° N, 70°13’ W), station 6 (43° N, 65°10’ W), and station 9 (40°30’ N, 66°40’ W). Sediment at stations 2 and 6 was composed of fine-grained material that is representative of deposits at other locations on the continental shelf of the NW Atlantic Ocean. In contrast, sediment at station 9 was composed of glacial detritus and similar to sand in texture. The methodology used to test these hypotheses is unique in that it combines two commonly used, but normally independent, approaches (i.e., inhibitor/promoter experiments and stable Hg isotope additions) to investigate the primary microbial functional groups that are responsible for Hg transformations under a variety of natural conditions.
FIGURE 3. Three sampling stations (STN) on the continental shelf and slope of the NW Atlantic Ocean.
II. METHODOLOGY

**Sample Collection and Preparation.** Sediment and overlying water were sampled with push cores from a box core of sediment on the continental shelf and slope. Sediment cores were transferred to a low-O$_2$ (N$_2$-filled, evacuated) glove-box promptly after sampling, where overlying water was removed and the upper 6 cm of material was transferred to a polyethylene beaker for homogenization by stirring. Aliquots of sediment (25 cm$^3$) were slurried with 25 cm$^3$ of an inhibitor or promoter solution in clean, 60-mL glass serum bottles.

**Inhibitor/Promoter Solutions.** Treatment solutions (Table 1) were made with 0.2-µm filtered overlying water that was de-oxygenated by purging with N$_2$. Water was collected from the respective site with trace metal-clean techniques (Gill and Fitzgerald, 1985). For each site, three replicate 60-mL serum bottles were prepared for each treatment. After amendment with an inhibitor or promoter solution, serum bottles were sealed promptly with a butyl rubber stopper and aluminum cap. The headspace was flushed with N$_2$ to remove O$_2$. Serum bottles were incubated with added inhibitors/promoters in the dark at *in situ* temperature (in a refrigerator) for 1 d, prior to Hg isotope additions.
**Control Kill Solutions**—The use of azide as a control kill treatment is potentially problematic. Neither azide nor cyanide inhibits anaerobic iron-reduction effectively (Arnold et al., 1986; Gorby and Lovley, 1991). Arnold et al. (1986) conducted inhibitor studies using pure cultures of FeRB and found dissimilative iron-reduction to be uncoupled from oxidative phosphorylation under low oxygen conditions. Furthermore, Woźniak et al. (2003) found a lack of inhibition of iron-reduction by azide in an isolated freshwater FeRB, *Aeromona hydrophila*. Specifically, azide failed to inhibit complex IV of the electron transport chain as intended. The use of azide could mask that FeRB are not significant MMHg producers in sediments, particularly if there is no difference in MMHg concentrations between the control and the control kill treatments. That is why a broad-spectrum antibiotic (i.e., chloramphenicol) was used as the main control kill treatment in my experiments.
Table 1. Inhibitor and promoter treatment solutions used in experiment, including intended functional group and purpose.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration</th>
<th>Control, Inhibitor, or Promoter</th>
<th>Affected Microbial Group</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubated, no amendments</td>
<td>----</td>
<td>Control</td>
<td>None</td>
<td>Establishes baseline net MMHg production under incubation conditions</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>1 mM</td>
<td>Control</td>
<td>All</td>
<td>Inhibits the (Eu)bacteria domain (all major functional groups targeted in this experiment), but does not affect the Eukarya or Archaea domains</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>37% sol.</td>
<td>Control</td>
<td>All</td>
<td>Control kill. Eliminates biotic component and, when compared to control, accounts for abiotic effects on net MMHg production during incubation</td>
</tr>
<tr>
<td>Sodium Azide</td>
<td>10 mM</td>
<td>Control</td>
<td>All</td>
<td>Serves as a control kill for all microorganismal groups, with FeRB as a possible exception</td>
</tr>
<tr>
<td>Acetylene (C₂H₂)</td>
<td>20% sol.</td>
<td>Inhibitor</td>
<td>nitrogen cyclers</td>
<td>Inhibits N₂O reduction</td>
</tr>
<tr>
<td>Potassium Nitrate (NO₃⁻)</td>
<td>1 mM</td>
<td>Promoter</td>
<td>nitrogen cyclers</td>
<td>Promotes NO₃⁻ reduction</td>
</tr>
<tr>
<td>Deferoxamine B (DFB)</td>
<td>1 mM</td>
<td>Inhibitor</td>
<td>FeRB</td>
<td>May inhibit FeRB by making the terminal electron acceptor, Fe(III), less bioavailable</td>
</tr>
<tr>
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<td>----------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Iron-III (Fe(III))</td>
<td>10 mM</td>
<td>Promoter</td>
<td>FeRB</td>
<td>Promotes FeRB</td>
</tr>
<tr>
<td>Sodium Molybdate (MoO$_4^{2-}$)</td>
<td>28 mM</td>
<td>Inhibitor</td>
<td>SRB</td>
<td>Inhibits SRB. Equimolar to ambient sulfate in marine sediments</td>
</tr>
<tr>
<td>Sodium 2-bromoethane-sulfonic acid (BES)</td>
<td>10 mM</td>
<td>Inhibitor</td>
<td>methanogens</td>
<td>Inhibits methanogenesis</td>
</tr>
<tr>
<td>Multiple (Multi)</td>
<td>10 mM BES + 28 mM MoO$_4^{2-}$ + 20% C$_2$H$_2$</td>
<td>Inhibitor</td>
<td>methanogens, SRB, and denitrifiers</td>
<td>Inhibits all major functional groups in experiment except FeRB and acetogens</td>
</tr>
</tbody>
</table>
**Isotope Additions.** In order to determine simultaneously which bacterial functional groups are the predominant producers and demethylators of MMHg, the inhibitor/promoter experiments were used in combination with a quantitative approach involving enriched, stable isotopes of Hg as described by Hintelmann and Evans (1997). The use of stable isotopes has greatly improved the understanding of Hg methylation and MMHg demethylation dynamics (Hintelmann et al., 2000). After the inhibitor/promoter amendments and a 1-d incubation, aliquots of $^{200}$Hg(II) and CH$_3$$^{199}$Hg$^+$ were added to each serum bottle. The amount of each isotope added was equal to about 50% of ambient concentration (Hintelmann and Evans, 1997).

