Quantitative Assessment of Mercury Methylation by Phylogenetically Diverse Consortia of Sulfate-Reducing Bacteria in Salt Marsh Systems

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Quantitative Assessment of Mercury Methylation by Phylogenetically Diverse Consortia of Sulfate-Reducing Bacteria in Salt Marsh Systems

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DEDICATION

This thesis is dedicated in loving memory of my mother,

Sandra Ann King

The love, guidance, and never-ending encouragement she gave continue to be an inspiration in my life. Thank you mom for everything.
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I would like to emphasize that this thesis is a product of the contributions of many people who provided help either directly or indirectly towards its completion. First, I would like to thank God for providing me with His grace, love, and presence throughout my life. I know that this would not be possible without His direction and strength.

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<tr>
<td>$\beta$</td>
<td>mercuric ion availability</td>
</tr>
<tr>
<td>$\gamma$</td>
<td>coefficient of microbial activity</td>
</tr>
<tr>
<td>$\mu$</td>
<td>net specific growth rate</td>
</tr>
<tr>
<td>$\mu g$</td>
<td>microgram</td>
</tr>
<tr>
<td>$\mu L$</td>
<td>microliter</td>
</tr>
<tr>
<td>$\mu m$</td>
<td>micrometer</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>CH$_4$</td>
<td>methane</td>
</tr>
<tr>
<td>CH$_3$Hg$^+$</td>
<td>methylmercury</td>
</tr>
<tr>
<td>cm</td>
<td>centimeter</td>
</tr>
<tr>
<td>Co</td>
<td>cobalt</td>
</tr>
<tr>
<td>CO$_2$</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>°C</td>
<td>temperature in Celsius</td>
</tr>
<tr>
<td>d</td>
<td>day</td>
</tr>
<tr>
<td>dH$_2$O</td>
<td>deionized water</td>
</tr>
<tr>
<td>Da</td>
<td>dalton (unit of mass precisely equal to 1.00000 on a molecular weight scale)</td>
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**DBACTER**

*Desulfbacter*

**DHHS**

United States Department of Health and Human Services
dissolved organic carbon

*Desulfobulbus*

*Desulfobacterium*

*Desulfococcus*

*Desulfovibrio*

electron potential

United States Environmental Protection Agency

$\hat{f}$ maximum value of incidence of mercury methylation (mole of methylmercury generated per mole sulfate reduced)

$f$ “Net” incidence of methylmercury generated relative to sulfate reduced and available mercury

g gravity

$H_2$ hydrogen gas

$H^+$ hydrogen ion

Hg mercury

Hg$^0$ elemental mercury (Hg(0))

Hg$^{2+}$ inorganic mercury (Hg (II))

hr hour

$k_e$ first-order hydrolysis of particulate organic matter

$K_{Hg}$ half-velocity constant for mercury

$K_s$ half-velocity constant for organic-carbon substrate

$K_{SO_4}$ half-velocity constant for sulfate
\( K_{sp} \)

solubility product constant

\( m \)
meter

\( \text{min} \)
minute

\( \text{mL} \)
milliliter

MMR
mercury methylation rate

NSMR
net specific mercury methylation rate

\( N_2 \)
nitrogen gas

\( P \)
phosphorous

\( \text{pg} \)
picogram

\( \text{pH} \)
\(-\log [H^+]\) with \([H^+] = \text{mole/L}\)

\( \text{ppt} \)
parts per thousand (salinity)

rRNA
ribosomal ribonucleic acid

\( S \) (of 16S)
sedimentation coefficient

\( S^{2-} \)
sulfide

\( \text{SO}_4^{2-} \)
sulfate

\([\text{SO}_4]\)
sulfate concentration (moles/L)

SRB
sulfate reducing bacteria

SRR
sulfate reduction rate (nmoles/g_{dry-hr})

\( S_s \)
organic-carbon substrate

\( Y \)
yield coefficient

\( X_B \)
microbial biomass
SUMMARY

The methylation of mercury by sulfate reducing bacteria in marine sediments has been confirmed with dynamic studies using intact sediment cores and anoxic slurry systems. Key factors influencing the incidence of mercury methylation were availability of soluble mercury in pore waters, microbial respiration measured as rates of sulfate reduction, and microbial speciation of sulfate reducing bacterial populations.

Dynamic studies with sediment cores and anoxic slurries (~12%) of sediments were used to establish the coupling of mercury methylation to sulfate reduction by consortia of sulfate reducing bacteria. The coupling was confirmed using temperature variations, substrate additions, and sulfate-reduction inhibitors.

The incidence of mercury methylation relative to sulfate reduction was dramatically affected by the concentration of soluble mercury in pore waters and followed a saturation-kinetic relationship when examined on the basis of applied mercury. The methylation of mercury was further demonstrated to occur at pore water concentrations of 30-230ng/L (0.15-1.15 x 10^-9 M) of soluble mercury.

The incidence of methylation varied significantly among species of sulfate reducing bacteria grown in pure culture. The phylogenetic groups Desulfo bacterium and Desulfo bacter demonstrated the greatest incidence of methylmercury production when normalized to sulfate reduction rate, i.e., the respiration rate of the microbial cells. Desulfo bulbus, Desulfovibrio, Desulfococcus species were shown to be present in significant quantities but were minor contributors to methylation of mercury in
sediments. Preconditioning sediments with selective organic substrates was used to successfully alter the composition of the microbial consortia in sediment slurries, as confirmed with 16S rRNA gene probes to identify sediment phylogenetic groups. Furthermore, the changes in the consortia were shown to affect the incidence of methylation in sediment systems using intact cores and sediment slurry reactors. Finally, results for methylation rates with pure culture of sulfate reducing bacteria and gene probe characterization of sediments were used to confirm the effects of microbial consortia composition on methylation in sediments.

The results of this project will lead to a quantitative approach to assessing mercury methylation processes in natural and contaminated systems and to the development of innovative ways of assessing and remediating contaminated sediments.
CHAPTER I
INTRODUCTION

Mercury is considered one of the most toxic metals found in the environment. Contaminated sediments containing mercury continue to generate serious health concerns due to the neurotoxic effects of mercury on mammalian systems. Lavaca Bay, Texas and Brunswick, Ga. (LCP site) have been identified as two sites in the United States with very high mercury concentrations in marine sediments. Mercury concentrations as high as 12,000 mg/kg were reported in sediments of waste settling ponds of a former chloroalkali plant located at the LCP site in Brunswick, GA. (Dept. of Interior, 1995). The mercury poisoning in Minamata Bay, Japan illustrated the potential hazards associated with chronic exposure to mercury, particularly methylmercury (Hosokawa, 1995). The lipophilic nature of methylmercury enhances its ability to be bioaccumulated when compared to inorganic mercury. This results in biomagnification of methylmercury in the food chain (Gardner, 1972; Matida et al., 1971; Heinz, 1974). The cited literature in this thesis documents sulfate-reducing bacteria (SRB) as the principal producers of methylmercury (Andersson et al., 1990; Compeau and Bartha, 1985; Compeau and Bartha, 1984; Blum and Bartha, 1980; Gilmour and Capone, 1987). Several studies have suggested that sulfate reduction by SRB correlates with methylmercury production. Microbial-inhibition studies utilizing molybdate, an inhibitor of sulfate-reduction,
effectively reduced the methylation of mercury by 95% (Compeau and Bartha, 1985). Other studies have demonstrated that SRB fermenting substrates in the absence of sulfate, do not generate methylmercury from available inorganic mercury (Pak and Bartha, 1998b). Based on these studies, an approach was postulated to explain the complex relationship that exists between sulfate reduction and mercury methylation by SRB.

**Problem Statements**

There are no quantitative relationships to predict MMR in marine sediments based on SRB, SRR and mercury concentrations. Since SRB utilize sulfate as a terminal electron acceptor in respiration and are principal methylators of mercury in sediment systems, a quantitative correlation should exist between mercury methylation and sulfate reduction rates.

There is limited information on the abilities of SRB species to methylate mercury. In the past, the SRB genus *Desulfovibrio* has been used in pure culture to demonstrate the mercury methylation potential of the SRB group under various environmental conditions (e.g., Compeau and Bartha, 1985; Pak and Bartha, 1998b; Pak and Bartha, 1998b). At the time of this publication (1999), there are nineteen recognized genera of SRB (Widdel and Bak, 1992; Brock *et al.*, 1994). Thus, the possibility exists that each genera may have different potentials to methylate mercury on a per cell basis. This problem becomes even more complex if the MMR of each individual genera is normalized in terms of the sulfate-reduction rate (SRR).
Objectives

The thrust of this research is to establish a quantitative understanding of the microbial populations that methylate mercury in marine sediments. Fundamental techniques for assessing biochemical processes in sediments were utilized in conjunction with advanced quantitative measurements of microbial populations. The following represent the objective of this dissertation.

(1) Quantify mercury methylation in active sulfate-reducing environments and establish a fundamental relationship between MMR and SRR for sediments using slurry batch reactors and intact cores.

(2) Establish gene-probe techniques to identify and quantify mercury-methylating SRB communities that exist in natural-environment sediment cores and slurry reactors.

(3) Quantitatively establish relationship between sulfate reduction and mercury methylation utilizing various species of SRB, organic substrates, and ionic mercury in slurry-based enrichment cultures.

(4) Demonstrate relevance of batch reactor and pure culture results to sediment column profiles by comparing to in situ microbial populations, SRRs, and methylmercury production/concentrations.
The proposed research is structured to provide a quantitative measure of the kinetic relationship between mercury methylation and sediment SRR. As mentioned previously, SRB have been implicated as the primary producers of methylmercury in anaerobic sediments. From an environmental engineering perspective, knowledge of in situ SRB populations and regions of enhanced methylmercury production would aide in remediation efforts. Moreover, an ability to predict MMR based on mercury concentrations, SRR profiles, and SRB profiles would impact the priority placed on the remediation of various contaminated sites.
CHAPTER II

LITERATURE REVIEW

Mercury in the Environment

Global Record of Mercury Emissions and Depositions

Mercury is a naturally occurring element found in rocks, soil, air, and water (Stein et al., 1996). The amount of mercury that has existed on earth has remained the same since the beginning of time. However, concerns have increased over the amount of mercury that is available for human exposure. Comparisons of recent findings to historical data suggest that total global atmospheric mercury burdens have increased since the beginning of the industrialized period by a factor of two to five (EPA, 1997). A study of peat cores resulted in evidence which suggests that present day mercury deposition is two to three times greater than pre-industrial levels (Zillioux, 1991). In addition, Lindqvist (1991) concluded that present day Swedish lakes contain five times greater mercury levels than pre-colonial times. Travis and Blaylock (1993) indicated that tree rings and soil/sediment core mercury levels have increased four to five fold since the beginning of the industrial revolution. Although it is generally recognized that atmospheric mercury levels have increased since the industrial revolution, it is uncertain what the trends are for the future. Analysis of remote areas of the Atlantic Ocean show that mercury concentrations
increased up until 1990 with a decrease observed from 1990-1994 (Slemr, 1996). Other remote regions located in northern Canada and Alaska show deposition rates that continue to increase (Lucotte et al., 1995; Engstrom and Swain, 1997). The increase in anthropogenic contributions could significantly affect global distribution and biogeochemical cycling of mercury. Conceivably, a greater concentration of mercury could be transformed into more readily bioaccumulated mercury species such as methylated mercury. The significance of methylmercury and associated health concerns will be discussed in subsequent sections.

**Historical Anthropogenic Sources of Mercury**

In 1968, the total release of mercury in the world was estimated to be 8000 metric tons, of which the United States produced 1000 metric tons (Faust and Osman, 1981). Until the mid 1970's electrolytic preparations of chlorine and caustic soda accounted for the largest industrial use of mercury and were considered one of the most significant sources of contamination in natural waters. Prior to the implementation of environmental regulations, wastewater discharges from these plants contained as much as 0.125-0.250 g of mercury per kg of soda produced (Faust and Osmand, 1981). Mercury has been used in the production of batteries, silent switches, high-intensity street lamps, and fluorescent lights. In addition, mercury has also been used in agricultural applications as fungicides and herbicides. Mercury has received considerable attention for its use as a catalyst in the manufacturing of organic materials and chemicals. Another major source of mercury discharge to the environment includes the burning of fossil fuels. The mercury content of
coal is highly variable with concentrations ranging from 70 to 33,000 µg/kg (Joensuu, 1971). Petroleum obtained from oil fields in California also had variable mercury content with values from 1.9 to 2.9 mg/kg being reported (Fleisher, 1970). In 1996, it was reported that the total annual global input of mercury to the atmosphere was 5000 kg, and as much as 70 to 80% of the total mercury emissions may be related to anthropogenic activities (Fitzgerald and Mason, 1996). Previous reports indicate approximately 80% of the anthropogenic sources of mercury are derived from fossil fuel combustion, mining, smelting, and solid waste incineration. Fifteen percent of anthropogenic mercury sources have been attributed to municipal waste, pesticide, and herbicide deposition. The remaining five percent is distributed by direct discharge of commercial effluent to bodies of water (DHHS, 1992).

Environmental Attenuation of Mercury

The cycling of mercury in the environment is determined by natural, as well as anthropogenic (human), activities. Natural processes that release primarily elemental mercury to the atmosphere are the volatilization of mercury in marine and aquatic environments, volatilization from vegetation, degassing of soils, and volcanic emissions (EPA, 1997). Industrial processes and combustion sources that release mercury into the atmosphere dominate anthropogenic mercury releases. Gaseous mercury emissions are thought to include both elemental and oxidized chemical forms. However, particulate mercury emissions are composed of primarily oxidized compounds due to the high vapor
pressure ($1.6 \times 10^{-4}$ kPa) of elemental mercury (Lindqvist et al., 1991). Natural levels of mercury found in ambient air are generally in the range of 1 to 10 ng/m$^3$ total Hg. However, total atmospheric mercury concentrations as high as 20,000 ng/m$^3$ have been reported at ground levels near mercury ore deposits (McCarthy et al., 1970). Sorption of greater than 90% of atmospheric mercury by particulate matter has been reported (Kothny, 1973). The mercury that is bound to particulate matter can be removed from the air by rainfall or dry deposition. It should be noted that precipitation is the predominant factor that mediates the removal of mercury from the atmosphere. As a result, mercury present in rainfall averages between 0 and 2 µg/L. Ionic mercury species are removed from the air at a significantly faster rate than the elemental form (EPA, 1997). It has been reported that the overall atmospheric lifetimes for mercury range from 90 d to 2 yr depending on the metal partitioning and the meteorological conditions (WHO, 1990). Others have reported an average residence time for elemental mercury to be one year (Porcella et al., 1996).

Once elemental mercury ($\text{Hg}^0$) is released to the atmosphere, it can be oxidized by ozone (O$_3$) to form Hg$^{2+}$ through gas-phase reactions or aqueous phase reactions in clouds or fog (Schroeder et al., 1991; Seigner et al., 1994). It has been hypothesized that the transformation of Hg$^0$ to Hg$^{2+}$ in cloud water demonstrates a possible mechanism by which natural and anthropogenic releases of Hg$^0$ to air can result in mercury depositions to the land and water (Fitzgerald, 1994). The conversion of elemental mercury to the mercuric species in the atmosphere is suspected in global mercury
pollution. However, direct wet deposition of anthropogenic Hg$^{2+}$ is most important in localized pollution where it is re-deposited in soil and surface waters through rainfall (Fitzgerald, 1994; Lindqvist et al., 1991).

Once mercury is deposited in soils, sediments, and surface waters the activity, mobility, and deposition of mercury can be influenced by a multitude of physical/chemical environmental factors. For example, the presence and amount of organic carbon, pH, oxidative-reduction potential (ORP), temperature, and presence of sulfide dictate the partitioning of mercury and types of mercury species found in a given environment. Biologically mediated processes which effect mercury speciation will be discussed in a later section. Background concentrations of mercury in the open ocean range from 0.5 to 3.0 ng/L, and "non-contaminated" coastal estuaries have been reported to have concentrations ranging from 2.0 to 15.0 ng/L (Schroeder, 1989). Elemental mercury can also be deposited in surface waters as well, although water solubility is extremely low e.g., 2.8x10^{-7} \text{ mol/L} or 56 \text{ \mu g/L} (Stein et al., 1996). However, under oxic conditions elemental mercury can be quickly oxidized to the mercuric ion (Seigneur et al., 1994).

The aqueous solubility of the mercuric ion is very low as well. Bodek et al. (1988) reported the aqueous solubility of Hg$^{++}$ was equivalent to 39 \text{ \mu g/L}. However, when the mercuric ion was allowed to form complexes with anionic species, the solubility increased significantly. Bodek et al. (1988) reported that the mercuric ion complexed to two OH$^{-}$ increased the solubility of Hg$^{2+}$ to 107 \text{ \mu g/L}. Similarly, the presence of other anions increased mercuric ion solubility even further. Chloride (Cl$^{-}$) added to an Hg(OH)$_{2}$
solution increased mercuric ion solubility by a factor of 55 (Bodek et al., 1988). In natural systems, which are under oxidizing conditions, the major concentrations of divalent mercury are with chloride ions and hydroxide ions. Bodek et al. (1988) have reported that HgCl\(_2\) and Hg(OH)\(_2\) are the major divalent mercury species found under oxidizing conditions in lakes. However, HgCl\(_4^{2-}\) is the dominant species in oxidized salt waters.

Under reducing conditions, the presence of sulfide in a system greatly affects the availability of inorganic mercury. In environments that have low redox potentials and high sulfide concentrations, divalent mercury will be precipitated as cinnabar (HgS). Cinnabar has an extremely low water solubility with a \(K_{wp}\) (solubility-product constant) reported at \(10^{-53}\) (Bodek et al., 1988). The complexing of mercury with the sulfide ion represents the primary mechanism by which mercury can be immobilized in reducing sediments (Faust and Osman, 1981; Bodek et al., 1988; Gilmour and Henry, 1991). Thus, reducing sediments that are rich in sulfide represent an important sink for mercury. However, mercury bound to sulfide may become resolubilized if the sediment or overlying water becomes re-oxidized (Stein et al., 1996).

Sorption phenomena also play a role in the partitioning of mercury in terrestrial and aquatic environments. Divalent mercury (Hg\(^{2+}\)) forms strong complexes with both humic and fulvic acids. Humic acids are major products formed during decomposition of organic matter. They are typically found in both liminic and marine sediments. Humic acids have been reported to comprise approximately 40% of the organic matter
contained in marine sediments (Nissenbaum and Swaine, 1976). The attractive forces between metal ions with soluble, colloidal, or particulate organic material range from weak bonds, which allow for the metal to be easily replaced, to strong bonds that result in metal chelation. This adsorption of metals onto organic surfaces has been attributed to the general negative charge associated with humics. Fulvic acids play an important role in the transport of heavy metals in water due to their low molecular weight, large number of functional groups, and the fact that fulvic acids are much more soluble than humic fractions (Goldberg, 1965). Andersson et al. (1990) reported that at a pH of 4.0 to 4.5, approximately 98% of mercury added to bulk water was associated with the sediments. Moreover, 50 to 75% of the mercury in the sediments was sorbed to humic acids. It has been suggested that a decrease in pH could allow for other cations to displace mercury that is associated with sulfide anions (Stein et al., 1996). Reimers and Krenkel (1974) reported that Hg$^{2+}$ binding in sediment increased with increasing sediment organic carbon content. At pH < 5 optimal sorption of HgCl$_2$ by organics occurred. At pH > 5 formation of soluble Hg-humate complexes lowered the sorption of mercury.

**Mercury Oxidation States and Speciation**

**Oxidation States**

As a general rule, mercury can exist in three oxidation states as elemental mercury, (Hg (0)), mercuric (Hg (II)), and mercurous (Hg (I)) (EPA, 1997). Elemental mercury is the most common form of mercury found in nature. Elemental mercury is a slivery-white
liquid at ambient temperatures and pressures. Because of its high volatility (i.e., vapor pressure of $1.6 \times 10^{-4}$ kPa), it is constantly being released to the atmosphere under ambient conditions. As a result, elemental mercury is not typically found in nature as a pure confined liquid. Mercurous and mercuric species of mercury can form various inorganic and organic chemical compounds although the mercurous form is thought to be relatively unstable under typical environmental conditions (EPA, 1997).