$^{200}$Hg(II) and CH$_3$$^{199}$Hg$^+$ were added to sediments slurries that had reacted for 1 d with specified inhibitor/promoter solutions. Samples were incubated with $^{200}$Hg(II) and CH$_3$$^{199}$Hg$^+$ for 10 h before termination by freezing ($\leq -20$ °C). Frozen sediments were transported on dry ice to Wright State University, where they were lyophilized and homogenized prior to extraction of MMHg by either aqueous distillation or with acid and methylene chloride.

**MMHg Distillation, Methylene Chloride Extraction, and Detection with ICPMS.** Gross potential Hg methylation was quantified by measuring how much $^{200}$Hg(II) was converted to CH$_3$$^{200}$Hg$^+$, whereas gross potential MMHg demethylation was determined as the amount of added CH$_3$$^{199}$Hg$^+$ remaining in the sediment. MMHg was distilled from station 6 sediment, following the methods of Horvat et al. (1993). In contrast, dilute acid and methylene chloride (i.e., CH$_2$Cl$_2$; Hammerschmidt and Fitzgerald, 2004) were used for stations 2 and 9. This method was selected because
distillation has been shown to generate artifact MMHg, especially in sediments with high concentrations of inorganic Hg(II) and organic matter (Bloom et al., 1997; Hintelmann et al., 1997; Hammerschmidt and Fitzgerald, 2001). Initial distillation tests with these two deposits indicated a large proportion of added $^{200}\text{Hg(II)}$ was labile and potentially susceptible to artifact $\text{CH}_3^{200}\text{Hg}^+$ production. Distillates and $\text{CH}_2\text{Cl}_2$-extracts of sediment were derivatized by ethylation (Bloom, 1989). Volatile species were collected on Tenax, separated by isothermal gas chromatography (GC), and detected with inductively coupled plasma mass spectrometry (ICPMS; Hintelmann and Evans, 1997). The retention time for measured MMHg isotopes was about 2 min (Figure 4). Many of the calculations used to determine the concentrations of the MMHg isotopes using ICPMS are described in Hintelmann and Ogrinc (2003).

![FIGURE 4. Chromatogram of five Hg isotopes (i.e., Hg-198, Hg-199, Hg-200, Hg-201, and Hg-202) and two species (i.e., methylmercury at about 2 min and diethylmercury, the Hg$^{2+}$ derivative, at about 3.5 min) from ICPMS detection.](image-url)
**Quality Assurance.** Quality of results was assessed from replicate analyses, recoveries of known additions, and analyses of blanks and standard calibration solutions.

*Field Quality Assurance*—Each inhibitor/promoter treatment was performed in triplicate.

*Laboratory Quality Assurance*—Sediment masses (± 0.001-g) were measured on a balance calibrated with ASTM-Class 1 certified masses. Chemical reagents were suitable for MMHg analysis (i.e., ACS-grade or better) and all water used was reagent-grade (i.e., > 18 MΩ·cm). The ICPMS was calibrated with a constant source of Hg\(^0\). The detection limit for the ICPMS (i.e., 3 standard deviation of \(n \geq 4\) procedural known additions) for ambient total mercury was between 173–425 pg for the three stations. Aqueous MMHg standards were calibrated before use against a NIST-traceable aqueous Hg standard. A known addition of internal standard (i.e., CH\(_3\)^{201}Hg\(^+\)) was added to all samples prior to distillation or extraction. A reverse isotope dilution equation was used to correct all Hg-isotope values based on the internal standard amendment and check standards, which contained varying concentrations of a natural isotopic abundance MMHg standard and internal \(\text{CH}_3^{201}\text{Hg}^+\) standard. Standard calibration curves for natural abundances of MMHg isotopes (\(r^2_{\text{STN} 2} \geq 0.9984\); \(r^2_{\text{STN} 6} \geq 0.9463\); \(r^2_{\text{STN} 9} \geq 0.9907\)) were used to correct for instrumental mass biases. Ten percent of analyses were procedural blanks that had been taken through the extraction process. About 5% of analyses were analytical blanks. MMHg levels in analyzed samples were as follows: Analytical blanks < procedural blanks < lowest natural abundance MMHg standard. Mean relative standard deviation for samples at station 2 was 22.5% for demethylation and 30.7% for methylation; 14.3% for demethylation and \(\geq 100\)% for methylation at station 6; and 31.7% for demethylation.
and 30.3% for methylation for station 9. The high degree of imprecision for methylation assays at station 6 was likely an artifact MMHg formation during the distillation process.
III. RESULTS AND DISCUSSION

**Ambient MMHg Concentrations.** After a 34-h total incubation, ambient MMHg concentrations were similar for all treatments and controls at station 2 (Figure 5a). This suggests that metabolic inhibitors and promoters had no effect on net MMHg production from ambient Hg over this brief incubation period. Furthermore, the concentration of MMHg (~0.7 pmol MMHg g\(^{-1}\) dw) is similar to those in surficial sediments of eastern Long Island Sound (1.0–2.1 pmol g\(^{-1}\) dw; Hammerschmidt et al., 2004) and deposits on the continental shelf (0.7–1.8 pmol MMHg g\(^{-1}\) dw; Hammerschmidt and Fitzgerald, 2006). This is important because sediment with these concentrations can account for a diffusive MMHg flux to overlying water that is sufficient to sustain levels in marine fish (Hammerschmidt et al., 2004).

Ambient MMHg concentrations at stations 6 and 9 were more variable and mean values were about 3–4x less than station 2 (Figures 5b–4c). These two stations are farther in distance from the coast than station 2; therefore, they are likely to have reduced Hg(II) and, possibly, different sources and/or quality of organic matter inputs (i.e., allochthonous versus autochthonous sources). As a result, stations 6 and 9 are likely to have reduced ambient MMHg concentrations compared with station 2.
While organic matter is presumably homogenous among treatments at each station, its source also may differ for the near-shore station (station 2) versus the remote continental shelf and slope stations (stations 6 and 9). That is, proportionately more of the organic matter in sediments at station 2 may be allochthonous compared to the other two remote stations where most of the organic matter is presumed to be autochthonous. Although $^{13}$C measurements would be required to verify these assumptions, a study in NY/NJ Harbor suggests that Hg(II) has a greater affinity for allochthonous organic matter (Hammerschmidt et al., 2008). Alternatively, Hollweg et al. (2009) illustrated that organic matter with low sulfide content found at sites on the remote continental shelf and slope had a lower binding capacity for Hg(II), which resulted in comparatively greater MMHg production.

If Hg(II) were bound more strongly to (allochthonous) organic matter at station 2, then it may have been less bioavailable for transformation, which could explain no net ambient MMHg production (Figure 5a). Compared with the killed control, MMHg levels in the inhibitor/promoter treatments were less at station 6 (Figure 5b), but often greater at station 9 (Figure 5c). This suggests that there was net MMHg demethylation at station 6 and net MMHg production at station 9. If Hg(II) were bound more strongly to organic matter at stations 6 and 9, then I would expect greater net Hg methylation, as observed for station 9. Explanations as to why station 6 had net demethylation of ambient MMHg remain elusive. It is possible that the problems associated with artifact formation during distillation confound interpretation of results from station 6. Although overlap in the standard deviations for station 2 must be considered, ambient MMHg concentrations from stations 2 and 9 might be explained by previous research which showed that Hg(II)
is less bio-available at near-shore sites with strongly binding allochthonous organic matter, but more bioavailable for Hg methylation at remote sites with autochthonous organic matter.
FIGURE 5a. Station 2 mean ambient MMHg concentrations, determined from ambient CH$_3^{202}$Hg$^+$, versus treatment.
FIGURE 5b. Station 6 mean ambient MMHg concentrations, determined from ambient CH$_3^{202}$Hg$^+$, versus treatment.
FIGURE 5c. Station 9 mean ambient MMHg concentrations, determined from ambient CH$_3^{202}$Hg$^+$, versus treatment.
**Hg Methylation.** Gross Hg methylation, measured as the conversion of added $^{200}\text{Hg(II)}$ to $\text{CH}_3^{200}\text{Hg}^+$ was generally between 0.1–1% for all stations, which means that about 0.3–3.0 ng of $^{200}\text{Hg(II)}$ were methylated during 10 h of incubation (Figures 6a–c). This is equivalent to about 1.6–16 pmol MMHg g$^{-1}$ dw, which is 1–2 orders-of-magnitude greater than the maximum ambient MMHg concentration produced over 34 h of incubation. Clearly, given the appropriate biogeochemical conditions and increased loadings of inorganic Hg(II), these sediments are have a large potential for MMHg production.

At all stations, there is a relatively large degree of uncertainty among replicates in many of the treatments, especially for the control and killed control treatments. Therefore, interpretations must be made with caution. Despite the uncertainty, it appears that more MMHg was produced in the control than killed control treatments for all stations. This implies that a portion of gross MMHg production was due to biotic factors. However, the killed control did not completely inhibit gross Hg methylation. There are two potential explanations for this observation. Complete inhibition of Hg methylation by chloramphenicol implies that a portion of gross methylation was due to abiotic factors. The maximum portion of gross Hg methylation that could be due to abiotic factors is 26%, 39%, and 39% for stations 2, 6, and 9, respectively (Figures 6a–c). Acetate, humic matter, some organic acids, and methylated metals are potential abiotic methylating agents common in aquatic environments (Craig and Moreton, 1985; Weber, 1993; Fitzgerald et al., 2007). It also could suggest that chloramphenicol was not completely effective at inhibiting all microbial groups and/or some bacteria developed resistance to it over 34 h of incubation. Chloramphenicol inhibits eubacteria, but not eukarya or archaea.
MMHg production has been attributed to archaea (e.g., methanogens; Wood et al. 1968) and eukarya (e.g., fungi; Landner 1971). If some biotic Hg methylation were occurring in the presence of chloramphenicol, then the contribution of abiotic factors to MMHg production would be less than estimated. Formaldehyde and sodium azide also were used as killed control treatments, but were less effective than chloramphenicol at inhibiting both MMHg production and demethylation.

The majority (up to 60%) of gross Hg methylation is due to biotic factors at these stations (Figures 6a–c). This is in agreement with research conducted at other freshwater and marine locations where most MMHg production is biological (Benoit et al., 2003). Methanogens probably were not major methylators of Hg at any of the stations; if they were, inhibition with 2-bromoethane sulfonate would have resulted in a distinguishable decrease in percent methylation versus the control, which did not occur (Figures 6a–c). This is notable, considering that methanogens have been shown to methylate Hg independently (Wood et al., 1968) and in association with or at rates comparable to SRB (Compeau and Bartha, 1987; Warner et al., 2004). FeRB do not appear to be primary methylators of Hg in continental shelf and slope sediments from the NW Atlantic Ocean. If they were, inhibition with DFB would have decreased gross methylation and promotion with labile Fe(III) would have increased gross methylation relative to the control (Figures 6a–c). This is notable given recent studies which have shown that FeRB are potentially important producers of MMHg (Fleming et al., 2006; Kerin et al., 2006). The prior studies were conducted on freshwater sediment and laboratory cultures, so it is not surprising that marine sediments, with different microbial communities and biogeochemical controls, produced contrasting results.
SRB are possibly important methylators of Hg at station 2. When SRB were inhibited (either individually by MoO$_4^{2-}$, or in combination with methanogens and N$_2$O reducers in the multi-inhibitor treatment), there was a decrease in gross methylation (Figure 6a). When their competitors (i.e., NO$_3^-$ reducers, FeRB) were promoted, there was a decrease in gross methylation (Figure 6a). Furthermore, inhibition of FeRB resulted in a substantial increase in gross methylation (Figure 6a), which may have given another functional group (e.g., SRB) a competitive advantage. Consequently, SRB appear to be primary methylators of Hg in station 2 sediments.

N$_2$O reducers also could be important producers of MMHg in station 2 sediments, because when they were inhibited with acetylene, gross methylation decreased (Figure 6a). Yet, contradictorily, when NO$_3^-$ was added, Hg methylation did not increase as expected, but was comparable to the killed control (Figure 6a). The denitrification process involves several steps (Table 1) and these trends highlight the distinction between N$_2$O reduction and NO$_3^-$ reduction.