**Mercury Speciation**

Inorganic species of mercury are predominantly mercuric salts and organomercurics which are defined by the presence of a covalent C-Hg bond (EPA, 1997). It is important to note that the existence of a covalent bond between a carbon and mercuric atom differentiates organomercuric compounds from inorganic mercury compounds that associate with organic material in the environment. Comparing mercury to other metals, mercury has a greater potential to form covalent bonds rather than ionic bonds.

Mercury compounds that are typically observed under ambient conditions are: elemental mercury ($\text{Hg}^0$); the mercuric salts (e.g. $\text{HgCl}_2$, $\text{Hg(OH)}_2$,) and mercuric precipitates (e.g. $\text{HgS}$); methylmercury ($\text{CH}_3\text{Hg}^+$); methylmercuric chloride ($\text{CH}_3\text{HgCl}$); and methylmercuric hydroxide ($\text{CH}_3\text{HgOH}$). The production and distribution of methylmercury in natural systems will be discussed in future sections. Other organomercurics such as dimethylmercury ($\text{(CH}_3)_2\text{Hg}$) and phenylmercury ($\text{(C}_6\text{H}_5\text{)}\text{Hg}$) have been observed in trace amounts (EPA, 1997). For each of the previously mentioned
species, several derivatives of soluble and insoluble mercury species exist. For example, chloride species such as HgCl$_4^-$ and HgCl$_3^-$ are found in seawater. In addition, soluble mercury thiol complexes such as HgS$_2^{2-}$ and HgS$_2^-$ are present in equilibrium with HgS in saturated systems (Stumm and Morgan, 1981). Figure 2.1 illustrates the various species of mercury found in anoxic seawater that is saturated with solid HgS. The diagram indicates that the species that are present vary as a function of pH. In general, the concentration of mercury species affiliated with the halide ions (Cl$^-$, I$^-$, and Br$^-$) decreases in anoxic seawater as pH increases from 6 to 10. The soluble thiol complexes represent the most abundant species of mercury present in anoxic saltwater. The HgS$_2^{2-}$ species of mercury continues to increase as pH increases while the Hg(SH)$_2$ species is maximum in the pH 6-8 range. Finally, the HgS$_2^-$ species is at a maximum concentration in the pH 4-7 range and then decreases with increasing pH (Figure 2.1). From this figure, the solubility of mercury species in anoxic seawater at Cl$^-$ of 0.6 M and S (II) of 0.01 mM is approximately 2-20 ng/L with Hg$^{2+}$ ion at a concentration of approximately $10^{-37}$ to $10^{-40}$ M. The implication with respect to methylation is that the speciation in soluble species is diverse and the “availability” of these species in the fortuitous methylation by microbial cells is a complex issue.

The dissociation tendencies of mercuric compounds depend on the nature of the ligand. Equation 2.1 and 2.2 illustrate dissociation reactions for mercury species in aquatic environments.
Figure 2.1 Mercury speciation in anoxic seawater that is in equilibrium with HgS (Stumm and Morgan, 1981).
In the equations, \( X \) is equated with any electron withdrawing ligand that forms an ionic bond with mercury. \( R \) represents an organic group such as phenyl or methyl that is covalently bound to the mercuric species.

\[
\text{HgX}_2 \leftrightarrow \text{Hg}^{2+} + 2X^- \quad \text{Equation 2.1}
\]

\[
\text{RHgX} \leftrightarrow \text{RHg}^+ + X^- \quad \text{Equation 2.2}
\]

The dissociation tendencies of mercuric compounds (\( \text{HgX}_2 \) or \( \text{RHgX} \)) have been defined based on the nature of the ligand \( X^- \). In general the dissociation of ligands proceeds in the following direction from least to greatest:

\[
\text{F}^- > \text{OCOCH}_3^- \ (\text{low weight organic acid}) > \text{HPO}_4^{2-} = \text{Cl}^- > \text{Br}^- > \text{NH}_3 > \text{OH}^- > \text{SR}^- > \text{S}^2^- \]

Thus, the thiol and sulfide compounds represent the most stable mercury compounds (Faust and Osman, 1981). Mercuric and organomercuric compounds can also undergo ligand exchange reactions with a multitude of ligands that exist in natural waters (Faust and Osman, 1981). These processes involve the interactions of mercuric or organomercuric complexes with other chemical species to form new complexes. Equation 2.3, 2.4, and 2.5 illustrate the ligand exchange reactions where \( X \) and \( Y \) represent two ligands.

\[
\text{HgX}_2 + Y \leftrightarrow \text{HgXY} + X \quad \text{Equation 2.3}
\]

\[
\text{HgXY} + Y \leftrightarrow \text{HgY}_2 + X \quad \text{Equation 2.4}
\]

\[
\text{RHgX} + Y \leftrightarrow \text{RHgY} + X \quad \text{Equation 2.5}
\]
Table 2.1 illustrates some of the various reactions and equilibrium constants for mercury-ligand interactions in natural waters (Faust and Osman, 1981). The information provided clearly indicates that ligand concentrations, pH, and ORP influence the mercury profiles found in natural environments. From the information provided, it is clear that the solubility and speciation of mercury is a complex issue. Clearly all processes discussed in this section must be considered in natural environments. Inevitably, the interactions of mercury with organic matter, anions, oxidative-reductive potential (ORP), pH, and other factors determine mercury speciation as well as aqueous solubility. This in turn is directly related to mercury availability for methylation by sulfate-reducing bacteria. Experiments that will be presented in this thesis demonstrate that the ability of SRB to methylate mercury is directly proportional to the amount of total soluble mercury present in sediment systems.

**Methylmercury in the Environment**

**Production of Methylmercury**

Methylmercury represents another species of mercury present in the environment that can be formed through both abiotic and biotic mechanisms. Although this thesis concentrates on the biologically mediated processes of mercury methylation, arguments have been made for abiotic processes that generate methylmercury as well.
Table 2.1
Equilibrium Constants for Formation of Mercury Species at 25°C and 1 atm.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Log $K_{eq}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{Hg}^{2+} + 2\text{Cl}^- = \text{HgCl}_2(s)$</td>
<td>17.96</td>
</tr>
<tr>
<td>$\text{Hg}^{2+} + 3\text{Cl}^- = \text{HgCl}_3^-$</td>
<td>15.35</td>
</tr>
<tr>
<td>$\text{Hg}^{2+} + 2\text{OH}^- = \text{Hg(OH)}_2(aq)$</td>
<td>21.7</td>
</tr>
<tr>
<td>$\text{Hg}^{2+} + \text{SO}_4^{2-} = \text{HgSO}_4(aq)$</td>
<td>1.42</td>
</tr>
<tr>
<td>$\text{HgS}_2^{2-} = \text{HgS}(s) + \text{S}^2-$</td>
<td>-4.57</td>
</tr>
<tr>
<td>$\text{HgS}(s) = \text{Hg}^{2+} + \text{S}^2-$</td>
<td>-52.37</td>
</tr>
<tr>
<td>$2\text{Hg}^{2+} + 4\text{NH}_3(aq) = 2\text{Hg(NH}_3)_2^{2+}$</td>
<td>19.28</td>
</tr>
</tbody>
</table>

The most convincing argument suggests that humic matter may play a role in abiotic methylation. Nagase et al. (1984) demonstrated that high concentrations of humic acid resulted in mercury methylation at 3.8 mM Hg$^{2+}$. In addition, Weber et al. (1985) observed methylation of Hg$^{2+}$ by fulvic acids at pH values between 4 and 6. Weber (1993) has suggested that the mechanism consist of an electrophilic attack by Hg$^{2+}$ on the fulvic acid. The fact that humic matter is ubiquitous throughout an aquatic environment suggests that the methylation of mercury by organic matter must be considered.
However, a majority of evidence presented in the literature indicates that sulfate-reducing bacteria (SRB) are the primary producers of methylmercury in natural environments, and inhibition of their metabolic activity inhibits methylmercury production. Future sections will focus on the biological production of methylmercury in sediments.

It has been reported that mercury methylation occurs to the greatest extent in the uppermost layers (i.e. top few centimeters) of sediments and decreases substantially with depth. Korthals and Winfrey (1987) demonstrated that the ratio of methylation rates to demethylation rates were less than 1 in the water column of an oligotrophic lake. However, a value of 5.8 was attained at the sediment surface, and this value was observed to decrease with increasing sediment depth. Elevated levels of mercury methylation in the upper sediment zone have been postulated to occur as a result of the presence of active bacterial populations, adequate temperatures, and an abundance of accumulated organic matter. Stein et al. (1996) suggested that methylation of mercury below a depth of 4 to 5 cm is minimal due to the lack of bacteria capable of methylation.

It has become clear that many physical and chemical processes associated with sediment can affect the rate which mercury gets methylated by sulfate-reducing bacteria. The pH, redox potential, salinity, presence of sulfide, organic matter content, and inorganic mercury content of a sediment directly affect mercury methylation rates. Obviously, significant concentrations of inorganic mercury must be made available for methylation. Likewise, an ample amount of organic matter must exist in the sediment. Studies conducted by Choi and Bartha (1994) demonstrated that mercury methylation rates correlated best with sediment organic matter content in saltwater sediments. In a
study of sediment cores, Choi and Bartha (1994) found that sulfate reduction rates and methylmercury concentration were maximum at the sediment/water interface and both proceeded to decrease with depth. Similarly, the organic carbon content of the sediments were also maximum at the surface/water interface and declined with increasing depth. Conceivably, the organic matter would provide a substrate for populations of bacteria capable of methylating mercury. In addition, binding of inorganic mercury (Hg$^{2+}$) with organic matter could result in longer, more relevant bacterial exposures. That is, mercury could be more readily transported in lake and runoff where it ultimately gets incorporated in bacterial rich sediments. In addition, the binding of mercury to organic matter could reduce the sedimentation rate of mercury. Thus, mercury (Hg$^{2+}$) cations would remain in environments that are more favorable to bacterial methylation. Conversely, arguments have been presented which suggest that some forms of dissolved organic carbon (DOC) may hinder mercury methylation. Miskimmin et al. (1992) found that increased concentrations of DOC resulted in decreased rates of mercury methylation in Canadian lakes. It has been hypothesized that the availability of free Hg$^{2+}$ could be reduced by complexing with DOC (Jackson, 1989). Miskimmin et al. (1996) suggested that DOC that was added to cultures could inhibit methylating activity. The data referenced suggest that there are several inconsistencies associated with mercury methylation rates and the organic carbon content of sediments.

The pH of sediments and pore waters can also have a significant effect on methylation rates. Andersson et al. (1990) stated that an increase in methylation rates occurred as the pH of sediment is lowered. It has been suggested that a decrease in pH
resulted in an increased number of protons, which can liberate Hg^{2+} from organic complexes through cation exchange. Miskimmin et al. (1996) also reported similar results with respect to pH and mercury methylation. A reduction in pH from 7.0 to 5.0 produced a significant increase in net mercury methylation rates. Miskimmin et al. (1996) suggested that the increased methylation rate could be attributed to increased protonation of anionic functional groups resulting in reduced complexation with Hg^{2+}. In addition, Miskimmin et al. (1996) suggested that a decrease in pH from 7.0 to 5.0 enhanced the activity of the microbial community and that this resulted in enhanced methylation activity. It has also been suggested that a low pH may catalyze enzyme transfer of methyl groups from methylcobalimin to Hg^{2+} (Andersson et al., 1990).

The electron potential (E°) of a given environment is significant in the methylation of Hg^{2+}. Compeau et al. (1984) effectively demonstrated that maximum production of methylmercury was observed at measured electrode potentials of -220mV. Conversely, minimum values of methylation were observed at electrode potentials of +110mV. Given the fact that most sulfate-reducing bacteria are obligate anaerobes, a reduced environment is indicative of an environment most suitable for sulfate reducing bacteria that, in turn, have been implicated in mercury methylation (Brock et al., 1994).

Compeau et al. (1984) also demonstrated that the salinity of an environment affects methylation rates in marine sediments. Sediments exposed to salinity levels of 4.0 ppt had the highest observed methylation rates while sediments with salinity of 25.0 ppt had the lowest. In addition, Blum and Bartha (1980) found that microbial methylation of mercury is inversely related to salinity in estuarine sediments. Compeau and Bartha
(1983) suggested that mercury methylation would decrease in full strength seawater where \( \text{HgCl}_2 \) is the dominant mercuric chloride, because the methyl group transferred by methylcobalimine in a negatively charged carbanion.

Sulfate concentrations present in sediments and pore waters can also affect the microbial methylation capacity. For example, sulfate concentrations in the water column of lakes have been reported at 0.01-0.02 mM (320 \( \mu \text{g/L} \)-640 \( \mu \text{g/L} \) as S), while ocean concentrations are approximately 28 mM or approximately 900 mg/L. (Holland, 1978). Consequently, sulfate-reducing bacteria have a lower activity in freshwater (Gilmour and Henry, 1991). Gilmour and Henry (1991) hypothesized that mercury methylation in sediments is optimum when concentrations of sulfate range from 200-500 \( \mu \text{M} \). Concentrations of sulfate greater than 500 \( \mu \text{M} \) would be expected to generate large concentrations of sulfide that would prevent methylation due to mercury complexation with the sulfide. The complexation with sulfide would precipitate the metal rendering it unavailable for methylation.

Other experiments conducted on freshwater sediments suggested that sulfate addition enhanced mercury methylation (Gilmour et al., 1992). Gilmour and colleagues demonstrated that additions of sulfate as low as 2 \( \mu \text{M} \) generated substantial increases in methylmercury production above background in freshwater systems (Gilmour et al., 1992). Gilmour and Capone (1987) hypothesized that mercury bound to sulfide in saltmarsh systems reduces the overall amount of mercury available for microbial methylation. It is interesting to note that the researchers observed a decrease in mercury
methylation rates as salinity and sediment sulfide content increased. However, in freshwater systems, concentrations of sulfate lower than 200 μM would limit the sulfate reducing bacteria by producing an environment that is depleted of the terminal electron acceptor. Thus, the number of sulfate reducing bacteria capable of growing in this media would be decreased which would theoretically reduce the net amount of methylmercury produced.

Bioaccumulation and Toxicity Associated with Methylmercury

Research pertaining to methylmercury production increased dramatically in the late 1960’s when more than 700 people in Minamata, Japan were unknowingly poisoned by methylmercury. In sediments, only 0.1% of the mercury which is deposited in sediments is converted to methylmercury (Stern et al., 1996). However, over 50% of the methylmercury found in aquatic systems is found in fish and other aquatic organisms (Anderson et al., 1990). Several experiments have been conducted in which the toxicity of mercury and methylmercury have been characterized in aquatic organisms. In a species of fish (Tricogaster tricopretus), median tolerance levels for a 24-, 48-, and 96-hr periods were reported at mercury concentrations of 0.10, 0.08, and 0.07 mg/L, respectively (Roales and Perlmutter, 1974). The toxicity of methylmercury and inorganic mercury in rainbow trout fry and fingerlings has also been conducted. Wobeser (1975) reported that the median tolerance by trout fry for methylmercury at 24, 48, and 96 hr were 0.084, 0.045, and 0.024
mg/L of methylmercury, respectively. The median toxicity for the fingerlings at the 24-, 48-, and 96-hr timepoints was 0.125, 0.066, and 0.042 mg/L of methylmercury, respectively. It is interesting to note that the median toxicity of inorganic mercury (Hg^{++}) in fingerling trout at 24-hr was 0.903 mg/L (Wobeser, 1975). Wobeser (1975) suggested that the toxicity of methylmercury was seven times that of inorganic mercury. The toxic action of inorganic and organic mercury was reported on the gills which showed morphological change in the epithelial cells which lead to necrosis (Wobeser, 1975). In other toxicological studies related to methylmercury, Matida et al. (1971) fed trout (Homoura mutabilis) and sea fish (Konosirus punctatus) with shellfish from Minamata Bay, Japan that contained 16 μg/g of methylmercury. Matida et al. (1971) reported that the fish showed toxic signs such as loss of appetite, dark color, rolling from side to side, collision with walls, and bending at rest. Autopsy results illustrated that the expression of the above mentioned symptoms occurred when mercury contents of the liver and brain were in the range of 26 to 68 μg/g and 16 to 20 μg/g, respectively.

The toxicity of methylmercury has also been examined in various species of birds. Gardiner (1972) demonstrated that consumption of methylmercury (33 μg/g) resulted in 90, 85, and 7.5% mortality in pheasants, mallards, and chickens, respectively. Doses of methylmercury as low as 3 μg/g caused a reduction in egg laying, as well as an increase in offspring mortality for all three bird species (Heinz, 1974). The bioaccumulation of methylmercury is due in part to its lipid solubility and ability to passively diffuse through plasma membranes. Once inside an organism, methylmercury can concentrate in fats and
muscle tissue. Following adsorption, methylmercury (CH$_3$Hg) can be oxidized to form reactive inorganic mercury, Hg$^{2+}$ (Stein et al., 1990). This results in a culmination of toxic effects ranging from neurological disorders to kidney and renal dysfunction (Casseret and Doull, 1992).

The mass poisoning of the population at Minamata Bay reflects the facilitation of methylmercury through the food chain. In 1968, the Japanese government proclaimed that the toxic-central-nervous system disease had been caused by the ingestion of fish and shellfish which contained methylmercury (Hosokawa, 1995). Over 2,000 people in the Minamata Bay area have been recognized as Minamata disease patients (Hosokawa, 1995). Due to a release of mercury by a chloro-alkali plant, a potential risk was found in Canada in the early 1970’s in two Northern Ontario Native Communities on the English-Wabigoon river system (Rudd et al., 1983). Mercury was found to have accumulated too significant levels (as high as 24 µg/g) in the fish which were the main food supply of the area. Consequently, many Aboriginal people living traditional lifestyles developed high blood mercury levels (Wheatley, 1994). Results of a twenty-year testing program that addressed methylmercury exposure levels in 514 native communities across Canada suggest that many people in the areas tested are at risk for mercury poisoning (Wheatley and Paradis, 1996). A total of 608 individuals were found to have blood levels of mercury over 100 µg/L. In that same group, over 30% of women who were tested had methylmercury levels over 10 µg/g with 16 µg/g the average for the study (Wheatley and Paradis, 1996). This represented an unacceptable risk for fetal exposure which sets the
"risk" levels at 10-20 μg/g (WHO, 1994). The above mentioned finding suggests that exposure to methylmercury continues to be a problem in many parts of the world. Clearly, reducing exposure to methylmercury would result in less chance of adverse health effects particularly during fetal development.

**Characteristics of Sulfate-Reducing Bacteria**

**Sulfate-Reducing Bacteria as Primary Producers of Methylmercury**

In the early studies of mercury methylation, Jensen and Jernolov (1969) successfully demonstrated the ability of biologically active bottom sediments to methylate mercury while sterilized sediments did not. Later, Olson and Cooper (1976) suggested that anaerobic sediments produced the greatest amount of methylated mercury as compared to the aerobic sediments. This finding has been substantiated by results that showed that methylmercury production is greatest in reduced environments (Compeau and Bartha, 1984).