SRB also were important producers of MMHg at station 6. To reiterate, methanogens and FeRB were not important Hg methylators at any of the stations. When SRB were inhibited at station 6 (either individually by MoO$_4^{2-}$, or in combination with methanogens and N$_2$O reducers in the multi-inhibitor treatment), there was a decrease in gross methylation (Figure 6b). Gross methylation in those two treatments was similar to the killed control, which suggests that SRB may account for up to 60% of total MMHg production and all of the biologically produced MMHg. These treatments also reinforce the possibility of abiotic Hg methylation. Specifically, inhibition of SRB individually
and in combination with other functional groups did not cease Hg methylation (Figure 6b). In contrast to station 2, nitrogen cycling microorganisms at station 6 do not appear to be important Hg methylators. Promotion of NO$_3^-$ reducers resulted in no gross methylation and inhibition of N$_2$O reducers did not decrease methylation compared with the control (Figure 6b). These two trends are consistent and the elimination of Hg methylation in this treatment demonstrates that another functional group (e.g., SRB) may be important producers of MMHg in station 6 sediments.

Gross Hg methylation at station 9 was the lowest of the stations, which might be expected because these deposits were sandy in texture with presumably less organic material to support microbial activity. There is some biotic Hg methylation taking place at station 9, but unequivocal determinations regarding which functional group is methylating Hg cannot be made given the high degree of uncertainty in the control. Determinations can be made with caution regarding nitrogen cycling microorganisms. Nitrogen cycling microorganisms are possible Hg methylators in these sediments from the continental slope. Promotion of NO$_3^-$ reducers increased gross methylation, but this increase was still within the range of the control (Figure 6c). Inhibition of N$_2$O reduction resulted in decreased gross methylation, similar to the killed control (Figure 6c).
FIGURE 6a. Station 2 percent gross Hg methylation, determined by conversion of $^{200}\text{Hg}^{II}$ to $\text{CH}_3^{200}\text{Hg}^+$, versus treatment.
FIGURE 6b. Station 6 percent gross Hg methylation, determined by conversion of $^{200}$Hg(II) to $\text{CH}_3^{200}$Hg$^+$, versus treatment.
FIGURE 6c. Station 9 percent gross Hg methylation, determined by conversion of $^{200}$Hg(II) to CH$_3$$^{200}$Hg$^+$, versus treatment.
**MMHg Demethylation.** Gross demethylation, measured as the loss of added \( \text{CH}_3^{199}\text{Hg}^+ \), was 1–2 orders of magnitude greater than gross methylation for sediments at all stations (Figures 7a–c). This could reflect that Hg isotopes were added at 0.5x ambient concentrations and both ambient and amended concentrations of MMHg were much lower than for Hg(II) (i.e., MMHg in coastal marine pore waters is about 10–30% of Hg\(_T\); Fitzgerald et al., 2007), resulting in a greater proportion of MMHg conversion. At stations 2 and 6, more MMHg was demethylated in the control than killed control treatments (Figures 7a–b). This implies that a portion of gross MMHg demethylation was due to biotic factors. However, the killed control did not completely inhibit gross MMHg demethylation, possibly due to reasons similar to Hg methylation. Interestingly, abiotic MMHg demethylation accounted for up to 60% and 68% for stations 2 and 6, respectively (Figures 7a–b). At station 9, however, it appears that biotic MMHg demethylation was nearly non-existent (Figure 7c). Indeed, all of the MMHg demethylation at station 9 could be attributed to processes not inhibited by chloramphenicol.

Methanogens probably were not major demethylators of MMHg at any of the stations; if they were, inhibition would have resulted in a distinguishable decrease in gross demethylation versus the control, which did not occur (Figures 7a–6c). Upon initial examination of the FeRB inhibitor treatments, FeRB do not appear to be primary demethylators of MMHg in continental shelf and slope sediments. If they were, inhibition with DFB would have decreased gross demethylation (Figures 7a–6c). However, it is questionable whether or not the FeRB inhibitor, DFB, effectively inhibits FeRB. As a siderophore, DFB chelates labile Fe\(^{3+}\), but FeRB have evolved ways to...
obtain Fe$^{3+}$ in Fe-oxyhydroxides. Kostka et al. (2002) found that FeRB strains implicated in Hg transformations (i.e., *Proteobacteria, Geobacteraceae*) can access Fe$^{3+}$ bound to clay particles common in sediments.

MMHg demethylation increased substantially at all stations when Fe(III) was added (Figures 7a–c). This, in combination with the questionable efficacy of DFB, implies that FeRB may be important demethylators in sediments from the NW Atlantic Ocean. MMHg demethylation, alternatively, may have been due to an abiotic mechanism in the FeRB promoter treatment. Fe$^{3+}$ may have stimulated FeRB and the production of Fe$^{2+}$. Fe$^{2+}$ is known to react with H$_2$O$_2$ to produce OH radicals via the Fenton reaction. OH radical is known to degrade MMHg in natural waters (Hammerschmidt and Fitzgerald, accepted). Generation of •OH via a Fenton-type reaction from added Fe(III) may explain the enhanced MMHg demethylation in the Fe$^{3+}$ amendments. Regardless of the mechanism, Fe(III) seems to have an important role in MMHg demethylation, either directly or indirectly.

Nitrate-reducing bacteria appear to be important demethylators of MMHg in continental shelf and slope sediments, as supported by the substantial increase in gross demethylation in samples amended with NO$_3^-$ (Figures 7a–c). An alternative explanation is that another functional group of microorganisms, capable of demethylating MMHg, may be limited by NO$_3^-$. When N$_2$O reducing bacteria were inhibited, I did not see a concomitant decrease in gross demethylation compared with the control (Figures 7a–c). Extracted sediments from stations 2 and 9 showed an *increase* in gross demethylation when N$_2$O reducing bacteria were inhibited. If acetylene inhibited N$_2$O reducing bacteria
effectively, a competing functional group of bacteria may have become the primary MMHg demethylator (e.g., SRB). This is plausible given that denitrification is a more energetically favorable redox reaction than sulfate-reduction. On the other hand, acetylene could have had a promontory effect on MMHg demethylation at stations 2 and 9. Culbertson et al. (1988) explain that acetylene inhibits methanogenesis and can be fermented into ethanol and acetate, which have promontory effects on sulfate-reduction (as demonstrated by sulfide accumulation). Furthermore, acetylene consumption was entirely biotic, as evidenced by zero acetylene consumption in autoclaved estuarine sediments (Culbertson et al., 1988). The anomaly in the acetylene treatment might be explained best by a promotion of SRB. However, application of this reasoning to Hg methylation does not fully uphold; for example, I would expect acetylene stimulation of SRB to result in increased Hg methylation, particularly in station 2 sediments.

There was a decrease in gross demethylation for all stations when SRB were inhibited either individually by MoO$_4^{2-}$, or in combination with methanogens and N$_2$O reducers in the multi-inhibitor treatment. SRB appear to be both important producers and demethylators of MMHg in sediments collected from the NW Atlantic continental shelf and slope. It also is possible that other functional groups of bacteria, including those limited by NO$_3^-$ or Fe$^{3+}$ demethylate MMHg.
FIGURE 7a. Station 2 percent gross MMHg demethylation, as measured by the loss of CH$_3^{199}$Hg$^+$, versus treatment.
FIGURE 7b. Station 6 percent gross MMHg demethylation, as measured by the loss of CH$_3^{199}$Hg$^+$, versus treatment.
FIGURE 7c. Station 9 percent gross MMHg demethylation, as measured by the loss of CH$_3^{199}$Hg$^+$, versus treatment.
Summary. It appears that SRB are primary methylators of Hg in continental
shelf sediments, which is in agreement with previous studies conducted in freshwater and
estuarine sediments (Compeau and Bartha, 1985; Gilmour et al., 1992; King et al., 1999;
King et al., 2000; Benoit et al., 2001). SRB do not seem to be important Hg methylators
in sandy sediments on the continental slope (station 9). It would be interesting to perform
these experiments on freshwater sediments with relatively low SO$_4^{2-}$, high Fe$^{3+}$, and high
organic matter to see if FeRB are primary methylators of Hg in those environments. SRB
also seem to have a role in demethylating MMHg. Nitrogen cycling bacteria and FeRB
also may demethylate MMHg. It seems more likely that bacterial MMHg demethylation
is limited or enhanced by NO$_3^-$ and Fe$^{3+}$, or MMHg demethylation is due to an abiotic
mechanism in Fe$^{3+}$ amendments. Methanogens do not seem to play a major role in
methylating Hg or demethylating MMHg.
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In a nutshell:

- Anthropogenic activities have increased human exposure to monomethylmercury (MMHg), which occurs primarily through fish consumption.
- Bacteria produce MMHg that is biomagnified in foodwebs.
- Sulfate-reducing bacteria (SRB) are thought to be the primary agents of Hg methylation. However, recent work suggests that other bacteria can transform MMHg.
- Policy decisions pertaining to future Hg deposition, carbon dioxide concentrations, and nutrient and acid deposition can potentially alter MMHg production and, ultimately, human exposure.
Abstract. Anthropogenic activities, especially fossil fuel combustion, contribute substantially to mercury (Hg) contamination of our environment. Hg undergoes multiple transformations—the most important being methylation, a process in which microorganisms convert Hg into toxic monomethylmercury (MMHg). MMHg bioaccumulates and biomagnifies in foodwebs, and often ends up in the fish that we consume. Sulfate-reducing bacteria (SRB) have long been hypothesized to be the primary agents of mercury methylation in aquatic ecosystems. Recent studies, however, demonstrate that Hg transformations may be more universal than thought previously. Short-term policy decisions aimed at altering Hg deposition, atmospheric carbon dioxide concentrations, and nutrient and acid deposition are likely to affect Hg methylation and MMHg burdens in the fish we consume for many decades.
Fate and transport of Hg

From the sky to our bodies

Mercury (Hg) is a pervasive, global contaminant that can have detrimental health effects for humans (Mergler et al. 2007) and wildlife (Scheuhammer et al. 2007). Humans are exposed to Hg mostly in a form called monomethylmercury (MMHg; Fitzgerald and Clarkson 1991). MMHg is the toxic, organic form of Hg that accumulates easily in organisms and biomagnifies in foodwebs (Wiener et al. 2003). Fish consumption is the most common route of human exposure to MMHg (Clarkson et al. 2003). Because of the ubiquitous distribution, high toxicity, and elevated levels of MMHg in fish, there are fish consumption advisories in 48 of the United States and a large portion of its coastal waters (U.S. EPA 2010; Figure 1a-d). In 2007, mercury contamination was the reason for 80% of all fish consumption advisories in the U.S. (U.S. EPA 2010). Because (1) Hg is important to human health and (2) there are myriad biogeochemical factors controlling the fate and transport of Hg, we are presenting this review of Hg as groundwork for evaluating how future policy decisions may affect human exposure to MMHg.

About one-third of environmental Hg is from natural sources (eg volcanism, weathering of mineral deposits; Fitzgerald and Lamborg 2003). The majority of environmental Hg is due to recent anthropogenic activities—principally fossil fuel combustion and, specifically, the burning of coal (Pacyna et al. 2006). Elemental mercury (Hg$^0$) is the predominant chemical species of Hg emitted anthropogenically (Pacyna et al. 2006). Hg$^0$ also has a long residence time in the troposphere (~1 year; Lamborg et al. 2002a), which results in global distribution and contamination of pristine environments, such as the Arctic (UNEP, 2002). Moreover, Hg loadings have increased
about 3 times since the onset of the Industrial Revolution. This is supported by Hg levels in sedimentary archives, such as lake sediment cores and peat bog cores (Fitzgerald et al. 1998). This increase implies that we have tremendous influence over Hg contamination, and that our actions can augment or reduce Hg contamination of our environment.

Hg has a complex biogeochemical cycle that includes a variety of transformations in the atmosphere, surface waters, and sediments (Figure 2a). It is removed from the atmosphere principally by oxidation to Hg$^{2+}$, which is deposited in wet and dry deposition (Fitzgerald and Lamborg 2003). A large portion of Hg$^{2+}$ that is deposited in aquatic ecosystems is sequestered in sediments. A key transformation is Hg methylation, where inorganic Hg$^{2+}$ is converted to an organic form through the addition of a methyl group (ie CH$_3$). This process produces toxic MMHg (ie CH$_3$Hg$^+$). Researchers hypothesize that one of the principal sources of MMHg in aquatic ecosystems is microbially-mediated Hg methylation in surficial sediments (Balcom et al. 2004). Once MMHg is produced, it is thought to be (1) taken up directly by benthic organisms, or (2) mobilized from sediments into the water column where it can biomagnify in foodwebs.