The methanogenic bacteria were ruled out as primary methylators of mercury in sediments after additions of a specific methanogen inhibitor (i.e., bromoethanesulfonate) increased methylmercury production in sediment samples (Compeau and Bartha, 1985). This result was hypothesized to reflect the reduction in competition for substrates. Recently, pure cultures of methanogens demonstrated an inability to methylate mercury (Pak and Bartha, 1998a). Presently, it has been accepted that sulfate reducing bacteria are the primary methylators of mercury in the environment (Stein *et al.*, 1996; Gilmour and
Henry, 1991; Anderson et al., 1990; Compeau and Bartha, 1985). Inhibition studies utilizing molybdate, a specific inhibitor of sulfate reduction, effectively reduced the methylation of mercury in sediments by 95% (Compeau and Bartha, 1985). Enrichment cultures of SRB, as well as pure cultures of Desulfovibrio, have also demonstrated an ability to methylate mercury in sulfate-rich environments (Pak and Bartha, 1998a; Pak and Bartha, 1998b; Compeau and Bartha, 1985). The elucidation of SRB mediated mercury methylation rates of some sediments has been attempted. Using contaminated sediments from the Hudson River and radiolabeled mercury ($^{203}\text{Hg}$), in situ mercury methylation rates in these sediments were estimated at 0.0-0.3 ng/g-d (Gilmour and Capone, 1987). It has been reported that the absolute methylation rate may increase with levels of mercury added to the system. In addition, the rate of mercury methylation also varies with mercury concentrations which makes it difficult to make comparisons between studies (Gilmour, 1991). It has been suggested that the concentration dependence of methylation is a function of both microbiological and chemical parameters. Bisogni and Lawrence (1975) described the net specific methylation rate in the conceptual equation (Equation 2.6):

$$\text{NSMR} = \gamma \beta^n [\text{Hg}_{\text{total}}]^n$$  \hspace{1cm}  \text{(Equation 2.6)}

In the equation, NSMR represents the net specific methylation rate, $\beta$ represents mercuric ion availability, $\gamma$ is a coefficient of microbial activity, $[\text{Hg}_{\text{total}}]$ is the total concentration of mercury in the system, and $n$ is a pseudo-order of the reaction. Data and equations
associated with this thesis will expand on the general premise put forth by this conceptual equation.

Speciation of Sulfate Reducing Bacteria

Sulfate reducing bacteria (SRB) inhabit the anaerobic zones of sediment in which sulfate is plentiful (Gilmour and Henry, 1991). Typically, these organisms utilize sulfate as a terminal electron acceptor and thus sulfate is reduced to sulfide. Mesophilic nonsporeforming species are the most pronounced sulfate reducers found in nature. Sulfate reducing bacteria play a primary role in the sulfur cycle of aquatic systems by serving as the primary means by which sulfate is reduced to sulfide (Gilmour and Capone, 1987). The typical habitats of sulfate reducers are the subsurface parts of aquatic environments, such as anoxic sediments or bottom waters. Marine sediments have the greatest variety of species (Widdel, 1988). This is due, in part, to the concentration of sulfate (approximately 17-28mM) which is thereby not a growth-limiting factor at these concentrations. In addition to marine sediments, sulfate reducers have also been found in rice paddies, freshwater lakes, and anaerobic digesters (Watanabe and Furusaka, 1980; Badziong et al., 1978; Gilmour et al., 1992). Sulfate reducing bacteria as well as sulfate reduction activity have been recorded in oxic freshwater zones as well as oxic marine sediments (Bak and Pfennig, 1991; Jorgensen and Bak, 1991). The existence of anoxic microniches within these oxic environments has not been ruled out (Jorgensen, 1977).

At the time of this manuscript publication (1999), nineteen genera of dissimilatory sulfate reducing bacteria were recognized (Rooney-Varga et al., 1998). The genera have
been placed into two families based on the phylogenetic tree. The Desulfovibrionaceae family includes Desulfovibrio and Desulfomicrobium genera. These bacteria can utilize lactate, pyruvate, fumarate, propionate, ethanol, and other organic acids as carbon and energy sources. The Desulfobacteriaceae family includes Desulfobulbus, Desulfobacter, Desulfooccus, Desulfoarcina, Desulfobacterium, and Desulfonema genera. These bacteria can utilize many of the same organic acids as the Desulfovibrionaceae family. However, species that comprise the genera Desulfobacterium, Desulfooccus, and Desulfobacter can also utilize acetate as carbon and electron source (Rooney-Varga et al., 1998). As a general rule, sulfate-reducing bacteria are considered obligate anaerobes. Sulfate-reducing bacteria are widespread in both aquatic and terrestrial environments that are anoxic (Brock et al., 1994). Using 16S rRNA sequence analysis, predominantly all of the sulfate-reducing bacteria are Gram-negative bacteria in the "delta" subdivision of the purple bacterial group. However, the endospore forming sulfate-reducing group, Desulfotomaculum, aligns more closely with the Clostridium subdivision of the Gram-positive bacteria. Members of the genera Desulfovibrio and Desulfobulbus are found to the same extent in both freshwater and marinewater habitats (Widdel, 1988; Bak and Pfennig, 1991). Bacteria such as Desulfobacter, Desulfobacterium, Desulfoarcina, and Desulfonema may be regarded as primarily marine or brackish water inhabitants (Widdel and Bak, 1992). High numbers of Desulfovibrio species have demonstrated an ability to ferment whey without added sulfate (Zellner et al., 1987). The authors have suggested that even in the absence of sulfate, Desulfovibrio is involved in anaerobic degradation by channeling lactate into methanogenesis. Given the growth kinetic data of
several species of sulfate reducing bacteria, *Desulfo bacter* is probably the main utilizer of acetate in brackish or marine sediments (Widdel, 1988). It has been suggested that the more prevalent sulfate reducing species (*Desulfobacterium* species) may utilize a variety of other electron donors produced during the primary breakdown of decaying biomass in sediments (Widdel, 1988).

As mentioned previously, the use of oligonucleotide probes, which are complimentary to the 16S ribosomal RNA of major SRB phylogenetic groups, has become a major tool in determining the species of sulfate reducing bacteria that reside at different depths of a sediment. Devereux *et al.* (1996) utilized 16S rRNA probes to ascertain the phylogenetic groups that occupied Santa Rosa Sound in Northwest Florida. Results indicated that the most concentrated populations of sulfate reducing bacteria occurred at depths between 2 and 6 cm. Sulfate reducing bacteria belonging to the *Desulfovibrionacea* group were found in the highest abundance while bacteria belonging to the *Desulfococcus* and *Desulfo bacter* species were also detected (Deverux *et al.*, 1996).

**Identification of Sulfate-Reducing Bacteria using 16S rRNA probes**

The 16S rRNA probes are relatively novel tools that allow for the characterization of SRB communities that exist in a microbial ecosystem. Stahl *et al.* (1988) indicated that the use of 16S rRNA probes was derived from the need to better define microbial ecosystems due to the fact that many microbial populations cannot be readily cultured and organisms that can be grown do not always fit existing determinative classification schemes. In the 1980's, comparative sequencing of rRNA, particularly 16S rRNA,
demonstrated the unique differences in nucleotide sequences in terms of microbial phylogeny (Fox et al., 1980; Olsen et al., 1986; Pace et al., 1986). These differences in sequence provide a means to illustrate bacterial ancestry between phylogenetic groups. In addition, these differences in 16S rRNA have been utilized as a means of identifying microorganisms in environmental studies. Stahl et al. (1988) provided the first assessment of bacterial compositions using 16S rRNA probes in studies of ruminal microbial ecology.

This same type of nucleotide sequencing was demonstrated in SRB as well. Devereux et al. (1989, 1990) successfully identified conservative regions of 16S rRNA unique to the phylogenetically defined groups of SRB. Using oligonucleotide probes that were complementary to the conserved tracts of 16S rRNA, Devereux et al. (1992) successfully demonstrated that the probes hybridized to the specific phylogenetic group that they were designed to identify. Four probes were genus specific and identified Desulfobacterium (DSBM), Desulfo bacter (DBACTER), Desulfo bulbus (DSB), and Desulfovibrio (DSV). One of the other probes was specific for the phylogenetic lineage composed of Desulfococcus multivorans (DSC), Desulfo sarcina variabilis, and Desulfo botulus sapovaorans. The final probe was specific for DSBM, DBACTER, DSC, Desulfo sarcina variabilis, and Desulfo botulus sapovaorans. Later, Devereux et al. (1996) illustrated the use of 16S rRNA probes in natural systems by characterizing the distribution of sulfate-reducing bacteria in anaerobic sediments in northwest Florida sediments. The use of oligonucleotide probes continues to be the most novel and robust method for identifying microbial species present natural systems. The materials and methods section of this thesis (Table 3.2) illustrates the probes that were designed by Devereux et al. (1992) that
were used in this study. Figure 5.20 illustrates the genetic homology between the SRB bacteria and the probe identification number used to quantitate the SRB 16S rRNA.

**Biochemistry of Sulfate Reducing Bacteria**

As the name implies, sulfate-reducing bacteria utilize sulfate as a terminal electron acceptor under anaerobic conditions. The reduction of sulfate (SO$_4^{2-}$) to sulfide (S$^{2-}$) is an 8-electron reduction. Because the sulfate anion is a relatively stable anion, it must first be coupled to the "high energy" molecule of ATP prior to being reduced. An enzyme known as ATP sulfurylase catalyzes the reaction. In this process, two phosphates are released in the triphosphate molecule producing adenosine-5′ phosphosulfate (APS). In dissimilative sulfate reduction, the addition of two electrons to the APS molecule results in the formation of sulfite (SO$_3^{2-}$). When sulfite is formed, the subsequent 6-electron reduction of sulfite proceeds readily to sulfide (Brock *et al.*, 1994). Sulfate-reducing bacteria reduce sulfate through a cytochrome-based electron transport process. The cytochrome molecules that compose the electron transport chain of sulfate reducing bacteria are ferredoxin, flavodoxin, and cytochrome C$_3$. Cytochrome C$_3$ is a low energy cytochrome molecule that is unique to the organisms that utilize sulfate as a terminal electron acceptor (Brock *et al.*, 1994). Species of sulfate reducers capable of reducing acetate and fatty acids also contain cytochrome b. For example, the oxidation of lactate to pyruvate is facilitated by the lactate dehydrogenase enzyme. This results in the release and transport of H$_2$ across the plasma membrane. A hydrogenase enzyme located in the plasma membrane oxidizes the H$_2$ to 2H$^+$ atoms. The two electrons are subsequently
transported down the electron transport chain that terminates with APS. It should be noted that sulfite can also accept electrons through the same process (Brock et al., 1994). The H⁺ ions located on the exterior of the plasma membrane can be utilized in the production of ATP energy molecules. Because of the concentration gradient and proton motive force which occurs due to the H⁺ difference on the plasma membrane, H⁺ ions can be funneled through an ATPase enzyme which utilizes the proton motive force to synthesize ATP (Brock et al., 1994). Growth yield studies with SRB have been utilized in order to determine the stoichiometry between ATP production and sulfate reduction. Studies suggest that three ATP molecules are produced per sulfate reduced to sulfide (Brock et al., 1994). However, the net ATP produced is only one molecule given that the conversion of sulfate to sulfite is associated with two high-energy bond equivalents being consumed.

Biochemistry of Mercury Methylation by Sulfate Reducing Bacteria

The mechanism which results in the methylation of mercury has been postulated to involve methyl corrinoid derivatives i.e., methylcobalmins (Ridley, 1977). These compounds are present in viable, proliferating cells and typically function to transfer carbanion ions to other cellular constituents (Ridley, 1977). In fact, isolated methylcobalamin has demonstrated an ability to methylate mercury (Bertilsson and Neujahr, 1971). S-adenosylmethionine and N⁵-methyltetrahydrofolate represent two other derivatives capable of methylating compounds. However, their role in the methylation of mercury has not been established. It should be noted that many B12 - producing bacteria
have been studied and results indicate that these bacteria, including some sulfate reducing bacteria, are not capable of generating methylmercury. Berman et al. (1990) demonstrated using radiocarbon that the methyl group that is conjugated to mercury is derived from the amino acid serine. Desulfovibrio desulfuricans, an anaerobic sulfate reducer, was grown in the presence of $^{14}$C-serine and mercury. Results obtained from this study indicated that the radiolabeled carbon on the functional group of serine is donated to mercury through a tetrahydrofolate-mediated reaction. Later studies conducted by Berman et al. (1990) actually traced the carbon flow in mercury biomethylation by Desulfovibrio desulfuricans. Radiolabeled pyruvate or serine in addition to a mercuric ion spike was incubated with Desulfovibrio desulfuricans. The authors found that approximately 95% of the specific activity associated with the serine, which was radiolabeled at the C-3 position, was found in the biosynthesized methylmercury. However, only 21% of the radiolabeled pyruvate (C-3 position) was observed to be transferred to biosynthesized methylmercury. From these studies, the authors concluded that the carbon located at the third position of serine is transferred to the mercuric ion. Although serine is synthesized from pyruvate, a greater percentage of radiolabel from the pyruvate was not observed in methylmercury production due to the large number of metabolic processes for which pyruvate is utilized as substrate (Berman et al., 1990). Thus, the primary use of serine as a methylating factor indicates its importance and relevance in mercury methylation. Other studies have attempted to provide a link between serine and methylcobalimins in the metabolic pathway of mercury methylation. Choi et al. (1994a) investigated the incorporation of radiolabeled $^{14}$C
compounds (serine and pyruvate) in methylmercury and the relevant enzyme activities in cell extracts. High rates of label incorporation from carboxyl-related compounds prompted the assay of enzymes in the acetylcoenzyme-A (CoA) synthase pathway. Based on the observed enzymatic activity and the fact that inhibitors of the acetyl-CoA pathway also inhibited methylmercury synthesis, Choi et al. (1994a) proposed that methylmercury synthesis by Desulfovibrio desulfuricans involved the methyl group transfer of CH$_3$-tetrahydrofolate utilizing methylcobalimins. The authors assert the fact that the methyl group may originate from the C-3 position of serine or formate via the acetyl-CoA synthase pathway (Choi et al., 1994a). In another study by Choi et al. (1994b), the actual enzymatic catalysis of mercury methylation by Desulfovibrio desulfuricans was investigated. Experiments using a $^{57}$Co label demonstrated that 95% of the label was associated with macromolecules rather than free cobalim. Further investigations conducted using gel filtration and electrophoresis implicated a single 40 kDa-corrinoid protein. Under reducing conditions, Choi and colleagues illustrated that cell extracts containing the corrinoid protein produced $^{14}$CH$_3$Hg$^+$ from Hg$^{2+}$ and 5-$^{14}$CH$_3$-tetrahydrofolate.

Demethylation of Mercury

Biologically Mediated Demethylation of Mercury

The demethylation of mercury and subsequent reduction of Hg$^{2+}$ to Hg$^0$ occur through microbial processes (Summers and Silver, 1978). It should be emphasized that much more information has been accumulated regarding the biochemical mechanism of
demethylation, as opposed to methylation. In sediments, demethylation occurs at a much faster rate than methylation when higher redox potentials are observed (Compeau and Bartha, 1984). The higher redox potentials are indicative of environments that are aerobic in nature. The demethylation of mercury has been observed in surface waters as well. Matilainen and Verta (1995) recorded demethylation rates in small-forest lakes at 66μg/L-d. It should be noted that high temperatures stimulated enhanced demethylation in surface waters. Martilainen and Verta (1995) also suggested that bacterial species were responsible for the observed demethylation due to inactivation when formaldehyde was added to surface waters. Sediments from mercury-contaminated and uncontaminated regions of the Carson River, Nevada were assayed for monomethylmercury degradation (OremLand et al., 1995). Demethylation of methylmercury was indicated by the use of \(^{14}\text{CH}_3\text{Hg}^+\) that resulted in the formation of \(^{14}\text{CO}_2\) and \(^{14}\text{CH}_4\) (OremLand et al., 1995). The authors reported that oxidized/reduced demethylation product ratios (\(^{14}\text{CO}_2/^{14}\text{CH}_4\) ratios) generally ranged from 4.0 in surface layers to as low as 0.5 at depth. Overall, demethylation activity was most pronounced in the upper surface layers (OremLand et al., 1995).

**Biochemical and Molecular Process of Demethylation**

Aerobic species of bacteria such as *Escherichia coli*, *Pseudomonas* spp., and *Staphylococcus aureus* have all demonstrated an ability to demethylate methylmercury (Robinson and Tuovinen, 1984). It should be noted that these bacteria have demonstrated a resistance to methylmercury associated toxicities.
The organomercurial-lyase (OML) and oxidative demethylation (OD) processes represent two pathways for microbial demethylation of mercury (Marvin et al., 1998).

The organomercurial-lyase (OML) pathway utilizes enzymes encoded for on the mer-B operon. The primary genes responsible for the demethylation of mercury are the lyase and reductase enzymes (Robinson and Tuovinen, 1994).

Initially, resistance to inorganic and organomercurial compounds involves the inducible synthesis of the organomercurial lyase and mercuric reductase enzymes by subtoxic levels of inorganic mercury. In the demethylation process, methylmercury is split into the mercuric ion and the methyl group by the organomercurial lyase enzyme that codes for the mer B operon. Subsequent reduction occurred via the mercuric reductase enzyme that is coded by the mer A gene (Robinson and Tuovinen, 1984). The mercuric reductase enzyme exists as a soluble flavoprotein located in the cytoplasm. This enzyme catalyzes the NADPH dependant reduction of mercuric ions to metallic mercury (Robinson and Tuovinen, 1984). It should be noted that this enzyme requires the presence of excess thiol compounds in order to have activity. L-cysteine was demonstrated to be the most effective thiol compound in accentuating the mercury reductase enzyme. The organomercury lyase enzymes have not been characterized in as much detail as the Hg(II) reductase enzyme. However, cysteine as well as cofactors like NADPH are necessary for their proper function (Robinson and Tuovinen, 1984). Future research in the area of OML demethylation involves the use of transgenic plants that express modified bacterial merA and merB genes. Rugh et al. (1996) has suggested that plants which contain the mer genes could convert the toxic form of mercury (Hg$^{2+}$ and
CH$_3$Hg$^+$) to the less toxic, elemental mercury (Hg$^0$). Thus, the use of these transgenic plants could provide an ecologically compatible approach for the remediation of mercury pollution.

Oxidative demethylation (OD) represents another means in which methylmercury can be demethylated (Marvin-Dispaaquale and Oremland, 1998). Although the enzymatic mechanism that determine OD are not known, the OD process results in the oxidation of the methyl group to CO$_2$. Studies conducted on sulfate-reducing bacteria as well as methanogens suggest a possible role in oxidative demethylation (Oremland et al., 1991).

It should also be noted that several marine bacterial isolates that did not contain typical mer genes have demonstrated abilities to volatize mercury (Reyes et al., 1999). Reyes et al. (1999) findings indicate that these mer-negative marine sediment isolates reduce Hg (II) to Hg(0). These findings suggest other microbial pathways exist which provide a mechanism of mercury resistance in marine microbial systems.

**Summary of Literature Review**

From the above literature review, it is clear that methylmercury represents the most bioavailable species of mercury with respect to human exposure and adverse health concerns. Limiting or reducing exposure to methylmercury is a primary concern for many of the researchers in this field. Collectively, these studies offer insight into the production, fate, and transport of methylmercury in sediment systems.

Studies presented in subsequent chapters seek to develop a fundamental understanding of the various kinetic parameters associated with the microbial production
of methylmercury is saltmarsh systems. In previous studies, the unique relationship between sulfate-reducing bacteria and mercury methylation was not defined. The studies presented here seek to couple mercury methylation rates to the microbial respiration of sulfate-reducing bacteria. In addition, various parameters discussed in the literature review that affect sulfate-reduction rates and mercury methylation rates were delineated based on simultaneous observations of both rates while manipulating the system. Moreover, the research presented will add to the present literature by distinguishing various potentials to methylate mercury relative to the SRB phylogenetic groups.
CHAPTER III

MATERIALS AND METHODS

The methods, materials, reactors, and analytical techniques utilized in the research are presented in this chapter.