MMHg demethylation reverses the Hg methylation process by removing the methyl group, which results in less toxic, inorganic forms of Hg. MMHg can be demethylated by abiotic processes (eg photodecomposition) or by microbially-mediated processing, which occurs largely in the benthos (Monperrus et al. 2007). The efflux of MMHg from sediments to the overlying water column can be measured as a proxy of net MMHg production (ie net methylation = Hg methylation - MMHg demethylation), which is crucial in determining the potential amount of MMHg available for bioaccumulation in fish (Hammerschmidt and Fitzgerald 2006a).
Connecting the dots

The global fate and transport of Hg (Figure 2a) have not been established unequivocally, in part because scientists cannot quantify directly all of the connections between anthropogenic sources of Hg and human exposure to MMHg. There is evidence that connects a few of the links simultaneously, and some of the links between these processes are stronger than others, allowing researchers to draw a plausible picture of the biogeochemical cycle of Hg. Furthermore, we can link anthropogenic sources of Hg to MMHg indirectly (Figure 2b–c). There is a coupling between atmospheric carbon dioxide (CO₂) concentrations and Hg deposition versus time for a location in the southern hemisphere, distant from sources of pollution (Figure 2b). The positive correlation between CO₂ and Hg (Figure 2c) allows us to extrapolate Hg deposition based on CO₂ projections from the Intergovernmental Panel on Climate Change (IPCC 2007). If unchecked, atmospheric CO₂ concentrations are expected to reach 650–970 ppm by year 2100, which implies that Hg deposition will be 3–6x the current amount.

What does this mean for MMHg concentrations in fish and potential human exposure? We can use current measurements of MMHg in fish versus Hg deposition to predict MMHg concentrations in fish in 2100 (Hammerschmidt and Fitzgerald 2006b; Figure 2d). If we assume a conservative increase in atmospheric CO₂ and Hg deposition (ie 650 ppm CO₂ and 3x present-day Hg deposition), many fish will exceed the U.S. EPA fish consumption advisory (0.3 ug g⁻¹) and most bass will have sufficient MMHg concentrations to exhibit reproductive problems (~ 0.8 ug g⁻¹; Hammerschmidt et al. 2002).
Given the current and projected exposure to Hg in our environment, we ask: What actions can reduce current and future human exposure to MMHg? To begin answering this question, we: (1) Review the known and unknown controls on Hg transformation, and (2) hypothesize how policies related to CO₂ emissions or atmospheric deposition might affect human exposure.

- The known and unknown controls on Hg transformations

While researchers have recently measured deposition rates and fluxes of Hg to and between ecosystems (Mason and Sheu, 2002), our understanding of the controls on these fluxes remains incomplete. Which microbial guilds are primarily responsible for Hg methylation? And MMHg demethylation? How and why do microorganisms carry out these processes? We review what is known about these questions, as well as some new work challenging the existing paradigms.

Proximal and distal controls on Hg transformations

A variety of environmental factors influence the mechanisms and rates of Hg methylation and MMHg demethylation by affecting: (1) The amount of inorganic Hg²⁺; (2) the chemical speciation, or form, of Hg; and (3) the composition and behavior of benthic organisms and bacteria (Figure 3). Inorganic Hg²⁺ inputs are a proximal control on net MMHg production globally (Hammerschmidt et al. 2004; Hammerschmidt and Fitzgerald 2006; Hammerschmidt et al. 2006a; Hammerschmidt et al. 2006b). The greater the inorganic Hg²⁺ input, the more substrate there is available to form toxic MMHg; therefore, changes in anthropogenic inputs of Hg into the environment directly affect the amount of MMHg produced. Another proximal control is the chemical species of Hg that forms, which depends on the presence of binding ligands and reduction-
oxidation (redox) conditions. Spatial and temporal variations in biotic factors also influence net MMHg production. Redox conditions and seasonality, for example, affect the composition and distribution of organisms, whose activity (eg bioturbation by benthic organisms; Benoit et al. 2009), in turn, affects net MMHg production.

These controls influence profoundly Hg transformations and mobility in different media; however, the cumulative effect on net methylation still is unresolved. Multiple, compounding environmental controls make it difficult to assess how individual factors affect net MMHg production—eg is bioturbation or a specific ligand more important for a given ecosystem?

**The microbial connection: The SRB paradigm**

Sulfate-reducing bacteria (SRB) have long been implicated as the principal methylators of Hg in various natural and laboratory settings (Compeau and Bartha 1985; Gilmour et al. 1992; Benoit et al. 1999; King et al. 1999; King et al. 2000). SRB use organic compounds as a source of (1) carbon for assimilation (ie building biomass) and (2) electrons. They use sulfate as an external electron acceptor; hence, they are called “sulfate-reducing” bacteria. Other forms of dissimilatory respiration are named accordingly; for example, iron-reducing bacteria (FeRB) use iron (ie Fe$^{3+}$) as an external electron acceptor, which is reduced to Fe$^{2+}$. In the aforementioned studies, scientists used molybdate to inhibit SRB, because it causes cell death (Wilson and Bandurski 1958). However, as we will explain later, researchers have modified this technique, revealing that other microbial guilds besides SRB may be important in transforming Hg.

How Hg enters bacterial cells and is methylated remains a “black-box” process. MMHg production by bacterial cells is associated with several methylating agents
(Landner 1971; Choi and Bartha 1993; Schaefer and Morel 2009) and metabolic pathways (Choi et al. 1994a, 1994b). The current hypothesis is that under sub-oxic conditions, a small, neutral, dissolved form of Hg (such as HgS\(^0\); Benoit et al. 1999) diffuses across the cellular membrane, and is methylated intracellularly and inadvertently (Ekstrom et al. 2003).