Sediments

Sediments that were used in slurry studies and sediment core studies were obtained from marine, intercoastal waterway marshes on the Skidaway River located at the Skidaway Institute of Oceanography in Savannah, Georgia. Sediments were collected during a period of low tide. Approximately eight hours separates the beginning of a new tidal cycle with an average 1.5-2.0 m difference between high and low tide readings. Sediments were collected on the Skidaway River at two locations. Location A was 60 m north of main dock at the Skidaway Institute of Oceanography (adjacent to fuel dock) and B was 80 m south of main dock (adjacent to marine science aquarium). Table 3.1 indicates the location at the Skidaway Institute of Oceanography where sediments were collected, sediment characteristics, and the experiments in which they were used.
<table>
<thead>
<tr>
<th>Sediment Location &amp; Experiment</th>
<th>Date</th>
<th>Slurry</th>
<th>Intact Core</th>
<th>Porosity*</th>
<th>Particle Density (g/cm$^3_{sed}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-1</td>
<td>2/97</td>
<td>X</td>
<td></td>
<td>0.76</td>
<td>2.34</td>
</tr>
<tr>
<td>A-2</td>
<td>3/97</td>
<td>X</td>
<td></td>
<td>0.76</td>
<td>2.34</td>
</tr>
<tr>
<td>A-3</td>
<td>4/97</td>
<td>X</td>
<td></td>
<td>0.77</td>
<td>2.25</td>
</tr>
<tr>
<td>A-4</td>
<td>6/97</td>
<td>X</td>
<td></td>
<td>0.76</td>
<td>2.38</td>
</tr>
<tr>
<td>A-5</td>
<td>8/97</td>
<td>X</td>
<td>&lt;5cm</td>
<td>0.77</td>
<td>&lt;5cm</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>&gt;5cm</td>
<td>0.76</td>
<td>2.30</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A-6</td>
<td>8/97</td>
<td>X</td>
<td></td>
<td>0.73</td>
<td>2.27</td>
</tr>
<tr>
<td>B</td>
<td>8/98-11/98</td>
<td>X</td>
<td></td>
<td>0.77</td>
<td>2.46</td>
</tr>
<tr>
<td>C</td>
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<td>X</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>&gt;5cm</td>
<td>0.71</td>
<td>2.10</td>
</tr>
</tbody>
</table>

* Porosity (void volume, cm$^3$ / total sediment volume, cm$^3$) was determined based on mass and density of Skidaway River water ($\rho = 1.02$ g/cm$^3$) that occupied the void volume of sediment.
Sulfate-Reducing Bacteria Pure Cultures

Desulfovibrio desulfuricans, Desulfobulbus propionicus, and Desulfooccus multivorans were freshwater cultures that were used in pure culture experiments and in the calibration of oligonucleotide probes. Dr. Joel E. Kostka at the Skidaway Institute of Oceanography (Savannah, GA) donated these cells. Desulfovibrio desulfuricans, Desulfobulbus propionicus, and Desulfooccus multivorans are representative of three distinct SRB phylogenetic groups designated throughout this thesis as DSV, DSB, and DSC, respectively.

BG-8 and BG-33 cells were derived from marine sediments and used in pure culture experiments as well as the calibration of oligonucleotide probes. These cells were donated by Richard Devereux at the Environmental Protection Agency Lab (Gulf Breeze, FL). BG-8 shared a 96.6 % sequence identity with Desulfo bacter curvatus. BG-33 shared a 96.6 % sequence identity with Desulfo bacterium niacini (Rooney-Varga et al., 1998). BG-8 (Desulfo bacter sp.) and BG-33 (Desulfo bacterium sp.) are representatives of two distinct SRB phylogenetic groups designated throughout this thesis as DBACTER and DSBM, respectively.

All cultures were maintained in anoxic media that is outlined in Appendix A. For the BG-8 and BG-33 species, the amount of NaCl added to component I was 7.0 g as opposed to 1g NaCl for the other 3 genera. Cultures not used for experimental purposes were allowed to grow for 30 d prior to 100 μL transfer of culture to fresh media.
Chemicals

Radioactive sulfate $^{35}$SO$_4^{2-}$; (2.2 mCi/µL, cat # 64040, ICN Biomedicals, Inc.) was used to determine sulfate concentrations and sulfate reduction rates (SRR) in pure cultures, sediment slurries, and sediment cores. Inorganic mercuric nitrate (Fisher Chemical) was added to pure cultures, sediment slurries, and sediment cores at specified concentrations in order to determine mercury methylation rates (MMR). All other chemicals used in the experimental protocols presented in this thesis were reagent grade unless otherwise specified.

Batch Sediment Slurry Reactors

Slurry reactors were constructed using 150 mL Fleaker Beakers® (Corning) with gastight caps. Three ports were placed in the cap and sealed with silicon sealant. One tube (15 cm) allowed for continuous addition of N$_2$/CO$_2$ (9:1) through the slurry at 40 mL/min. This served to evacuate the reactor of any oxygen that might be present. The second tube (8 cm) served as a gas release port while the remaining tube (15 cm) reached the bottom of the reactor and served as a sample port.

Sediment Core Device

Sediment core devices were donated by Dr. Richard A. Jahnke at the Skidaway Institute of Oceanography. Sediment cores (10 cm) were collected using a core barrel (12 cm long and 1.90 cm diameter) with a Teflon plunger. Specified incubations of sediment cores were started within 1 hr of retrieval. Teflon plungers were fitted into the
top and bottom of core barrels to maintain anoxic environment of sediment. Injection ports for inorganic mercury addition and $^{35}$SO$_4$ were placed 1 cm apart for the entire length of the core barrel and then sealed with silicon sealant.

**Experimental Procedures**

**SRB Pure Cultures**

Cells were grown in a multipurpose media similar to those of Widdel and Bak (1992). The composition of the media can be found in Appendix A. In brief, each component was made and autoclaved separately before combining the fractions at 50 °C under a gas stream of 90 % N$_2$ and 10 % H$_2$. The media was purged with the mixed gas for approximately 45 min prior to the individual additions (Widdel and Bak, 1992). Once the media was anoxic as indicated by reazurin dye, the media was placed in an anaerobic chamber (90 % N$_2$ and 10 % H$_2$) where it was aliquoted in 50 mL amounts to 100 mL butyl rubber-fitted, gas-tight bottles (Belloco, Inc.). Cells were transferred from stock vials to media bottles using 1mL syringes fitted with 20 gauge ($^{1/2}$) needles. All transfers were done in an anaerobic chamber (90 % N$_2$ and 10 % H$_2$). Cells were allowed to grow in the dark at 25 °C.

In pure culture experiments on methylation, inorganic mercury was added to 100 mL media bottles 120 hr after the initial inoculations of SRB such that the calculated inorganic mercury concentration in the reactors was 100 ng/mL (0.1 mg/L). For each of the phylogenetic groups, four media bottles were constructed. The use of 4 parallel media bottles allowed for the following measurements per reactor: (bottle 1) turbidity, cell
numbers, sulfate concentrations; (bottle 2) sulfate-reduction rate (SRR); (bottle 3) total soluble Hg in filtered samples; and (bottle 4) methylmercury. The $^{35}$SO$_4^{2-}$ (ICN Biomedical, Inc.) was added to media bottle (2) 12 hr prior to inorganic mercury addition. At specified timepoints, aliquots were removed for the analysis of turbidity (1 mL), cell counts (1 mL), sulfate concentrations (1 mL), SRR (3 mL), total Hg in filtered samples (8 mL), and methylmercury concentrations (4 mL). Media bottles were sampled at the initial inoculation time point, as well as every 24 hr after initial inoculation with inorganic mercury for a total of 96 hr.

**Sediment Slurry Reactor Systems**

The use of 3 parallel reactors allowed for the measurements of (reactor 1) total soluble Hg in filtered samples and organic acids; (reactor 2) SRR; and (reactor 3) methylmercury and sulfate concentrations. At specified timepoints aliquots were withdrawn for total soluble Hg in filtered sample (50 mL), organic acid content (5 mL), SRR (3 mL), methylmercury concentration (3 mL), and sulfate concentration (1 mL). Sampling was achieved using a 15 mL syringe for organic acids, SRR, methylmercury, and sulfate concentrations. A 60 mL syringe was used to collect samples for total mercury analysis. At the end of the experiment, slurry samples from reactor 1 (15 mL) and reactor 3 (15 mL) were pooled for 16S rRNA determinations and cell counts. Reactors contained approximately 140 g/L of sediment (on a dry weight basis) in Skidaway River water with a salinity of 17.39 ppt (parts per thousand).
Skidaway River water was purged with nitrogen gas for 30 min prior to sediment addition and the entire reactor was continually shaken at 100 rpm (Junior Orbital Shaker, Labline Inc.) and maintained at 25 °C throughout the incubation unless otherwise specified. After an initial 12 hr stabilization period, 8 μL of 1 μCi/μL radioactive $^{35}$SO$_4^{2-}$ (2.2 mCi/μL, cat #. 64040, ICN Biomedicals, Inc) was added. Inorganic mercury additions were performed 12 hr after $^{35}$SO$_4^{2-}$ addition to avoid any short-term artifacts that may be associated with the tracer addition. For substrate/inhibitor experiments, the substrate or inhibitor was added during initial reactor construction. The pH was stable in the slurry reactors, being within limits of 6.8 to 7.2.

To determine the effects of temperature at 4, 25 and 37 °C, reactors were held at a constant temperature in incubators. Reactors were allowed to reach specified temperatures 12 hr prior to $^{35}$SO$_4^{2-}$ addition. After an additional 12 hr, approximately 955 ng/g$_{\text{dry weight}}$ of Hg$^{2+}$ was added to the slurries.

In heat-inactivated slurries, slurries were heated at 100 °C for 30 min. Slurries were then cooled for 2 hr prior to reconstituting with anoxic Skidaway River water (25 mL) and addition of $^{35}$SO$_4^{2-}$ 12 hr prior to addition of 955 ng/g$_{\text{dry weight}}$ of Hg$^{2+}$ addition.

In molybdate treated reactors 100 mM sodium molybdate (Fisher Scientific) was added 12 hr prior to $^{35}$SO$_4^{2-}$ addition. After an additional 12 hr, approximately 955 ng/g$_{\text{dry weight}}$ of Hg$^{2+}$ was added to the slurries. In substrate amended reactors, 100 mM of acetate or pyruvate was added 12 hr prior to $^{35}$SO$_4^{2-}$ addition. After an additional 12 hr, approximately 955 ng/g$_{\text{dry weight}}$ of Hg$^{2+}$ was added to the slurries.
Preconditioned slurries were used to alter the population of microbial species present in the sediment. Reactors that were preconditioned with 20 mM lactate or 10 mM acetate received an aliquot of $^{35}\text{SO}_4^{2-}$ 19.5 d after substrate addition. After an additional 12 hr, 950ng/g[dry weight] of Hg$^{2+}$ was added to the slurries.

**Intact Cores**

Intact cores were maintained at 27 °C and sectioned after specified treatments in 1 cm intervals for analysis. SRR incubations were performed by injection of 1 μL of 1 μCi/μL radioactive $^{35}\text{SO}_4^{2-}$ (ICN Biomedicals, Inc.) into intact sediment cores at 1 cm intervals. The core was allowed to incubate for 12 hr at 27 °C after which it was sectioned into depth intervals and analyzed as described below. For MMR incubations, 2 μL of 0.5 μg/μL inorganic mercury (Hg$^{2+}$) were made at 1 cm intervals. Intact cores were sampled immediately (i.e., zero hr) or after 12 hr. After incubation, the cores were sliced at 1 cm intervals using a spatula sterilized with 70 % ethanol and cleaned between sectioning.

**Sediment Density and Dry Weight**

For sediments used in reactor systems, a 5 mL volume of the homogenized sediment was collected in a 10 mL syringe designed for sediment collection (Becton Dickinson & Co., Cat # 309604). In order to obtain the density of the sediment (g/cm$^3$), the weight of the specified volume of sediment was determined by subtracting the weight of the syringe from the weight of the sediment and syringe. The dry fraction of sediment was then
determined by ejecting the sediment from the syringe into a tin weigh pan and heating at 75 °C for 48 hr. The density and dry sediment fraction of the sediment used in reactor systems was determined in duplicate measurements for each sediment (Table 3.1). In order to determine the density and dry fraction of sediment in intact cores, a 10 cm intact core was sectioned into 1 cm intervals (Each 1 cm interval had a volume of 2.85 cm³) and each 1 cm interval was weighed. Dry sediment fraction was determined heating sediment intervals at 75 °C for 48 hr. The density and dry sediment fraction was determined for each sediment used (Table 3.1). Porosity (void volume, cm³ / total sediment volume, cm³) was determined based on mass and density of Skidaway River water (ρ = 1.02 g/cm³) that occupied the void volume of sediment. The particle density of the sediment was based on Equation 3.1.

\[
\left(\rho_b - \phi \rho_w\right) \left(\frac{1}{1-\phi}\right) = \rho_{sed}
\]

Equation 3.1

In Equation 3.1, ρb is the bulk density (g/cm³), ρsed is the particle density (g/cm³sed), ρw is the density of Skidaway River water, and ϕ is the porosity of the sediment (cm³void/cm³bulk).

Sample Analysis

Sulfate Concentration

Sulfate concentrations present in slurry water were determined based on a method developed by Tabatabi (1974). In brief, a barium-gelatin reagent was utilized in the
turbidimetric analysis of sulfate. The gelatin solution consisted of 1.5 g of gelatin in 500 mL of de-ionized water. This solution was stored for 24 hr at 4 °C before use. To 150 mL of the gelatin solution, 1.5 g of barium chloride (Allied Chemical) was dissolved and incubated 1 hr at 25 °C before use. Standards consisted of 0, 10, 20, and 30 mM SO\textsubscript{4}\textsuperscript{2-} concentrations (Na\textsubscript{2}SO\textsubscript{4}, Sigma Chemical) and were utilized in the development of a calibration curve. Slurry samples were placed in micro-centrifuge tubes and stored at −78 °C until analysis. Samples were allowed to warm to room temperature then centrifuged for 5 min at 10,000 x g (Eppendorf Centrifuge, model 5415) and the overlying water extracted. To 10mL of de-ionized water, 500 µL of 1N HCl was added followed by 40 µL of slurry water or standard. At 1.5 min intervals, 500 µL of the barium/gelatin reagent was added to a sample tube. The tube was mixed gently. After 30 min incubation at 25 °C, the samples were analyzed in 1.5 min intervals at 420 nm in 4 cm quartz, spectrophotometric cells. The standard curves developed for sulfate concentration determination generally had r\textsuperscript{2} values greater that 0.995.

Sulfate Reduction Rate (SRR)

The amount of sulfate reduced in sediment slurry reactors and sediments were determined using the analytical procedures developed by Fossing and Jorgenson (1989). At the specified sampling periods, aliquots of the slurry, intact core, or pure culture that were treated with \textsuperscript{35}SO\textsubscript{4} were removed and immediately placed in 10 mL of 20 % (w/v) zinc acetate (ZnAc) solution. The sediment slurry was then centrifuged at 2500 rpm or
1350 x g (International Clinical Centrifuge, IEC) and washed with N₂ purged deionized H₂O (dH₂O) three times. The sample was transferred to a distillation apparatus that consisted of a gas-tight reaction flask that was supplied with a gas-bubbling tube, a condenser, and ZnAc trap. This unit was continually purged with N₂ gas. After the sample was transferred to the reaction flask, 16mL of 1 M Cr²⁺ in 0.5 N HCl and 8 mL of 12 N HCl were added together using a 50 mL syringe through a gas-tight port located on top of the reaction flask (Fossing and Jorgenson, 1989). The procedure for reduction of chromium can be found in Appendix B. The slurry was gently boiled for 40 min. During this distillation the total reduced inorganic sulfur was dissolved and carried as H₂S to the ZnAc traps which contained 10mL of a 5% w/v ZnAc solution. The calculation for the amount of sulfate reduced was determined using Equation 3.1.

\[
\text{Sulfate reduced} = \frac{(\text{SO}_4^{2-}) \cdot 1.06}{(A + a)} \text{nmol SO}_4^{2-}/\text{cm}^3
\]

Equation 3.2

In Equation 3.2, \(a\) is the total radioactivity of Zn\(^{35}\)S expressed in counts per minute (CPM), \(A\) is the total radioactivity of \(^{35}\text{SO}_4^{2-}\) after incubation expressed in CPM, \((\text{SO}_4^{2-})\) is the sulfate concentration in nmol/cm\(^3\) and 1.06 is a correction factor for the expected isotope fractionation (Jorgenson and Fenchel, 1974). The values for \(A\) and \(a\) (i.e., total \(^{35}\text{SO}_4^{2-}\) radioactivity and Zn\(^{35}\)S radioactivity, respectively) were determined using a liquid-scintillation counter (Beckman LS6500) with 10 mL Ecoscint® (cat# LS-271,
Manville, NJ) scintillation cocktail. The scintillation counter was programmed for counting of $^{35}$S for a period of 5 min.

For intact core experiments, SRR was expressed as nmol sulfate reduced per gram dry sediment per hour (converted from nmol/cm$^3_{sediment}$ by calculating the density of the sediment and dry sediment fraction of sediment). For sediment slurries, the amount of sulfate reduced was expressed as nmol sulfate reduced per gram dry sediment (converted from nmol/cm$^3_{slurry}$ by calculating density of slurry and dry sediment fraction of slurry) at specified timepoints.

For pure culture analysis, 3 mL aliquots were removed from a slurry reactor over time and placed in 30 mL of an anoxic 20 % ZnAc solution. A fraction of the ZnAc/sample solution (100 μL) was aliquoted for total radioactivity calculations. The remaining sample was distilled to determine the amount of radioactive sulfide present at a specified timepoint.

To obtain SRR values, the amount of sulfate reduced was plotted as a function of time. Linear regression analysis was performed on the data set to determine the average SRR in slurries or pure culture.

**Total Soluble Mercury Determination**

For total soluble mercury analysis of slurry water samples, the entire slurry mixture was centrifuged at 2500 rpm or 1350 x g (International Clinical Centrifuge, IEC). The aqueous fraction was collected (approximately 50 mL) into Polytetrafluorethylene
(PTFE) containers (cleaned with hot concentrated HNO₃ for 24 hr and rinsed with water). Extracted water samples were placed in PTFE containers and acidified with 250 μL of concentrated HNO₃ and 20 μL of BrCl (0.54 g of KBr added to 50 mL of concentrated HCl and 0.76 g of KBrO₃ was added 1 hr after KBr). Samples were incubated at 27 °C for 48 hr. Samples were then filtered using a 0.45 μm capsule filter (Gelman Sciences, Acrodisc® cat #. 4184) prior to analysis. Analysis was performed by placing the sample (50 mL) into a 250 mL gas washing bottle fitted with 6 mm input and output tubes, fritted gas bubbler, and an injection port. A continuous stream of nitrogen (350 mL/min) was fed through the reaction vessel to a cold-vapor UV detector (Laboratory Data Control, Inc.). A moisture trap containing 20 g of magnesium perchlorate, MgClO₄ (Ficher Scientific) was fitted immediately after the reaction vessel and before the detection unit. The MgClO₄ was held in the trap using cotton plugs. Concentrated nitric acid (1 mL) was then added to the sample in the reaction vessel. Once a consistent base line had been established, 500 μL of 10 % stannous chloride, SnCl₂ (J.T. Baker, Inc.), in 3 % H₂SO₄/dH₂O was added through the injection port to the sample. The sample was then monitored using a Cold-Vapor UV detector with a 30 cm cell (Laboratory Data Control, Inc.). Concentrations were determined based on a standard curve which was generated using inorganic mercury standards diluted in filtered Skidaway river water (approximately 17.39 ppt) and digested exactly as samples. For pure culture analysis, 8 mL samples were filtered through a 0.45 μm capsule, prepared and analyzed as previously described.
The correlation coefficient ($r^2$) of the standard calibration was always greater than 0.99. A continuing calibration standard was run after every 10 samples to verify that the instruments remained calibrated. The precision of the method was based on the digestions and ethylations of different samples receiving the same treatment. Generally, data sets acquired in triplicate had relative percent differences (RPD) that averaged 10.2% ($n = 17$) for slurry systems. Instrument accuracy was based on spike recoveries. This involved addition of 10 ng Hg^{2+} to 45 mL of Skidaway River water (45 mL) followed by acidification and filtering water 30 min after treatment. Recoveries averaged 98.7 $\pm$ 10.2% ($n = 9$) of the treatment value. The method detection limit (MDL) for total Hg determination in sediment slurries and pure cultures was approximately 10 ng/L for sediment slurries and 62.5 ng/L for pure cultures. This is based on the total volume of sample available for complete digestion and total soluble mercury quantification.

**Methylmercury Concentration**

Methylmercury in sediment slurries and sediment samples were analyzed using modifications of the method described by Horvat *et al.* (1993) and Liang *et al.* (1994). For sediment slurries and sediment cores, a 1.0-2.0 g sample was weighed into a Teflon (PTFE) vial followed by the addition of 5 mL of dH$_2$O, 0.2 mL of 20 % KCl and 0.5 mL of 8 M H$_2$SO$_4$. The mixture was diluted to 10 mL with dH$_2$O, and distillation was started after addition of reagents. The reaction vial was sealed onto a distillation system, maintained at 140 °C, and an argon carrier gas was applied at a flow rate of 60mL/min. The distillate collection rate was approximately 7 mL/hr. The distillate was collected in
30 mL PTFE vials that were kept in an iced water bath. Prior to distillation, 5 mL of dH₂O was placed in the collection vial.

An aliquot volume of 100-1000 μL (exact volume depended on methylmercury concentration) of the distillate was added to 100 mL of dH₂O in a 250 mL ethylation reaction flask. The sample was buffered to pH 4.9 with 2 M acetic acid-sodium acetate solution (0.2 mL) which was followed by the addition of 50 μL of a 1.0 % aqueous sodium tetraethylborate solution. The flask was immediately closed and connected to a collection trap (Tenax®) on one end and argon on the other. The mixture was allowed to react without bubbling for 15 min. After the reaction period, the solution was purged for 12 min at a flow rate of 250 mL/min with Hg-free, high purity argon. The outflowing gas stream was passed through a Tenax trap (100 mg packing). After the sample was purged, dry argon was flushed through the Tenax trap for 5 min to remove traces of condensed water vapor. The mercury species on the trap was released by thermal desorption into an isothermal gas chromatograph U shaped, silanized glass column filled with 15% OV-3 Chromosorb WAW-DMCS (Supelco, Bellefonte, PA) at 100°C. Under the argon flow, the eluted mercury species was converted into elemental mercury by thermal decomposition at 900 °C and then detected by cold vapor atomic fluorescence spectrophotometry, CVAFS (Model 2500, Tekron Inc., Ontario). The output from the detector is quantified using an integrator (Model HP3394A. Hewlett-Packard, PA). Methylmercury was quantified by comparing peak areas of standards with those in
samples. For pure-culture methylmercury analysis, 15 mL of dH₂O was added to 5 mL of sample. The rest of the analysis was identical to what has previously been described.

The quality assurance protocol for methylmercury detection was included because of the novel technique used in quantification. Quality assurance included instrument calibration using certified standards and the analyses of matrix spikes, certified reference material (CRM) and reagent blank, according to Environmental Monitoring and Assessment Program–Environmental Protection Agency (EMAP-EPA) criteria (Heitmuller and Peacher, 1995). The correlation coefficient of the standard calibration was always greater than 0.99. A continuing calibration standard was run after every 10 samples to verify that the instruments remained calibrated. The precision of the method was based on the duplicate digestions and ethylations of the same sample. The relative percent difference averaged 8.4% for sediments and sediment slurries (n = 3) and 4.5% for pure cultures (n = 3). In order to test the accuracy of the procedure, matrix spikes were determined on samples. Sediment slurry samples were spiked with approximately 10 times the instrument detection limit of methylmercury (e.g., 10 pg) in order to examine the percent recovery of the matrix spike. Accuracy was also assessed using a certified reference material (IAEA-356 marine sediment certified for methylmercury, National Research Council, Canada) spiked into slurries. Recoveries of standard reference materials, as well as spiked sediment slurries, averaged 95.3 ± 3.2% (n = 12 recoveries). Calibration curves were prepared daily with 5, 10, 25, and 50 pg levels of methylmercury ($r^2 > 0.99$). The instrument detection limit, three times the instrumental
noise, was 6.0 pg/g dry sediment for sediments and sediment slurries and 0.3 ng/L for pure cultures.

**Organic Acid Analysis**

A Dionex, DX-500, ion chromatography system equipped with a conductivity detector was used to analyze for organic acid content in preconditioned reactors. Organic acids were resolved on an IonPac ICE-AS6 ion exclusion column (Dionex part number 46023). Heptafluorobutyric acid (HFBA) was used as the eluent that had a flow rate of 1.0 mL/min (Appendix C). The instrument was fitted with an Anion-ICE Micromembrane Suppressor using Tetrabutylammonium hydroxide (TBAOH) as the regenerant solution that had a flow rate of 5 mL/min (Appendix C). A 50 µL sample loop was used for sample injection using an AS40 automatic sampler. The ion chromatograph also utilized helium gas (5.5-6.8 atm) to inject samples. Samples were taken from the reactors and centrifuged at 5000 x g (Eppendorf Centrifuge 5416) for 10 min. Samples were then filtered using a 0.22 µm filter. The filtered samples were then passed through OnGuard-Ag and OnGuard-H cartridges (Dionex P/N 039637, 039596 respectively) in series. Chloride ion is precipitated as AgCl and any soluble Ag is removed from the samples with the OnGuard-H cartridge. Since a 3 mL void volume exists within the cartridges, samples are diluted with dH2O prior to preparation. Organic acid standards were prepared in similar chloride concentrations as the samples (approximately 17.0 ppt) and processed through the cartridges for standard curves. Sample curves generally had r² values greater than 0.995. Calibration of the column with known lactic acid and acetic
acid were performed daily to assure the reproducibility of the method. Standards were analyzed every 10 samples to ensure calibration. Acetic acid was calibrated over the range of 30 μM to 1 mM. Lactic acid was calibrated from 2 μM to 1 mM. Identity of peaks were substantiated by running samples and standards at two pH ranges in order to change the retention times of the organic acids.

**Sulfate Reducing Bacteria (SRB) Enumeration**

**Optical Density**

Optical density measurements were conducted on cell cultures to determine when cell growth was initiated. Cells analyzed for optical density were not grown in media that contained reazurin because of possible absorbance interference with indicator solution. Approximately 1mL aliquots of media that contained cells were placed in quartz cuvettes and analyzed with a spectrophotometer (660nm) every 24 hr including initial time of inoculation into media. The spectrophotometer was zeroed with dH2O prior to sample analysis. This was done so that media that did not contain cells could be analyzed for changes in optical density ensuring that contamination did not occur.

**Cell Counts**

Cells were collected in 1 mL volumes from pure culture reactors and 100 μL of 37% formaldehyde (3.4 % final concentration of formaldehyde) was added in order to preserve cells. Cells were then stored at 4 °C. For sediment slurries, the cell extraction
protocol was adapted from methods published by Velji and Albright (1985). 500 µL of 37% formaldehyde was added to approximately 4.5 mL of sediment slurry (3.7% final concentration of formaldehyde). Sodium pyrophosphate (PPI) and was then added to the sediment slurry to a final concentration of 0.01 M. A 3 µL addition of concentrated polyoxyethylenesorbitan monoleate (Tween-80) was added to a final concentration of 0.06%. The sediment slurry was vortexed for 1 min followed by a 30 min incubation at room temperature. The sample was vortexed for 15 sec, and centrifuged at 2000 rpm (500 x g) for 2 min. After centrifugation, the supernatant was collected and removed. An additional 10 mL of Artificial Sea Water, ASW (See appendix D), was added to the sediment tube. The sediment was again vortexed for 15 sec followed by centrifugation at 2000 rpm (700 x g, IEC Clinical Centrifuge, International Equipment Co.) for 2 min. Following centrifugation, the supernatant was extracted and combined with the previous supernatant. The buffer containing the cells was centrifuged at 12,000 x g (Avanti j-25, Beckman) for 10 min. After centrifugation, the supernatant was removed and cells diluted in 3 mL of ASW.

After cells were collected in pure culture or sediment slurries, serial dilutions of 1:10, 1:100, and 1:1000 were accomplished by adding 10 mM MgSO₄ (pH = 6.4) to sample aliquots. Total volume of diluted cells was 1 mL. 4′6′-diamidino-2-phenylindole, DAPI, stain (Fisher Scientific) was added (60 µL volume) to samples that were subsequently vortexed for 15 sec prior to incubation at room temperature in the dark for 30 min. Flurochrome stock solution of DAPI was prepared at 50 µg/mL in dH₂O, filtered with
0.2 μm GS filter (Millipore), and stored in the dark at 4 °C. Final mass of DAPI used was 0.3 ng (60 μL aliquot in 1 mL sample). After incubation, cells were fixed to slide for counting. Once stained with the DAPI stain, cells were then placed on a slide (Williams et al., 1998). In brief, a 0.2 μm, 25 mm GFF filter (Millipore) was placed on top of a vaccum bottle. Approximately 250 μL of dH₂O was added to the GFF filter in order to secure it to the vacuum bottle. A 0.2 μm black polycarbonate filter was then placed on the GFF filter and clamped to a column that led to the vacuum bottle. A vacuum (< 1 atm) was pulled on the apparatus and 1 mL of the cell suspension was carefully pipetted onto the top of the filter. Samples were then washed 3 times with 1 X SET (150 mM NaCl, 20 mM Tris-HCl, 1 mM EDTA, [pH 7.8]). Filter with cells was removed and placed on a slide with low fluorescence oil for counting. One drop of oil (immersion oil Type II, R.P. Cargille Laboratories, Inc.) was placed between the slide and cover slip. Total cell counts were determined under Wide UV filter set (U-M536), with exciter filter BP 330-385, dichroic mirror DM 400, and barrier filter BA 420. An Olympus BX-60 fluorescence microscope equipped with a 100X UPLANFL NA 1.3 oil objective was used for cell counts. The actual concentration of cells was then determined using Equation 3.3:

\[
\text{Cells/mL} = \frac{(\text{Avg.#cells/field}) \cdot (25,220) \cdot (\text{dilution})}{\text{volume filtered}}
\]  

Equation 3.3
The number 25,220 is the number of fields per filter. This value was calculated by dividing the area of the filter (radius = 15.09 mm) by the area of the field (radius = 9.5 \times 10^{-2} \text{ mm}).

**Quantification of SRB 16S rRNA**

The following sections describe the protocol used for the extraction and identification of SRB 16S rRNA from sediments and slurries.

**Extraction and Purification of 16S rRNA**

The methods for extraction and purification of microbial 16S rRNA from sediment and sediment slurries were based on those reported by Moran *et al.* (1993). Sediment cores (2.84 cm² X 10 cm) were collected using 12-cm core barrels with Teflon plungers. Samples were partitioned in 2-cm length segments and weighed into a 50 mL polypropylene centrifuge tube (Nalgene 3110-0500). Sediment samples were then stored at −78 °C at least 2 d prior to extraction. Sediment slurry samples (10mL) were collected from reactors and stored at −78 °C for at least 2 d prior to extraction. All plasticware and glassware was autoclaved at 1.02-1.36 atm for 1 hr prior to use. All solutions were made with deionized water (dH₂O) that had been autoclaved for 1 hr. The sediment samples were thawed immediately before the RNA extraction and allowed to reach room temperature. Once the samples reached a slurry consistency, 20 mL of 120 mM sodium phosphate buffer (pH 5.2) was added to each tube. After shaking the tubes on a rotary shaker for 15 min at 150 rpm, the tube contents were then centrifuged in a swing-bucket
centrifuge at 6,000 x g (IEC Clinical Centrifuge, International Equipment Co.) for 10 min. The resulting supernatant was decanted and discarded. The remaining pellet was washed and centrifuged a second time with 20 mL of 120 mM sodium phosphate buffer (pH 5.2). After the second supernatant was removed, the pellet was resuspended in 7 mL of Tris lysing buffer (50 mM Tris, 0.25 mM EDTA, 25% (w/v) sucrose [pH 8.0]). Lysozyme (Sigma, cat. # L-7651) was added to the Tris Lysing Buffer (5 mg/mL) immediately before use. The tube contents were incubated for 15 min at room temperature and then centrifuged at 6,000 x g (IEC Clinical Centrifuge, International Equipment Co.) for 10 min. The resulting supernatant was removed and then the pellet was resuspended in 7 mL of ACE buffer (10 mM sodium acetate, 10 mM NaCl, 3 mM EDTA [pH5.1]). The resuspended pellet was heated in a water bath to 60 °C for approximately 20 min. Then 250 μL of ACE buffered phenol-chloroform-isoamyl alcohol and 500 μL of 20 % (w/v) sarcosyl (warmed to 60 °C) were added to the warm pellet.

ACE buffered phenol-chloroform-isoamyl alcohol was prepared under the hood by adding 5 mL of ACE buffer per 25 mL of phenol-chloroform-isoamyl alcohol (75:24:1 volume basis). For 25 mL of phenol–chloroform-isoamyl alcohol: 18.75 mL of phenol (Sigma, P-4557 Lot# 48H0590), 6 mL of chloroform (American Burdic and Jackson, high purity), and 250 μL of isoamyl alcohol (J.T. Baker) were combined in an erlymeyer flask and shaken prior to 5 mL of ACE buffer addition. This mixture was always vortexed prior to use.
After the 20 min incubation with 20 % (w/v) sarcosyl and phenol-chloroform-isoamyl alcohol, tube contents were vortexed briefly and then incubated at room temperature for 5 min. After incubation, 300 µL of 2 M NaCl (at 60 °C) was added to the tube contents which was then vortexed briefly. A volume of 6 mL of ACE-buffered phenol-chloroform-isoamyl alcohol was added, and then the tube contents were vortexed for 30 sec and incubated at room temperature for 5 min under the hood. Following the incubation, the tube contents were centrifuged at 12,500 x g (Avanti J-25, Beckman) for 15 min at 4 °C. Separation of the aqueous (top) and organic (bottom) layers could be observed after centrifugation. As much of the aqueous phase (8 to 13 mL) as was possible was transferred to a sterile conical centrifuge tube. Approximately 10 % v/v of ice-cold 3 M sodium acetate was added to the extract. The tube contents were vortexed briefly prior to the addition of 3 times the extract volume of ice-cold 100 % ethanol. Samples were then vortexed and stored at -78°C for 2hr.

After that period, the tubes were taken out of the freezer and warmed at room temperature for 30 min. The tube contents were then centrifuged at 6,000 x g (Eppendorf centrifuge 5804) for 15 min. The supernatant was then decanted and discarded. The remaining pellet was thoroughly dried again with N₂ gas. The pellet was washed once in 1mL of ice-cold 70% ethanol –30% TE buffer (10 mM Tris , 1 mM EDTA [pH 8.0]). The tube contents were centrifuged again at 6,000 x g for 15 min and the resulting supernatant was decanted and discarded. Once again the remaining pellet with thoroughly dried with N₂ gas. The pellet was then resuspended in 500 µL of 10 mM Tris lysing buffer. The
DNA was removed from the extracted nucleic acid solution, by adding 50 μL of 10x DNase buffer (100mM sodium acetate, 500mM NaCl, 10mM MnCl₂) and 50 μL of 100 U RNase-free DNase (cat # M6101 Promega) to the extract. The tube contents were incubated for 1.5 hr at 37 °C. DNase was removed by phenol-chloroform-isoamyl alcohol extraction (same concentrations as previously defined. The tube contents were split into two sterile Eppendorf tubes (approximately 250 μL aliquots). An aliquot (500 μL) of ACE-buffered phenol-chloroform-isoamyl alcohol was added to each tube. The tubes were vortexed then centrifuged in a microcentrifuge at 12,500 x g for 5 min (Eppendorf Centrifuge 5415). The top fractions were extracted with autoclaved Pasteur pipettes and put back into sterile 50-mL conical centrifuge tubes. RNA was precipitated by adding 100 μL of 3 M sodium acetate and 10 mL of ice-cold 100 % ethanol. The tube contents were centrifuged and decanted. The remaining pellet was completely dried with N₂ gas. Lastly, the RNA pellet was resuspended in 500 μL 10 mM Tris lysing buffer. Extracts were stored at −78 °C.

Organic components were removed from the RNA extracts by spun-column gel filtration. First, 2.5 mL Sephadex G-75 (cat # G-75-120, Sigma Chemical Co.) per sample was saturated with distilled water and equilibrated over night in a 50 mL conical centrifuge tube. The end of the plunger was removed from a sterile 3 mL syringe, and the syringe packed with glass wool plug (0.5 mL). The Sephadex gel was centrifuged in a swing-bucket centrifuge for 5 min to remove excess dH₂O. After the excess water was decanted, 2.5 mL of Sephadex gel was placed in the syringe. The column was pretreated
by centrifugation in a swing-bucket centrifuge at 1,400 \times g (IEC Clinical Centrifuge, International Equipment Co.) for 4 min. The crude RNA extract (approximately 500 \mu L) was then added to the top of the sephadex in the syringe. The syringes were then centrifuged with the Eppendorf tube fitted at the bottom of the syringe. The syringe and Eppendorf tube were centrifuged at 2,500 \times g for 3 min (Eppendorf centrifuge 5804). The purified RNA collected in the Eppendorf tube during centrifugation and was then stored at 
\(-78 \, ^\circ C\).

Absorbance values were then performed on the RNA to determine purity. An A_{260}/A_{280} ratio of 2.0 is indicative of samples that are pure RNA (Sambrook \textit{et al.}, 1989). The A_{260}/A_{280} ratio averaged 1.68 for crude RNA preparations and 1.91 for purified preparations. RNA concentrations in extracts were determined spectrophotometrically in a 1 cm quartz cuvette. To determine the concentration, 5 \mu L of extract was diluted to 350 \mu L and the A_{260} determined. An absorbance value of 1 was assumed to equal 40 \mu g/mL of RNA (Sambrook \textit{et al.}, 1989).

Approximately 1\mu g of extracted RNA from samples was blotted onto Zeta-Probe\textsuperscript{\textregistered} Nylon filter blotting membranes (cat. \# 162-0165, Biorad) using a Minifold II slot-blot system (Scheicher & Schuell). Nylon filters were washed with 20 x SSC buffer (0.3 M sodium citrate, 3 M sodium chloride, and 20 \mu L of 37 \% formaldehyde) and a vacuum applied to the Minifold II slot-blot system (< 1 atm) prior to sample addition. After the application of the sample, the sample wells were rinsed twice with 100 \mu L of 2x
SSC. The 16S rRNA was cross-linked to the nylon filters by heat treatment (80 °C) under vacuum (< 15 mm Hg) for 2.5 hr. The blots were stored at −20 °C until use.

Probes Utilized for Hybridization with 16S rRNA

Oligonucleotide probes were utilized for determining quantities of SRB 16sRNA present in sediments and sediment slurries (Devereux et al., 1992). Oligonucleotide probes were synthesized at the Molecular Genetics Facility of the University of Georgia using an ABI DNA/RNA synthesizer (model 394). Table 3.2 illustrates the oligonucleotide sequence and numerical identification of each probe. A name was also designated for each oligonucleotide probe. This name indicates a specific genus within an SRB phylogenetic group that the oligonucleotide was hybridized. Although these names are indicative of a specific genus within a phylogenetic group, the names are used to identify the phylogenetic groups that were detected in sediment cores and sediment reactor systems.

Labeling and Probing with [³²P] Labeled Oligonucleotide Probes

Oligonucleotides received from the University of Georgia were quantitated based on optical densities reported by manufacturer (Sambrook et al., 1989). The oligonucleotides were constituted in sterilized dH₂O to a concentration of 100 ng/μL. The labeling of oligonucleotides was similar to the method of Frischer et al. (1996); Stahl and Amann (1991).
<table>
<thead>
<tr>
<th>Probe name</th>
<th>Probe #</th>
<th>Sequence of Oligonucleotide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Universal SRB Probe</td>
<td>SRB-100</td>
<td>CGY-GCG-CER-CTY-TAC-T</td>
</tr>
<tr>
<td>DSV*</td>
<td>687-704</td>
<td>TAC-GGA-TTT-CAC-TCC-T</td>
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<td>DSC*</td>
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<td>ACC-TAG-TGA-TCA-ACG-TTT</td>
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<tr>
<td>DBACTER*</td>
<td>129</td>
<td>CAG-GCT-TGA-AGG-CAG-ATT</td>
</tr>
<tr>
<td>DSBM*</td>
<td>221</td>
<td>TGC-GCG-GAC-TCA-TCT-TCA-AA</td>
</tr>
</tbody>
</table>

* Indicates abbreviations for sulfate-reducing bacteria. DSV is *Desulfovibrio*, DSB is *Desulfobulbus*, DSC is *Desulfooccus*, DBACTER is *Desulfobacter*, and DSBM is *Desulfobacterium*. Pure culture DBACTER and DSBM used to calibrate probes are saltwater species. The others represent freshwater species.