In contrast, the mechanisms of MMHg demethylation are better understood. Microbial MMHg demethylation comes in two flavors—oxidative and reductive. Generalized oxidative demethylation typically takes place in surficial sediments with little Hg pollution and is marked by the production of CO\(_2\) (Oremland et al. 1991; Marvin-Dipasquale et al. 2000). Reductive demethylation, on the other hand, is thought to be a detoxification mechanism, because it occurs in highly contaminated deposits (Marvin-Dipasquale et al. 2000). The genetic processes involved in detoxifying Hg have been explored thoroughly (Robinson and Tuovinen 1984; Nazaret et al. 1994; Barkay et al. 2003; Schaefer et al. 2004). The main genetic component is the mer operon, which produces enzymes that (1) cleave the methyl group from MMHg and reduce it to methane, and (2) reduce the remaining Hg\(^{2+}\) to Hg\(^0\) (Robinson and Tuovinen 1984). Hg resistant strains can confer resistance to Hg susceptible cells through horizontal gene transfer (Robinson and Tuovinen 1984; Ehrlich and Newman 2009) of the mer operon. Horizontal gene transfer may explain partly why MMHg demethylation takes place among many different bacterial guilds and, perhaps, why Hg methylation also is universal.
Challenging the SRB paradigm

Recent data challenge the so-called SRB paradigm. Fleming et al. (2006) inhibited SRB in iron-rich, Hg-polluted, freshwater sediments with “environmentally appropriate” levels of molybdate. The key difference between this study and past studies is that Fleming et al. (2006) added molybdate at concentrations equimolar to ambient sulfate concentrations. Previous studies often used excessive molybdate concentrations, which probably inhibited many microbial processes and may have lead to an incorrect interpretation of findings. Fleming et al. (2006) found that a strain of iron-reducing bacteria (FeRB) produced a significant portion of MMHg in their sediments. Results of Fleming et al. (2006) lead us to question whether (1) FeRB might be important producers of MMHg, and (2) microorganisms other than SRB may contribute substantially to MMHg production.

Based on Fleming et al.’s (2006) work, we might refine our understanding of microbial MMHg production to “SRB versus FeRB.” However, not all SRB or FeRB methylate Hg. Kerin et al. (2006) isolated 2 species of FeRB that could methylate Hg and another that could not. Moreover, they discovered that active iron-reduction is not necessary for MMHg production (Kerin et al. 2006). Broader classification schemes do not explain variation in the ability to methylate Hg. Carbon metabolism alone does not explain MMHg production capability (Ekstrom et al. 2003). In terms of respiration type, aerobes, anaerobes, and facultative anaerobes (eg denitrifiers; Cleckner et al. 1999) can produce MMHg (Robinson and Tuovinen 1984). The syntrophy, or cooperation, between SRB and methanogens may play a key role Hg methylation (Pak and Bartha 1998a). Organisms in domains other than bacteria—such as archaea (eg methanogens)
and eukarya (eg fungi)—can methylate Hg (Wood et al. 1968; Landner 1971). The ability to methylate Hg may be better explained by genetic relationships, because some strains of SRB and FeRB are closely related (Longergan et al. 1996); however, this must be explored further.

Researchers know little about the primary demethylators of MMHg in various ecosystems. Bacteria capable of producing MMHg also can demethylate it, which means that a bacterial cell could carry out both processes simultaneously. Compeau and Bartha (1984) found that MMHg demethylation is favored at a redox potential of +110 mV, which is near the zone of iron-reduction. Oxidative demethylation is prominent in zones of sulfate-reduction and methanogenesis (Pak and Bartha 1998b; Oremland et al. 1991). Reductive demethylation is carried out by both aerobes and anaerobes (Ehrlich and Newman 2009), and the genetic marker (ie mer operon) has been found in both bacteria and eukarya domains (Ehrlich and Newman 2009). Again, broad classification schemes are unable to explain variation in the ability to demethylate MMHg.

- **Policy implications**

The controls on Hg transformations are unresolved and the ability to transform Hg is more universal than we thought previously. Despite these uncertainties, how might changes in environmental policies affect Hg cycling and, ultimately, human exposure? Specifically, how can we create a disconnect between atmospheric carbon dioxide emissions, Hg deposition, and MMHg levels in fish? Based on Figure 2b, we must (1) decrease Hg deposition, and/or (2) reduce atmospheric carbon dioxide concentrations.

Decisions that affect nutrient and acid deposition also will have feedback on Hg cycling and MMHg levels in fish. Emissions of sulfur and nitrogen—which are normally
in the forms of sulfur dioxide and nitrogen oxides (ie SO\textsubscript{2} and NO\textsubscript{x}) from electric utilities and vehicles—are converted into sulfuric and nitric acid, which cause acidification, or a lowering of pH (Driscoll \textit{et al.} 2001). Acidification can enhance Hg deposition and exacerbate bioaccumulation of MMHg in fish (Driscoll \textit{et al.} 1994). Changes in nutrient deposition also affect electron acceptor availability (eg of sulfate and nitrate), which can stimulate or inhibit microbial Hg methylation (Gilmour \textit{et al.} 1992; Gilmour \textit{et al.} 1998; Benoit \textit{et al.} 2003).

The most recent policy initiatives intended to affect Hg cycling, CO\textsubscript{2} emissions, and nutrient and acid deposition include: (1) The U.S. Clean Air Mercury Rule (CAMR; a part of former President Bush’s Clear Skies Initiative), which called for a cap-and-trade on Hg emissions, but was abandoned in 2008 and awaits response from the U.S. Supreme Court; (2) The United Nations Framework Convention on Climate Change’s Conference of the Parties 15 (UNFCCC COP15), which sought to establish an international climate change agreement following the expiration of the Kyoto Protocol; and (3) the U.S. EPA’s Clean Air Interstate Rule (CAIR; also a part of former President Bush’s Clear Skies Initiative), intended to control SO\textsubscript{2} and NO\textsubscript{x}, which was remanded without vacatur in 2008, meaning that it remains in effect until the U.S. EPA devises another rule.

\textbf{Decreasing Hg deposition}

Previous reductions in upstream Hg emissions illustrate positive impacts. In the northeastern U.S., for example, decreased regional Hg deposition resulted in decreased MMHg burdens in organisms—such as zooplankton, fish, and common loons—over short timescales (Driscoll \textit{et al.} 2007a; Evers \textit{et al.} 2007). This suggests that future
reductions in Hg emissions can reduce MMHg levels in fish for certain ecosystems. We can achieve this through international and national agreements and specific technologies. In 2009, the U.S. and 140 nations agreed to an international treaty to tackle global Hg pollution (Burton 2009). Although CAMR was vacated in 2008, many of the technologies employed to meet CAIR will help remove Hg indirectly. Furthermore, many states are implementing their own Hg monitoring programs, and if and when new federal requirements for Hg are enacted, it will likely result in more stringent requirements and the inclusion of new technologies (Levin and Yanca 2008). Future regulations should target all chemical species of Hg. About 25% of U.S. coal-fired electric utilities are equipped with wet scrubber technologies, which capture 90% of Hg\(^{2+}\) and practically no Hg\(^{0}\) (Pavlish et al. 2003). Other technologies can improve removal of Hg (eg electrostatic precipitators, baghouses, and sorbents; Olson et al. 2000) and several can remove SO\(_2\), NO\(_x\), Hg\(^{2+}\), and Hg\(^{0}\) simultaneously (Hutson et al. 2008). Future regulations also should include measures to reduce Hg deposition in areas susceptible to Hg bioaccumulation (ie biological hotspots of Hg bioaccumulation; Evers et al. 2007), particularly under a Hg cap-and-trade scenario (Driscoll et al. 2007b).