In brief, an oligo cocktail was made when 2 μL of oligonucleotide was combined sequentially with 30 μL of sterilized dH2O, 10 μL of 10 mM Spermidine, 5 μL of 10x polynucleotide reaction buffer (cat. # C1313, Promega), 3 μL of [32P]-ATP (3000 Ci/mmol; DuPont/NEN), and 2 μL of T4 polynucleotide kinase (Promega, cat. # M4101).
The cocktail was allowed to incubate in a water bath at 37 °C for 2 hr. After incubation, the percent incorporation of \(^{32}\text{P}\)-ATP onto the oligonucleotide was determined as described by Frischer \textit{et al.} (1996). In brief, 2 μL of labeled oligo cocktail was mixed with 98 μL of 0.2 M EDTA pH 8.0. Three μL of diluted oligo cocktail was spotted onto each of four 2.5 cm DE81 filters (cat. # 1001325, Whatman). Filters were dried under infrared lamp for 3 min. Two filters were immediately placed into scintillation vials to represent total counts. Counts per minute (CPM) were determined using a liquid-scintillation counter (Beckman LS6500) with 10 mL Ecoscint\textsuperscript{©} (cat# LS-271, Manville, NJ) scintillation cocktail. The other two filters were washed two times in 50 mL of 0.5 M NaPO\textsubscript{4} (pH 6.8) at 25 °C (5 min each wash). The washed filters were then dried under lamp (3 min) and placed in scintillation vials (washed counts). Both sets of filters were counted for 5 min in the liquid scintillation counter using a automatic counting program specific for \(^{32}\text{P}\). The percent incorporation is defined by Equation 3.4:

\[
\text{% incorporation} = \frac{\text{cpm WASHED}}{\text{cpm TOTAL}} \times 100 \quad \text{Equation 3.4}
\]

In general, all probes had 20-60 % incorporation. However, it was necessary to adjust the specific activities of all probes to within 0.5 % of each other by adding an appropriate amount of unlabeled oligonucleotide following the end labeling as described by Frischer \textit{et al.} (1996).
Pre-hybridization and Hybridization of 16S rRNA Probes

Blots containing 16S rRNA were placed in heat-sealable bags (KAPAK®/scotchpak, 0.5L pint size). A volume (15 mL) of pre-hybridization solution (See Appendix A) was placed in bag with blots and then sealed using a Touch Sealer (KAPAK® Corp.) Sealed bags were then placed in a Tupperware dish and allowed to incubate in a circulating waterbath for 2 hr at 39 °C. Following the two-hour incubation, the pre-hybridization solution was removed. The appropriate [³²P] labeled probe was added to the blots followed by the addition of 15 mL of fresh pre-hybridization solution that had been warmed to 55 °C. Bags were then sealed and replaced in a Tupperware dish. Hybridization was allowed to occur over 18 hr in a shaking circulating water bath at 55 °C. After 18 hr, probe was removed from hybridization bags and saved in 45 mL tubes that were stored in a plexiglas shield at 4 °C.

Wash of Nylon Filters

Blots for each probe were washed three times (20 min each wash) in deep dishes that contained 50 mL of wash buffer (See Appendix C) at 55 °C. After final washes, the blots were dried for 3 min under infrared lamps and taped to blotting paper (cat. # 28303-100, VWR 238, VWR Scientific). Blots were then exposed to film (Fuji Medical X-Ray film, 20.3 x 25.4 cm, Fuji Medical Systems U.S.A.) in autoradiograph cassettes (FBXC 810, Fisher Scientific) with enhancement screen at – 80 °C.
Film was taken out of cassettes and placed in D-19 developer (Kodak) for 2.5 min. Film was then rinsed in dH$_2$O for 1 min. Finally, film was washed in fixer solution (Kodak) for 5 min and air dried on racks.

Quantification of 16S rRNA

The specificity and quantification of probes were determined using a densitometer (420 OE, PDI Inc.) that quantitated band signal intensity. In brief, film was scanned using the densitometer. The background signal of each blot was removed by subtracting the non-specific binding regions of that blot. Band intensities where then determined based on peak areas that reflected the peak intensities. Concentrations of 16S rRNA in samples were then quantitated based on standard curves of 16S rRNA isolated from pure cultures of DSV, DSB, DSC, DBACTER, and DSBM. Standard curves consisted of 16S rRNA that ranged from 100 to 12.5 ng. The $r^2$ values for DSV, DSB, DSC, DBACTER, and DSBM were greater than 0.950.
CHAPTER IV

COUPLING MERCURY METHYLATION RATES TO SULFATE REDUCTION RATES IN MARINE SEDIMENTS

Anoxic, slurry incubations were performed to examine the relationship between mercury methylation rates (MMR) and sulfate reduction rates (SRR) in saltmarsh sediments located at the Skidaway Institute of Oceanography. Various stimuli or inhibitors were introduced to batch reactors in an effort to enhance or suppress SRR. Production of methylmercury was then monitored in parallel slurry reactors over time. Total soluble mercury concentrations found in slurry waters over time were also characterized in the anoxic reactor systems. Manipulations in reactor environments were generated by changes in temperature, addition of low molecular weight organics, and microbial inhibitors. Various concentrations of inorganic mercury were also added to reactors in order to determine if saturation kinetics occurred.

Intact Core SRR and MMR

The SRR and MMR of sediment cores taken from Skidaway Island marshes are illustrated in Figure 4.1.
Figure 4.1 Mercury methylation rates (MMR) and Sulfate-reduction rates (SRR) observed in sediment cores located at the Skidaway Institute of Oceanography (Sediment A-3)
Both the SRR and MMR maxima are located in the top 4 cm of the core with a continual decrease observed in SRR and MMR with respect to depth. The results in Figure 4.1 suggest that a correlation existed between SRR and MMR in sediment cores. These results suggested further investigation of this phenomenon by utilizing more controlled environments in anoxic sediment slurries.

**Temperature Effects on Sulfate Reduction Rate and Mercury Methylation**

In order to substantiate a relationship between SRR and methylmercury production, sediment slurries were incubated at 4 °C, 25 °C, and 37 °C. The various temperatures provided a means of manipulating the system while assessing the sulfate reducing bacteria (SRB) response in SRR and methylmercury production from parallel reactors operated at a specified temperature (Figure 4.2). MMR estimated for the initial 12-hr period increased with temperature. MMR at 4 °C, 25 °C, and 37 °C were 117.4, 274.3, and 4,892 pg/g_dry-hr, respectively. Rates observed at 25 °C and 37 °C exceeded that measured at 4 °C by factors of 2.3 and 41.7, respectively (Figure 4.2). Incubation temperature also affected the SRR in slurry reactors with average rates at 25 °C (SRR = 4.41 nmol/g_dry-hr) and 37 °C (SRR = 41.5 nmol/g_dry-hr) exceeding those measured at 4 °C (1.04 nmol/g_dry-hr) by factors of 4.2 and 39.9, respectively (Figure 4.3). The data presented in Figures 4.2 and 4.3 indicate that temperature influenced methylmercury production and SRRs similarly.
Figure 4.2 Methylmercury production in sediment slurry reactors operated at 4 C, 25 C, and 37 C (Sediment A-4).
Figure 4.3 Sulfate reduced in sediment slurry reactors operated at 4C, 25C, and 37C (Sediment A-4).
Isaksen et al. (1994) reported similar SRR trends for slurries derived from sediments at Aarhus Bay, Denmark. Isaksen et al. (1994) reported SRR activity for non-substrate added slurries to be minimum at temperatures near 0 °C and maximum at temperatures of approximately 35 °C (1994). The results published by Isaksen et al. (1994) indicate that variations in temperature can affect SRB respiration. Results from our studies indicate that the methylation of mercury is also affected by changes in temperature. Moreover, the responses in mercury methylation and SRR follow the same trend with respect to the temperatures that reactors were incubated.

**Effects of Inhibitors on Sulfate Reduction and Mercury Methylation**

To examine further the relationship between SRR and mercury methylation, a series of incubations were performed under conditions that inhibited bacterial sulfate reduction. Methylmercury concentrations in untreated control incubations reached values of 11-13 ng/g in the initial 12 hr with a MMR (12 hr) of 916.7 pg/g-hr (Figure 4.4). In contrast, MMR (initial 12 hr) in sterilized sediments and sediments to which 100mM molybdate was added were approximately 78.7 and 73.5 pg/g-hr, respectively (Figure 4.4). Molybdate is a known inhibitor of SRB (Compeau and Bartha, 1985). Concentrations for sterilized and molybdate-treated sediments remained below 1.2 ng/g throughout the incubation (Figure 4.4). The SRR measured for each treatment had similar trends.
Figure 4.4 Methylmercury production in sediment slurry reactors that were heat-inactivated or treated with 100mM molybdate (Sediment A-3).
Relative to the untreated controls, Figure 4.5 illustrates that the sterilized slurries and 100 mM molybdate treated slurries display SRR of approximately 1.4 and 1.09 nmol/g-hr, respectively.

**Effects of Substrate Addition on Sulfate Reduction and Mercury Methylation**

Pyruvate and acetate were added to reactors in order to further substantiate a positive correlation between the sulfate reduction rates (SRR) and methylmercury production using these substrates to stimulate SRR. Incubations in which SRR were stimulated by the addition of added substrate (100 mM-pyruvate or 100 mM-acetate) resulted in elevated MMRs when compared to controls. Large concentrations of substrate provided reactor environments that were dominated by pyruvate or acetate as a non-limiting, electron source.

In the initial 12 hr incubation period, the MMR for the control, 100 mM pyruvate, and 100 mM acetate reactors was approximately 916.7, 2983, and 2668 pg/g-hr. This indicates that at the end of the initial 12 hr, reactors containing 100 mM pyruvate produced 3.25 fold more methylmercury than controls. Similarly, acetate treated reactors yielded 2.9 fold more methylmercury than controls (Figure 4.6). The reactors that were treated with pyruvate and acetate had increases in methylmercury concentrations that corresponded to observed SRR (Figure 4.6). In the initial 12 hr of reactor operation, SRR for acetate (38.15 nmol/g-hr) and pyruvate (46.76 nmol/g-hr) reactors were approximately 2.0 and 2.5 fold higher than control values (Figure 4.7).
Figure 4.5 Sulfate reduction rate for sediment slurry reactors that were heat-inactivated or treated with 100mM molybdate (Sediment A-3).
Figure 4.6 Methylmercury concentrations in sediment slurry reactors that had been treated with 100mM pyruvate and 100mM acetate (Sediment A-3).
Sulfate Reduced (nmol/g<sub>dry</sub>)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Equations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>SRR = 18.07 nmol/g&lt;sub&gt;dry&lt;/sub&gt;·hr (std. error = 1.13 nmol/g&lt;sub&gt;dry&lt;/sub&gt;·hr)</td>
</tr>
<tr>
<td>Acetate</td>
<td>SRR = 38.15 nmol/g&lt;sub&gt;dry&lt;/sub&gt;·hr (std. error = 1.54 nmol/g&lt;sub&gt;dry&lt;/sub&gt;·hr)</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>SRR = 46.76 nmol/g&lt;sub&gt;dry&lt;/sub&gt;·hr (std. error = 0.44 nmol/g&lt;sub&gt;dry&lt;/sub&gt;·hr)</td>
</tr>
</tbody>
</table>

Figure 4.7  Sulfate reduction in reactors treated with 100mM acetate and 100mM pyruvate (Sediment A-3).
The amount of methylmercury produced within pyruvate treated reactors is greater over time than those treated with acetate. The reason for this phenomenon is unclear. Literature suggests that SRB can be divided into acetate and non-acetate users (Brock et al., 1994). The results illustrated in Figure 4.6 suggest that pyruvate can be utilized by a larger population of SRB which allows for greater methylmercury production, while acetate can be utilized by only a fraction of the SRB resulting in less methylmercury production. Also equimolar concentrations of acetate and pyruvate are not equal in terms of electron equivalents. Pyruvate is 1.25 times higher in electron equivalents than acetate when both low weight organics are completely oxidized. This may also be an explanation of the 1.2 fold higher SRR for pyruvate over acetate and the corresponding 1.12 fold increase in MMR for pyruvate treated reactors over acetate treated reactors.

**Sequestering or Precipitation of Inorganic Mercury in Batch Reactors**

An initial study with mercury additions of 955 ng/g dry weight of Hg$^{2+}$ was conducted to monitor slurry water mercury concentrations in a reactor with a dry sediment concentration of approximately 140 g/L. The variation in methylmercury and aqueous mercury concentrations observed during slurry incubations is displayed in Figure 4.8. Following Hg$^{2+}$ addition at the zero timepoint, slurry water mercury concentrations were 160 ng/L which indicated that greater than 99.8% of the inorganic mercury added to the reactors was associated with the solid phase (i.e., 0.02% was in solution).
Figure 4.8 Methylmercury concentrations and total soluble mercury concentrations in sediment slurry reactors (Sediment A-6).
Methylmercury concentrations increased in a linear fashion over the initial 10-12 hr period followed by the attainment of a plateau value.

Slurry water mercury responded in a similar but opposite manner demonstrating the strong correlation of MMR to slurry water Hg\(^{2+}\) concentration (Figure 4.8). Since methylmercury concentrations effectively reached a plateau value by the 12hr timepoint in all non-manipulated slurry systems, it was concluded that a 12hr determination of MMR represented an appropriate estimate of mercury methylation rates. Additional incubations were performed in which two sequential doses of inorganic mercury were administered 24 hr apart to determine if MMRs could be reproducibly stimulated (Figure 4.9A). Methylmercury production was greatest in the 12 hr after each addition of inorganic mercury (Figure 4.9A). The reactor that received two doses of mercury, with one at 0 and one at 24 hr, had MMR for the following 12 hr of 273.67 pg/g-hr and 420.25 pg/g-hr, respectively. The SRR remained constant over the 48 hour time period which indicated that changes in the bacterial respiratory processes were not the cause of the observed spike in mercury methylation at the 0 hr and 24 hr timepoints (Figure 4.9B).

These results suggest that the higher aqueous mercury concentrations present in the early stages of the incubations correlated with higher MMR. Subsequent data analysis in this dissertation will therefore focus on the initial 12 hr period. The MMR decrease relative to decreases in slurry water Hg\(^{2+}\), suggested that sequestering of inorganic mercury is a possible factor in the decrease in MMRs over time.
Figure 4.9 (A) Methylmercury production in a reactor receiving two sequential doses of 955 ng/g dry inorganic mercury with a second dose added 24 hr after the first dose. (B) SRR in reactors receiving a single and double dose of inorganic mercury (Sediment A-2).
In conjunction, Figure 4.9 illustrates that reactors which received a second addition of 955 ng/g of Hg$^{2+}$ resulted in a subsequent increase in methyl mercury production for the following 12 hr. It is important to note that the MMR for the 12 hr following the second dose is comparable to the MMR observed in the initial 12 hr for both reactors. This result, in addition to no variations in SRR between the two reactors, suggests that the matrix of sediment can influence the amount of inorganic mercury that is available for methylation. Inorganic mercury has a strong affinity for sediment (Jackson, 1989). Whether or not mercury that is associated with the sediment can be methylated remains to be elucidated.

**Inorganic Mercury Additions to Sediment Slurries**

Results of sediment slurries treated with various concentrations of a single dose of inorganic mercury are reported in Figure 4.10A. These results illustrate a concentration dependency between inorganic Hg$^{2+}$ added and the MMR observed in the 12 hr after inorganic mercury addition. It should be noted that the net methylmercury production in reactors treated with 955 ng/g is different in the two experiments illustrated in Figures 4.8 (April, 1997) and 4.9A (October, 1996). This is attributed to seasonal variations in sediment components and bacterial SRB populations. This point will be illustrated in subsequent experiments and chapters.
Figure 4.10 (A) Methylmercury concentrations in reactors receiving various doses of inorganic mercury. (B) Sulfate reduced in reactors receiving various doses of inorganic mercury (Sediment A-1).
Interpretation of Data Sets

Initial observations indicate that significantly higher concentrations of methylmercury were produced when known substrates of SRB were added to reactors (Figure 4.6). This correlated well with SRR results observed in parallel reactors receiving the same treatment (Figure 4.7). Inactivation of SRB by extreme heat treatment or inhibitor addition dramatically reduced methylmercury production (Figure 4.4). The methylmercury results correlated well with SRR results observed in parallel reactors receiving the same treatment (Figure 4.5). Similarly, a correlation in methylmercury production and SRR was observed when reactors were incubated at different temperatures (Figure 4.2 and Figure 4.3). Collectively, this series of experiments demonstrated that methylmercury production is dependent on the microbial activity, as indicated by sulfate-reduction rates of SRB.

Results presented in Figures 4.8, 4.9, and 4.10 also suggest that mercury availability is a critical component in the microbial production of methylmercury. Figure 4.8 demonstrated that methylmercury concentrations approached a plateau value as the length of the experiment increased. This phenomenon occurred simultaneously with an exponential decrease in total soluble mercury concentrations. A plot of MMR, derived from methylmercury concentrations obtained at sequential timepoints, and corresponding slurry water mercury concentrations is illustrated in Figure 4.11. The data indicate that the MMR is linearly dependent on the amount of slurry water mercury present in anoxic reactors at this applied dose of inorganic mercury (i.e., 955 ng/g dry sediment).
Figure 4.11 MMR as a function of total soluble mercury concentrations. Data were developed from Figure 4.8 with MMR calculated as a running two-point rate and total soluble mercury as mid-point averages.
However, data reported in Figure 4.10 indicate that the amount of inorganic mercury applied to the slurry reactors does influence the observed concentration of methylmercury at the 12 hr timepoint. Figure 4.12 illustrates calculated MMR that were derived from the 0 and 12hr methylmercury concentrations of Figure 4.10 and plotted as a function inorganic mercury applied to the reactors. These results demonstrate that saturation kinetics occurred with respect to the amount of inorganic mercury applied to the system. Thus, in well-mixed systems that are not diffusion limited, a maximum mercury methylation rate should exist. This rate is approached as the concentration of dissolved inorganic mercury increases. This result can also be mathematically represented similar to the Michaelis-Menten equation in Equation 4.1.

\[
    MMR = \frac{MMR_{\text{max}} \cdot [Hg^{2+}]}{K_{Hg^{2+}} + [Hg^{2+}]} \tag{Equation 4.1}
\]

The equation was developed as a means to explain observed conversion rates that are directly proportional to the compound concentration when concentrations are low. In addition, Michaelis-Menten kinetics define a maximal rate, independent of compound concentration when concentrations are high (Stryer, 1988). Michaelis Menten kinetics has been used to describe enzyme kinetics in biological systems. Therefore, the use of Michaelis-Menton kinetics in these systems was justified since the production of methylmercury production is thought to be an enzymatic process (Choi et al., 1994b).
Figure 4.12 Mercury methylation rate (initial 12 hr) plotted with respect to inorganic mercury applied to slurry reactors (Data derived from Figure 4.10).
MMR indicates the mercury methylation rate, $\text{MMR}_{\text{max}}$ is the maximum mercury methylation rate, $[\text{Hg}^{2+}]$ is the aqueous inorganic mercury concentration, and $K_{\text{Hg}^{2+}}$ is equal to the inorganic mercury concentration at which the reaction is half the maximum value. Increasing $[\text{Hg}^{2+}]$ effectively saturates the system such that MMR approaches $\text{MMR}_{\text{max}}$. Comparing data in Figure 4.11 to data in Figure 4.12 suggests that mercury applied at 955 ng/g$_{\text{dry}}$ levels in this sediment slurry resulted in linear methylation, well below any perceived maximum or saturation rate. It would also indicate that methylation was well below a half-velocity saturation value and that naturally-occurring, mercury perturbations in this range would dramatically increase methylation rates. In terms of correlation of these data to variable-order kinetic data in Figure 4.12, the sediment samples for the two experiments were different and were collected in different seasons, having significantly different SRR, e.g., 5.0 nmol/g-hr (sediment A-1) vs 21.1 nmol/g-hr (sediment A-6). The MMR values obtained from Figure 4.12 are based on relative mercury levels (i.e., $[\text{Hg}]_{\text{added}}$, expressed in terms of mass mercury per mass of sediment) and followed a saturation kinetic-like response. The linear response in Figure 4.11 is analogous to the pseudo-first order or linear response at low levels of added mercury in Figure 4.8 (e.g., $[\text{Hg}]_{\text{added}}$ levels of 95-955 ng/g) and is not indicative of those rates at higher mercury addition levels for the sediment in Figure 4.12. The kinetic data for the two experiments however are in conceptual agreement and indicate the critical role of mercury availability on methylation processes and they collectively establish the hypothesis that fundamental microbial-process kinetic relationships can be used to determine in situ processes in sediments.
Proposed Model for SRB Methylation of Mercury

Data interpretation of preliminary slurry-reactor data demonstrated that MMR was a function of microbial respiration by SRB and mercury availability. The following equations are introduced as a method of predicting mercury methylation rates relative to SRR and mercury availability and for use in experimental design.

Terminology

In a sediment slurry system, the following parameters are defined. Note that all terms are presented in terms of equivalents for this system to allow for a common terminology for organic and electron-acceptor components. This is driven by the focus on sulfate reduction and its role as final electron acceptor. Conversion to more common or conventional units of measure (e.g., mass and number of microbial cells; and mass or molar values for sulfate and mercury) will be addressed later.

\[ X_B = \text{microbial biomass} \]
\[ S_S = \text{organic-carbon substrate in aqueous phase} \]
\[ X_S = \text{particulate organic (unavailable for direct microbial metabolism)} \]
\[ K_S = \text{half-velocity constant for available organic-carbon substrate} \]
\[ Y = \text{yield coefficient} \]
\[ \mu = \text{net specific growth rate} \]
\( \hat{\mu} \) = maximum specific growth rate

[SO\(_4^–\)] = sulfate concentration

\( k_c \) = first-order rate of hydrolysis for particulate organic matter

\( K_{SO_4} \) = half-velocity constant for sulfate

\( K_{Hg} \) = half-velocity constant for Hg

\( \hat{f} \) = maximum production of CH\(_3\)Hg\(^+\) per sulfate reduced to sulfide

**Fundamental microbial processes**

The growth of microbial cells is modeled as a simple, first-order reaction.

\[ \frac{dX_B}{dt} = \mu X_B \]  
\text{Equation 4.2}

where \( \mu \) = net specific growth rate

The utilization rate of organic-carbon substrate is expressed as follows.

\[ \frac{dS_i}{dt} = -\frac{1}{Y} \mu X_B \]  
\text{Equation 4.3}

where \( Y \) = cell yield coefficient.
The final electron acceptor is sulfate for the sulfate-reducing bacteria (SRB) in these marine sediment systems. The electron flux is to (i) the production of cell mass (through equation 1) and (ii) respiration of the final electron acceptor (i.e., sulfate-S), thereby

\[
- \frac{d[S_{O_4}]}{dt} = \frac{1}{Y} \frac{Y}{\mu} X_B
\]

Equation 4.4

These three equations address the overall growth of the cells and associated anaerobic respiration processes in organic-rich, sulfate-saturated sediments.

The availability of organic matter in pore waters is assumed driven by the hydrolysis of sediment organic matter (Xₜ) by extracellular enzymes to provide available substrates (Sₚ), i.e.,

\[
\left( \frac{dX_t}{dt} \right)_h = - k_e X_t X_B
\]

Equation 4.5

This reaction is furthermore assumed to limit bio-growth, since pore-water levels of available organics do not accumulate to excessive levels.

In the research with sediment systems and given the excess level of particulate organic matter (Xₜ), monitoring of biological processes with SRB is through the rate of sulfate reduction. Thereby equation 4.4 is the pivotal one for expanded kinetic analysis.
Using equation 4.4 to demonstrate process variable effects, key sediment variables are \([\text{SO}_4^\text{-}], [\text{O}_2],\) and \([S_\text{z}].\) Using the Michaelis–Menten approach, \(\mu\) can be defined as:

\[
\mu = \frac{\hat{\mu} S_z}{K_z + S_z \left( \frac{[\text{SO}_4^\text{-}]}{[\text{SO}_4^\text{-}] + K_{\text{SO}_4^\text{-}}} \right) \left( \frac{K_{\text{O}_2}}{[\text{O}_2] + K_{\text{O}_2}} \right)} \quad \text{Equation 4.6}
\]

Combining equations 4.4 and 4.6, results in

\[
-r_{\text{SO}_4} = \left( \frac{1 - Y}{Y} \right) \frac{\hat{\mu}}{K_z + S_z} \left( \frac{[\text{SO}_4^\text{-}]}{[\text{SO}_4^\text{-}] + K_{\text{SO}_4^\text{-}}} \right) \left( \frac{K_{\text{O}_2}}{[\text{O}_2] + K_{\text{O}_2}} \right) X_b \quad \text{Equation 4.7}
\]

In active marine sediment layers, the slow rate of enzymatic hydrolysis of particulate organics and the elevated levels of sulfate ([SO\(_4^\text{-}\)]) will typically result in a constant rate of sulfate reduction, given the rate of production of \(S_z\) (equation 4.7) is the controlling variable. Also, oxygen levels are typically negligible eliminating the effects of oxygen as a toxicant (and electron acceptor). Equation 4.6 and 4.7 indicate that an increase in the concentration of oxygen essentially decreases the SRR.
Mercury Assimilation

Mercury is assimilated by SRB resulting in the formation of methylmercury from inorganic mercury, Hg^{2+}, with the apparent reaction being:

\[ R - CH_3 + Hg^{2+} \rightarrow R^+ + CH_3 - Hg^+ \]  \hspace{1cm} \text{Equation 4.8}

It is proposed that this process of methylation is proportional to biomass production and anaerobic respiration and that methylation proceeds in parallel with sulfate reduction. The methylation reaction and its relation to sulfate reduction are central to this research. The hypothesis is that the incidence of methylation is variable between the phenotypes of SRB. That is, the phenotypes of SRB have varying potentials to methylate mercury through possible enzymatic and/or detoxification processes. Furthermore, it is proposed that the rate of methylation is affected by the concentration of available Hg^{2+}. In coupling methylation to sulfate reduction, the following is proposed.

\[ \frac{d[CH_3-Hg^+]}{dt} = \hat{f} \left[ \frac{[Hg]}{[Hg]+K_{Hg}} \right] \left[ \frac{1-Y}{Y} \right] \mu \left( \frac{S_i}{K_i+S_i} \right) \left[ \frac{[SO_4]}{[SO_4]+K_{SO4}} \right] \left( \frac{K_{O_2}}{[O_2]+K_{O2}} \right) (X_B) \]  

where \( \hat{f} = \) maximum methylmercury produced per unit of sulfate reduced
This equation can then be simplified and coupled with equation 4.7 as follows:

\[ r_{CH3Hg} = (-r_{SO4}) \hat{f} \frac{[Hg]}{[Hg] + K_{Hg}} \]  \hspace{1cm} \text{Equation 4.10}

Since \( r_{CH3Hg} \) and \( -r_{SO4} \) represent in-situ rates for a particular sediment slurry, normalizing \( r_{CH3Hg} \) to \( -r_{SO4} \) would represent a net incidence of methylation, \( \hat{f} \), and can be expressed as:

\[ \frac{r_{CH3Hg}}{-r_{SO4}} = \hat{f} \frac{[Hg]}{K_{Hg} + [Hg]} \]  \hspace{1cm} \text{Equation 4.11}

This equation uniquely indicates the equivalent fraction of electrons driven by anaerobic respiration processes that are directed to methylation of mercury. It is proposed herein that this “incidence” term \( \hat{f} \) is a phenotype-specific variable and accounts, in fact, for variation in methylation rates in sediments.

The above modeling included equivalents in assessing substrates, nutrients, and other reactants to minimize the use of conversion factors in the equations. In developing fundamental relationships from experimental systems, it is more convenient to initially use more common units for reactants. In that this research is focused on sulfate reduction and mercury methylation rates, it is appropriate to simplify the terminology for the subsequent
experimental assessment. Therefore, $-r_{SO_4}$ is expressed in units of moles of sulfate per number of cells or normalized to sediment mass (e.g., mole/g-hr). Accordingly, methylation rates will be expressed per number of cells or normalized to sediment mass (e.g., pg/g-hr). The incidence term will be initially assessed as pg methylmercury per nmol sulfate reduced.

Data Interpretations Based on Proposed Model

In examining the data presented in Figures 4.4 and 4.7, note that mercury "concentrations" were expressed in terms of applied doses. In evaluating the data with the above model, a non-linear, hyperbolic fit computer program was applied to the data and is illustrated utilizing equation 4.1 (Sigma Plot version 4.0 © 1997, SPSS inc.). The $K_{Hg}$ value was determined at 1.575ng/g Hg$^{2+}$ added and $MMR_{max}$ at 648.5pg/g-h. If it is assumed that $MMR_{max}$ is proportional to SRR,

$$MMR_{max} = \hat{f}(SRR) \quad \text{Equation 4.12}$$

it is possible to estimate MMR from SRR and the dose of inorganic mercury added to the reactor. In Figure 4.10, the observed SRR remained relatively constant for the dose-response reactors and was approximately 4.94 nmol/g-hr. Normalizing the $MMR_{max}$ by the observed SRR allowed for the calculation of $\hat{f}$ as 131.3 pg/nmol. Since all the variables for the reactor kinetics have been defined excluding the MMR and SRR, Equation 4.1 can now be written in terms of the SRR by substituting the known constant term and SRR for the $MMR_{max}$. Equation 4.13 illustrates the mathematical model for
predicting MMR in terms of the SRR in the initial 12 hr after inorganic mercury addition. This model utilized the dose of inorganic mercury concentration added to the slurry rather than aqueous [Hg$^{2+}$] concentrations. This value is represented by [Hg$^{2+}$]$_a$. Equation 4.13 illustrates the mathematical relationship for predicting MMR in terms of the SRR in the initial 12 hr after inorganic mercury addition.

$$\text{MMR} = \left[131.3 \text{ pg/nmol}\right] \left(\text{SRR}\right) \frac{[\text{Hg}^{2+}]_a}{[1575 \text{ ng/g}] + [\text{Hg}^{2+}]_a}$$  \text{ Equation 4.13}

An attempt was made to assess the relationship by predicting MMR reported in temperature and substrate/inhibitor experiments using the reported SRR. Figure 4.13 illustrates the observed and predicted MMRs based on the SRRs determined for the batch reactors in the temperature and substrate/inhibitor experiments (Figures 4.2, 4.3, 4.4, 4.5, 4.6, and 4.7). The graph illustrates that the model equation provided a good correlation for data reported with an SRR less than 30 nmol/g-hr. Predicted MMRs for the aforementioned reactors were within 5% of the observed MMRs. The accuracy of the model decreases for the three highest SRRs. The SRR determined in the acetate-treated reactors produced a predicted MMR that was 65% of the observed MMR value. Likewise, the predicted MMR for the pyruvate-treated reactors was 80% of the observed value. The reactor for 37°C had the largest discrepancy between observed and predicted MMR with a predicted value that was only 45% of the observed value (Figure
4.13). Figure 4.13 also indicates the range of $\dot{f}$ values reported for the substrate-amended reactors, inhibited reactors and temperature reactors for the initial 12 hr of reactor operation. Although the $\dot{f}$ values for the acetate reactor and control treated reactor (from substrate experiment) are different, the slopes of both $\dot{f}$ values are in agreement with the observed data plotted in Figure 4.13. That is, $\dot{f}$ defined for one reactor system can be used in conjunction with various SRR to generate a calculated MMR that is similar to the observed MMR. That further illustrates the coupling of the MMR to SRR since Equation 4.11 defined $\dot{f}$ as the MMR normalized to the SRR. Table 4.1 illustrates the $\dot{f}$ values for the previously reported sediments and experiments. Excluding values obtained for 4 °C and 37 °C, and examining experiments at 25 °C, the $\dot{f}$ reported for all reactor system was similar among with values ranging from 35.3 to 76.4 pg/nmol. It should be noted that the variations in the $\dot{f}$ term can be attributed to the different sediments being tested. However, values obtained for sediment A-3 demonstrated $\dot{f}$ values that are relatively consistent for the same sediment that received various treatment protocols. The variations in the $\dot{f}$ term for 4 and 37 °C are not understood. All other experiments (conducted at 25 °C) demonstrate similar values in the $\dot{f}$ term.
Figure 4.13 Plot of observed vs. predicted values of MMR demonstrating predicted MMR based on saturation kinetics and $f^*$ values of acetate and control reactors.
Table 4.1

*f* values observed in initial 12 hr for sediment reactors receiving 950ng/g of inorganic Mercury

<table>
<thead>
<tr>
<th>Sediment Experiment</th>
<th>Reactor Designation</th>
<th><em>f</em> value pg/nmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-1</td>
<td>Dose Response</td>
<td>50.1</td>
</tr>
<tr>
<td>A-2</td>
<td>Single Hg(^{2+}) dose</td>
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</tr>
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<td>A-3</td>
<td>Control</td>
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</tr>
<tr>
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<td>Acetate</td>
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<tr>
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<td>Molybdate</td>
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<td>Heat-Inactivated</td>
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</tr>
<tr>
<td>A-4</td>
<td>4 °C</td>
<td>112.9</td>
</tr>
<tr>
<td></td>
<td>25 °C</td>
<td>62.1</td>
</tr>
<tr>
<td></td>
<td>37 °C</td>
<td>117.8</td>
</tr>
<tr>
<td>A-6</td>
<td>Single Hg(^{2+}) dose</td>
<td>35.3</td>
</tr>
</tbody>
</table>
Conceivably, the change in temperature from 25 °C could change the amount of mercury available for methylation. The larger \( f \) term for the 4 and 37 °C reactor was due to an increase response in MMR relative to SRR as compared to other reactor systems (Table 4.1). Figures 4.8 and 4.10 demonstrated that mercury availability is a fundamental component in the ability of SRB to methylate mercury. Conceivably, the availability of mercury could increase or decrease with changes in temperature. Thus, \( f \) would be affected by changes in temperature.

The data presented in this chapter suggests that a correlation does exist between slurry SRR and the amount of methylmercury produced in continuously mixed reactors. In both the variable temperature and substrate/inhibitor experiments, a change in SRR produced a similar, corresponding trend in the amount of methylmercury produced. In all experiments, the greatest amount of methylmercury produced occurred in the first 12 hr after inorganic mercury addition.

An attempt was also made to predict MMR based on SRR. The results demonstrated an ability to reasonably estimate the slurry MMR based on the SRR for the initial 12 hr after inorganic mercury addition. It should be noted that the greatest discrepancy between measured and predicted MMR occurred in reactors that were exposed to conditions that are not encountered in situ. For example, the use of high concentrations of substrates, such as acetate and pyruvate, could affect normal cellular processes. Moreover, high concentrations of substrate could initiate cellular proliferation that could affect the coupling process. In any event, the data presented in this chapter
clearly demonstrate that the SRB mediated methylmercury production is a function of the microbial sulfate-reduction rate and mercury availability. Future studies will assess microbial speciation with respect to potential to methylate mercury both in pure culture and preconditioned sediment slurries.
CHAPTER V

METHYLMERCURY PRODUCTION BY PHYLOGENETIC
GROUPS OF SULFATE REDUCING BACTERIA IN PURE CULTURE

To date, very little information is known regarding the potential of individual phylogenetic groups of sulfate-reducing bacteria (SRB) to methylate mercury. In this study, bacteria representing five phylogenetic groups of sulfate-reducing bacteria (SRB) were tested to determine if the ability to methylate mercury varied among the phylogenetic groups. In previous studies, Desulfovibrio was utilized as a known genus of SRB capable of producing methylmercury (Pak and Bartha, 1998a; Pak and Bartha, 1998b; Compeau and Bartha, 1985). This genus was utilized in pure culture experiments as a positive control that implicated all SRB phylogenetic groups as methylmercury producers.

Pure cultures of SRB were used to represent five phylogenetic groups. Desulfovibrio (DSV), Desulfobulbus (DSB), Desulfo bacterium (DSBM), Desulfo bacter (DBACTER), and Desulfococcus (DSC) of SRB were utilized for methylmercury production comparisons between species. Demonstrating similarities or differences in MMR among
pure cultures of SRB provided pertinent information regarding various phylogenetic
groups most suspected to methylate mercury in natural environments. Moreover, this
novel experimental framework provided a method to compare SRB phylogenetic groups
and their potential to methylate mercury under anoxic conditions in pure culture.

It should also be noted that model equations in chapter 4 introduced mercury
methylation rates (MMR) as a function of sulfate-reduction rates (SRR). In an attempt to
further validate this relationship derived in chapter 4, pure cultures of SRB were grown in
sulfate-depleted systems. Growing SRB in sulfate-depleted systems resulted in cultures
of SRB that fermented organic substrates as a means of providing electrons. SRB
cultures (DSV and DSBM) grown under these conditions were then exposed to inorganic
mercury and resulting methylmercury concentrations quantified over time.

The reproducibility of the experimental protocol was also validated through triplicate
studies performed on phylogenetic groups that represented the greatest (DSBM) and
least ability (DSV) to methylate mercury when normalized to the sulfate-reduction rate.

Figure 5.1 illustrates the experimental protocol followed for SRR and MMR
determinations in pure culture.

**Optical Density Determination for Survey of Phylogenetic Groups**

Cells grown in pure cultures were monitored to ascertain when cells had equilibrated
to the media and had initiated growth. Cells were grown in a multi-purpose media (see
Materials and Methods).
Figure 5.1 Experimental protocol for cell incubations, $^{35}\text{S}$ addition, and inorganic mercury addition. Cells were allowed to grow for 108hrs prior to $^{35}\text{S}$ addition and inorganic mercury was added 120 hr after initial cell inoculation.
Figure 5.2 illustrates the optical density results of continued monitoring of cell cultures when no mercury is applied to cells. Growth, as indicated by the optical density measurement, begins to increase approximately 96 hr after initial inoculation in all cultures. The optical density of *Desulfovibrio*, *Desulfococcus*, and *Desulfobulbus* was higher than that of *Desulfo bacter* and *Desulfobacterium* at > 160 hr (Figure 5.2). From 0 to 264 hr, the optical density of *Desulfovibrio*, *Desulfococcus*, and *Desulfobulbus* increased approximately 130, 128, and 110 fold, respectively (Figure 5.2). For the same time period, *Desulfo bacter* and *Desulfobacterium* increased 20 and 35 fold, respectively (Figure 5.2). Optical density measurements collected at 100-120 hr suggested an initial time period of cellular activity/proliferation. Thus, the 120 hr timepoint was chosen for addition of inorganic Hg into the pure cultures of SRB. In subsequent experiments, cells were exposed to 100 ng/mL of Hg$^{2+}$ at 120 hr and analyzed for total soluble mercury, sulfate-reduction rates, total cell counts, and methylmercury production. Figure 5.3 illustrates the optical density measurements of pure cultures that were exposed to 100 ng/mL of Hg$^{2+}$ at 120 hr after initial cell inoculation. From 0 to 264 hr, the turbidity of *Desulfovibrio*, *Desulfobulbus*, and *Desulfococcus* increased approximately 180, 145, and 140 fold, respectively (Figure 5.3). For the same time period, both *Desulfo bacter* and *Desulfobacterium* increased approximately 65 fold (Figure 5.3). Media that was not inoculated with cells increased in turbidity when 100 ng/mL Hg$^{2+}$ was added.
Figure 5.2 Optical density assessment of pure SRB cultures grown at 27°C for 264 hr.
Figure 5.3  Growth curves of SRB at 27C with addition of 100 ng/mL of inorganic mercury at 120 hr.
Optical density measurements increased approximately 30 fold from 0-264 hr for mercury added to media (no cells) at the 120 hr timepoint (Figure 5.3).