Decreasing atmospheric CO\(_2\) concentrations

Since the 1990s, there have been several international efforts to reduce CO\(_2\) emissions. Only recently, there have been more national attempts to control green house gases. The 1997 Kyoto Protocol, which the U.S. signed but never ratified, was the first international treaty aimed at stabilizing atmospheric green house gases (GHGs). It also laid the groundwork for GHG cap-and-trade systems in many countries, including the entire European Union. The COP 15 international climate talks of 2009 were supposed to build
off of the Kyoto Protocol, but fell short of global expectations. The same year, the U.S. EPA declared CO$_2$ and 5 other GHGs as national pollutants and mandated nationwide reporting by large emitters (U.S. EPA 2010). A cap-and-trade bill on GHGs was passed in the U.S. House of Representatives following the COP 15 meeting; however, without a strong international framework, the bill in the U.S. Senate failed. The future of controlling CO$_2$ and other GHGs in the U.S. Congress remains unclear. However, U.S. GHG emissions from other sources are being targeted—e.g. CAFE vehicle emission standards must reach 35.5 mpg by 2016 (U.S. EPA, 2010).

**Decreasing nutrient and acid deposition**

The 1970 and 1990 Clean Air Act Amendments are considered successful, because SO$_2$ concentrations in wet deposition and freshwaters decreased markedly and NO$_x$ concentrations in wet deposition increased only slightly (Driscoll *et al.* 2001). Nevertheless, levels remain above background levels and many aquatic organisms are still threatened by low pH (Driscoll *et al.* 2001). The Acid Rain Program served as a model for CAIR, which calls for deeper cuts in SO$_2$ and NO$_x$ emissions (U.S. EPA, 2010). Reductions from CAIR are likely to reduce nutrient and acid deposition, as well as Hg deposition and MMHg levels in fish indirectly, moreso than without its implementation. Similar to the cap-and-trade on Hg emissions from electric utilities, precaution must be taken to avoid hotspots of nutrient and acid deposition in areas downwind from larger emission sources. Improving CAFE standards also will be important in controlling NO$_x$ inputs into the environment (Driscoll *et al.* 2001).
Where do we go from here?
Even though CAMR, CAIR, and COP 15 were less than ideal, we should use them as platforms in improving future policies on Hg, GHGs, SO₂, and NOₓ. Many of the ways to reduce human exposure to MMHg can be achieved indirectly by controlling other biogeochemical constituents, whose reduction will benefit humans in other ways. Control of CO₂ emissions, for example, will help reduce Hg deposition and mitigate climate change. There are clearly “win-win” scenarios that we can, and must, pursue.

- Conclusions
Anthropogenic activities, namely fossil fuel combustion, contribute substantially to Hg contamination of the environment. Inorganic Hg is converted by bacteria in sediments to toxic MMHg, which biomagnifies in aquatic foodwebs and ends up in the fish that humans consume. While SRB have long been thought to be the primary agents of MMHg production in sediments, recent findings demonstrate that microbial Hg transformations (ie Hg methylation and MMHg demethylation) are more universal than we thought previously. In addition, many of the biogeochemical factors that control Hg transformations are not fully understood. Therefore, once inorganic Hg is released into the environment, we cannot accurately or completely predict how it will affect Hg transformations, MMHg burdens in organisms, or human exposure. We suggest, however, that human exposure can be minimized by reducing Hg deposition, atmospheric CO₂ concentrations, and/or nutrient and acid deposition. Recent international and national policy initiatives are aimed at reducing these factors. While there are weaknesses in these initiatives—eg cap-and-trade poses the possibility of hotspots of Hg
bioaccumulation or nutrient and acid deposition—we must recognize that the likelihood of reducing human exposure to MMHg is greater than under a scenario of inaction.

**Literature Cited**


APPENDIX A, FIGURES

(1a) Total Mercury in Wet Deposition, 2008

(1b) Mercury in Fish

Source: Kamman et al. 2005
Figure 1. (a) National trends in total mercury concentration in wet deposition (i.e. precipitation) for 2008 (NADP/MDN); (b) Hg levels in fish in relation to mean length and comparison to U.S. EPA human health criterion (Driscoll et al. 2007); (c) sign warning against consumption of fish due to Hg contamination; (d) number and type of fish consumption advisories for each of the United States and its territories (U.S. EPA 2007).
Figure 2. (a) Mercury cycle with emphasis on sources, transformations, and biomagnification in food webs; (b) atmospheric CO$_2$ concentrations (Etheridge et al. 1996; Keeling and Whorf 2008) and Hg deposition — both normalized to pre-industrial levels—versus time based on sedimentary cores from New Zealand (Lamborg et al. 2002b); (c) measured and predicted correlations between atmospheric CO$_2$ concentrations (Etheridge et al. 1996; Keeling and Whorf 2008) and Hg deposition based on sedimentary cores from New Zealand (Lamborg et al. 2002b); (d) measured and predicted MMHg levels in bass versus wet atmospheric Hg deposition; brown dotted line is U.S. EPA fish consumption advisory and blue dotted line refers to reproductive effects (i.e., inhibited/delayed spawning and reduced egg production; Hammerschmidt et al. 2002).
Figure 3. Proximal and distal controls on Hg transformation processes (ie Hg methylation and MMHg demethylation).
Figure 4. Hg methylation (purple) and MMHg demethylation at bacterial cell level. Demethylation processes: General oxidative demethylation (green to orange), reductive demethylation (green to red) and reduction of mercury (blue).