Cell Counts of Phylogenetic Groups

Figure 5.4 illustrated the number of cells found in the culture over time after inorganic mercury addition. In the experimental protocol, a 1 mL aliquot of stock cells was injected into the reactor that contained 50 mL of media. Pure culture reactors were allowed 120 hr for equilibration and growth. Cells were quantitated starting at the time of initial Hg\textsuperscript{2+} exposure (120 hr). Following the 120 hr period and inorganic mercury addition, the number of cells present in each culture remained relatively constant for the duration of the experiment (Figure 5.1). The average total number of cells reported over the length of the experiments is reported for each of the phylogenetic groups that were grown under sulfate-rich conditions in Table 5.1. Values for DSV and DSBM consist of average values for three separate reactors (Table 5.1).

<table>
<thead>
<tr>
<th></th>
<th>DSV</th>
<th>DSB</th>
<th>DSC</th>
<th>DBACTER</th>
<th>DSBM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.08x10\textsuperscript{8}</td>
<td>2.44x10\textsuperscript{8}</td>
<td>1.68x10\textsuperscript{7}</td>
<td>1.27x10\textsuperscript{6}</td>
<td>1.21x10\textsuperscript{6}</td>
</tr>
<tr>
<td></td>
<td>(± 6.4 %)</td>
<td>(± 13.0 %)</td>
<td>(± 15.9 %)</td>
<td>(± 5.0 %)</td>
<td>(± 5.7 %)</td>
</tr>
</tbody>
</table>
Figure 5.4 Cell counts of SRB grown in sulfate-rich pure culture with addition of 100 ng/mL inorganic mercury at 0 hr. Average cell counts and percent standard error in Table 5.1.
Cell counts of DSBM and DSV Grown in Sulfate-Depleted Cultures

Following an initial assessment of the phylogenetic groups, two groups (DSV and DSBM) were selected to determine the ability of sulfate-reducing bacteria to methylate mercury under fermentative conditions, as well as determine the reproducibility of the results observed in the survey of pure cultures. Figure 5.5 illustrates the number of cells counted in each group. The data illustrate that the DSV cultures grown in the presence of sulfate were the most robust. Cell numbers for sulfate-respiring DSV averaged $1.05 \times 10^8$ cells/mL. DSV cultures that contained no sulfate and were obligated to ferment organic acids and had a lower number of cells with an average value of $1.97 \times 10^7$ cells/mL. DSBM cultures were approximately two orders of magnitude lower in number than DSV cells grown on the same media. In general, the average number of DSBM cells growing in the sulfate-rich media averaged approximately $1.10 \times 10^6$ cells/mL and average concentrations for the DSBM sulfate-depleted cultures were $4.13 \times 10^5$ cells/mL (Figure 5.5.). The effects of sulfate depletion on mercury methylation will be discussed in a subsequent section.

Sulfate-Reduction Rates for Phylogenetic Groups

Sulfate-reduction rates (SRR) were assessed for each of the phylogenetic groups grown in pure culture. The slope of a linear regression of sulfate concentration reduced over time indicated the average SRR for each culture over a 108 hr period.
Figure 5.5 Cell counts for DSV and DSBM grown in pure culture at 27°C under sulfate-rich and sulfate-depleted conditions with addition of 100 ng/mL inorganic mercury at 0 hr.
The zero-hr timepoint was the time in which \(^{35}\text{SO}_4\) was added to the culture, and the 12 hr timepoint was the time point where inorganic mercury was added to the cultures). Figure 5.6 and 5.7 illustrate the sulfate reduced over time for DSV and DSB cultures, respectively. Linear regression analysis performed on DSV data, derived from triplicate studies, indicated that an average SRR of 15.226 nmol/mL-hr existed in the pure culture (Figure 5.6). Similarly, linear regression analysis conducted on the DSB pure culture indicated that the average observed SRR was approximately 16.98 nmol/mL-hr (Figure 5.7). Figure 5.8 illustrates the amount of sulfate reduced over time in pure cultures of DSC. Linear regression analysis performed on DSC resulted in an average SRR of 4.58 nmol/mL-hr. Linear regression analysis on DBACTER resulted in an SRR of 1.76 nmol/mL-hr, respectively (Figure 5.9). Linear regression analysis performed on DSBM data that was derived from triplicate studies indicated that an average SRR of 1.36 nmol/mL-hr existed in the pure culture (Figure 5.10). The culture of DSBM that was heat-killed had no observable sulfate reduction rate and was 0.0 nmol/mL-hr.
Figure 5.6 Sulfate reduced in pure cultures of DSV grown at 27°C.
Figure 5.7 Sulfate reduced in pure cultures of DSB grown at 27°C.
Figure 5.8 Sulfate reduced in pure cultures of DSC grown at 27°C.
Figure 5.9  Sulfate reduced in pure cultures of DBACTER grown at 27C.
Figure 5.10  Sulfate reduced in pure cultures of DSBM grown at 27°C.
Methylmercury Production in Pure Culture

The amount of methylmercury found in pure culture was determined over time for each of the phylogenetic groups. The pure culture containing the *Desulfovulbus* species of SRB accumulated the least amount of methylmercury over time. Methylmercury concentrations in DSB cultures increased for the duration of the experiment with final concentrations of 85.0 pg/mL (Figure 5.11). The amount of methylmercury that accumulated in the pure culture of *Desulfobacter* also continued to increase throughout the experiment. Concentrations of methylmercury at the 96 hr timepoint in DBACTER cultures were 110.0 pg/mL. Methylmercury accumulated in the *Desulfovibrion* culture and methylmercury concentrations at 96hrs were approximately 289.0 pg/mL. The pure culture of *Desulfooccus* also demonstrated an ability to generate methylmercury over time. Concentrations of methylmercury were approximately 334.0 pg/ml at the 96 hr timepoint for *Desulfooccus*. Finally, the *Desulfobacterium* species of bacteria illustrated a pronounced ability to accumulate methylmercury in pure culture with a maximum concentration 472.5 pg/mL observed at 96 hr (Figure 5.11).

Methylmercury for DSV and DSBM in Sulfate-Rich and Sulfate-Depleted Systems

Methylmercury concentrations were also determined in the DSV and DSBM cultures that were sulfate-rich, as well as sulfate-depleted in a second experiment (Figure 5.12).
Figure 5.11  Methylmercury concentrations observed over time in pure culture reactors grown at 27°C in which 100ng/mL of inorganic mercury was added at 0 hr.
Figure 5.12 Methylmercury concentrations observed over time in sulfate-rich and sulfate-depleted cultures of DSV and DSBM grown at 27°C. 100ng/mL of inorganic mercury was added at 0 hr.
The average methylmercury concentrations for DSBM growing in a sulfate-rich medium ranged from 52.0 pg/mL to 647.3 pg/mL at the 24 hr and 96 hr timepoints, respectively. Average methylmercury concentrations for DSV growing in a sulfate-rich medium ranged from 55.10 pg/mL to 404.1 pg/mL at the 24 hr and 96 hr timepoints, respectively. Both DSBM and DSV cultures growing on sulfate-depleted media generated minimal quantities of methylmercury. The sulfate-depleted DSV and DSBM cultures generated concentrations of methylmercury that did not vary significantly from blank controls over the 96 hr period (Figure 5.11). DSBM cells that were autoclaved produced trace levels of methylmercury with no evident trend in production. In general, concentrations of methylmercury in the autoclaved sample averaged 14.7 pg/mL (Figure 5.12). Media that contained no cells produced trace amounts of methylmercury over the incubation period with concentrations of methylmercury ranging from 14.21 to 33.65 pg/mL (Figure 5.12).

Establishment of Mercury Methylation Rates in Pure Culture

Data presented in Figures 5.11 and 5.12 demonstrated that all phylogenetic groups of SRB generated methylmercury when grown in a sulfate-rich environment. In both Figure 5.11 and 5.12, the observed methylmercury production in the initial 24 hr is slower compared to methylmercury production in the remaining time series (24-96 hr). This apparent lag phase in methylmercury production can be attributed to the re-establishment of equilibrium conditions with respect to mercury in the media environment. The fact
that many chemical speciation reactions occur with mercury and other components of the media suggests that the chemistry of the media may impact the mercury that is available for methylation in the initial 12 hr. The impact that chemical speciation has on aqueous species of mercury in this pure culture environment will be addressed in the soluble mercury section of this chapter. It is also possible that enzymes necessary to sustain a consistent MMR for the methylation of mercury were not present in sufficient quantity during the initial 24 hr of mercury exposure. The induction of metal scavenger proteins such as metallothioneins due to high concentrations of cadmium being introduced in reticulocytes has been documented (Tanaka et al., 1985). It should also be noted that the initial flux of inorganic mercury into the cell could have been inhibited due to active transport processes. Thus, the concentration of mercury available for methylation within the cell could have been limiting in the initial 24 hr, as opposed to later time intervals where cellular inorganic mercury concentrations were saturated. Based on the fact that all five phylogenetic groups tested demonstrated a lag in methylmercury production in the initial 24 hr, MMR was assessed for each phylogenetic group based on a plot of methylmercury concentrations over the 24 to 96 hr time period (Figures 5.13-5.17). Figure 5.13 illustrates a plot of methylmercury concentrations observed in triplicate cultures of DSV. Linear regression analysis of the data resulted in an MMR of 4.48 pg/mL-hr (Figure 5.13). Figure 5.14 illustrates methylmercury concentrations for DSB over the 24 - 96 hr time period. Linear regression analysis of the data resulted in an MMR of 1.05 pg/mL-hr.
Figure 5.13 Mercury methylation rates (MMR) in DSV cultures. MMR based on linear regression analysis of methylmercury concentrations observed in pure culture over time.
Figure 5.14 Mercury methylation rates (MMR) in DSB cultures. MMR based on linear regression analysis of methylmercury concentrations observed in pure culture over time.

\[ MMR = 1.05 \text{ pg/mL-hr} \]
\[ \text{Std. Error} = 0.32 \text{ pg/mL-hr} \]
Figure 5.15 Mercury methylation rates (MMR) in DSC cultures. MMR based on linear regression analysis of methylmercury concentrations observed in pure culture.
Figure 5.16  Mercury methylation rates (MMR) in DBACTER pure culture. MMR based on linear regression analysis of methylmercury concentrations observed over time in pure culture.
Figure 5.17 Mercury methylation rate (MMR) in DSBM culture. MMR based on linear regression analysis of methylmercury concentrations observed over time in pure culture.
Figure 5.15 illustrates methylmercury concentrations for DSC observed over the 24 to 96 hr time period with linear regression analysis resulting in an MMR of 4.62 pg/mL-hr. A plot of methylmercury concentrations in DBACTER over the 24 to 96 hr time period is illustrated in Figure 5.16. Linear regression analysis resulted in an MMR of 1.55 pg/mL-hr. Finally, linear regression analysis of methylmercury concentrations observed in triplicate cultures of DSBM resulted in an MMR of 7.53 pg/mL-hr (Figure 5.17). In all cultures tested, linear regression analysis resulted in correlation coefficients \( (r^2) \) greater than 0.85, and the standard error of the slopes were generally less than 25 %. This implies that a linear response in methylmercury production occurred after the initial 24 hr and further validated the use of linear regression analysis to determine MMR based on methylmercury concentrations observed from 24 to 96 hr.

**Total Soluble Mercury in Pure Culture Experiments**

Total mercury concentrations were quantitated in the pure culture reactors and are presented in Figure 5.18. There were fluctuations in values obtained over time within the same reactor. However for all cultures, standard errors calculated for the soluble mercury species over the duration of the experiment were less than 10 % of the mean value. This suggests that the soluble mercury concentrations did not change given the minimal degree of variability observed within a given culture. Excluding media that did not contain cells, there was no significant difference in soluble mercury among all phylogenetic groups using t-test statistical analysis (Sigmamplot® version 4.0 (P > 0.05)).
Figure 5.18 Total soluble mercury concentrations present in pure cultures of SRB. Inorganic mercury (100ng/mL) was added at 0hr.
In general, values for total mercury in the media ranged between a maximum of 17.93 ng/mL and a minimum of 8.75 ng/mL in all reactors. An average was taken of all reactors over the duration of the experiment resulted in a value of approximately 10.65 ng/mL (std. error ± 0.64 ng/mL) (Figure 5.18). Reactors that did not contain cells but received an aliquot of inorganic mercury (100 ng/mL) had an average value of 59.89 ng/mL (std. error ± 1.39 ng/mL) of total soluble mercury observed in the media (Figure 5.18).

The concentrations of mercury in the aqueous phase of the pure culture media are on average three orders of magnitude higher relative to the aqueous mercury concentrations of 20-60 ng/L reported in marine systems contaminated with mercury (Bloom et al., 1999). However, it should be noted that porewater mercury concentrations have been reported as high as 2.7 ng/mL in mercury contaminated sediments in BERM mesocosms located at the Skidaway Institute of Oceanography. A review of the components that comprise the anoxic pure culture media (appendix A) indicates that aqueous sulfide concentrations, derived exclusively from additions of Na₂S, were approximately 1.5 mM. In reduced environments, sulfide species are the principal components that influence the speciation and solubility of mercury (Biester et al., 1998). Faust and Osman (1981) have reported that in anoxic waters with a pH of 8.0, aqueous mercury concentrations were approximately 25 ng/L. However, Paquette et al. (1997) have demonstrated that polysulfides can bind with mercury to produce mercury polysulfide species (HgSₙ(SH)⁻, where n = 2 to 6). It has been demonstrated that the presence of such polysulfides can increase the solubility of mercury as much as 1000 fold (Paquette et al., 1997). Paquette
et al. (1998) report soluble mercury concentrations of approximately 20-65 ng/mL (pH = 7) in media that contained polysulfide complexes derived from a saturated solution of sulfur powder and 2-6 mM total sulfide. The soluble mercury concentrations, reported by Paquette et al. (1998), were very similar to the average soluble mercury concentrations observed in this study (10.65 ng/mL for media with cells and 59.88 ng/mL for media without cells). It should be noted that in this chapter, thiosulfate (Na$_2$S$_2$O$_3$) was added to the media to a final concentration of 3.3 mM and this compound could influence mercury chemistry in the pure culture. Other thiol containing reductants such as thioglycolic acid (10 μM final concentration) may also increase mercury solubility through the formation of polysulfide-mercury complexes.

The media has other anion components, such as chloride (Cl$^-$) and sulfate (SO$_4^{2-}$), acetate, lactate, and citrate that contribute to the speciation of mercury as well. However, equilibrium constants reported for mercury speciation with these components (Table 2.2) results in aqueous concentrations on the order of parts per trillion which suggests that these anions do not contribute significantly to the large soluble concentration of inorganic mercury observed in this study. It should be noted that the media used to grow these SRB cells is not indicative of natural systems. Therefore, the levels of aqueous mercury reported in this chapter cannot be compared to soluble mercury levels observed in natural systems. Reducant compounds such as thioglycolic acid and thiosulfate are not typically encountered in marine systems to the degree with which they are added to this system. These two components in addition to the sulfide
chemistry in the pure cultures are offered as possible mechanisms for the increased solubility of mercury.

It should be noted that a difference of approximately 50 ng/mL of soluble mercury exists between cultures that contain cells and those that do not. It is possible that mercury bound or incorporated into cells is effectively removed from the aqueous phase resulting in lower concentrations. It is also possible that SRB cells incorporate or metabolize thioglycolic acid, thiosulfate, or other media components such that mercury speciation is altered to some degree resulting in decreased solubility of mercury relative to media that does not contain cells. In any event, the concentrations of soluble mercury in cultures containing cells were not statistically different from each other (P > 0.05), and the average value is reported here as 10.65ng/mL (std. error = 0.345 ng/mL).

SRR and MMR Determination Per Cell

**Cell Counts in Pure Culture**

Cell cultures in this chapter were reported as having approximately the same number of cells over time after the addition of inorganic mercury. Cells cultures that consistently have the same number of cells over time are characterized as being in a stationary phase of growth. The stationary phase of growth is defined by no net increase or decrease in cell number (Brock et al., 1994). In the stationary phase, cells may cease to proliferate while cellular functions (cell transport processes, secondary metabolite production, and energy metabolism) continue. It has also been illustrated that some cell growth may
continue to occur in the stationary phase. However, cell death and eventual lysis of dead cells occurs at the same rate (Brock et al., 1994).

The number of cells counted varied between phylogenetic groups (Figure 5.4). This could be attributed to inherent differences in the metabolism of the organic acids present in the media. It should also be noted that the low number of Desulfovibrio and Desulfobacterium could be attributed to the salinity of the media. Species of Desulfovibrio (BG8) and Desulfobacterium (BG33) grow more rigorously in media that contains 8 ppt of NaCl. It was necessary to use 1 ppt of NaCl to allow the fresh water species that represented the other three phylogenetic groups to grow. In addition, this experiment required the use of the same medium for all SRB groups in order to maintain consistent profiles in mercury speciation within the culture.

As mentioned in the results section, both Desulfovibrio and Desulfobacterium were grown in a sulfate-depleted medium. Results in Figure 5.5 suggest that growth of the bacteria were consistently lower in the sulfate-depleted cultures for both phylogenetic groups when compared to the sulfate-rich cultures. It should be mentioned that the initial transfer of bacteria from the stock culture to the sulfate-depleted cultures allowed for the introduction of a very small concentration of sulfate (< 200μM). However, concentrations of sulfate were not quantifiable prior to inorganic mercury addition. Replicate studies conducted on Desulfovibrio and Desulfobacterium grown in sulfate-rich media suggest a high degree of reproducibility in the total number of cells found in three different pure culture reactors for the respective phylogenetic group. The observed standard error for cell counts was typically 5-12% of the average value. Comparing cell
numbers obtained for each timepoint suggests that virtually no difference existed between the timepoints. Thus, the average number of cells observed over the 96 hr was used to normalize data on a per cell basis.

SRR on a Per Cell Basis

Figures 5.6, 5.7, 5.8, 5.9, and 5.10 illustrated the SRR on a volumetric basis for each SRB phylogenetic group based on the linear regression of sulfate reduced over time. In order to compare the SRR of a select group of SRB to the other groups, values of SRR (nmol/mL-hr) were normalized on a per cell basis. The average number of cells observed in the cultures over time was utilized to normalize SRR. Table 3.2 illustrates the SRR for each phylogenetic group on a per cell basis.

| Table 5.2 Calculated SRR of Pure Cultures nmmol/cell-hr (± % std. error) |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| DSV                  | DSB                  | DSC                  | DBACTER             | DSBM                  |
| 1.41x10⁻⁷ (± 7.9%)   | 6.96x10⁻⁸ (± 14.6%) | 2.73x10⁻⁷ (± 20.4%) | 1.39x10⁻⁶ (± 13.7%) | 1.12x10⁻⁶ (± 8.7%) |

In general, the data calculated here are similar to the data reported by other groups. An SRR of 2.62 x10⁻⁷ nmoles/cell-hr was reported for free-living Desulfovibrio desulfuricans in pure culture (Fukui and Takii, 1994). In addition, an SRR of 7.04 x 10⁻⁷
nmoles/cell-hr was observed for *Desulfovibrio postgatei* in pure culture (Ingvorsen et al., 1984). Still other species such as *Desulfovibrio salexigens* and *Desulfovibrio sapovorans* had calculated SRR values of $5.20 \times 10^6$ and $1.60 \times 10^6$ nmol/cell-hr, respectively (Jorgenson et al., 1984).

Figure 5.12 illustrates that sulfate-depleted cultures did not generate significant concentrations of methylmercury when compared to reactors that did not have cells. Thus, methylmercury concentrations in sulfate-depleted cultures were not reported on a per average cell basis. Similar results have been reported in cultures of *Desulfovibrio desulfuricans* LS which were unable to generate methylmercury in the absence of sulfate (Pak and Bartha, 1998b). Significant methylmercury concentrations were observed only in cultures that were sulfate-rich which implies that a "coupling" exists between the mercury methylation process and the sulfate reduction process. Choi and colleagues have proposed that methylmercury synthesis by *Desulfovibrio desulfuricans* involved the methyl group transfer of CH$_3$-tetrahydrofolate utilizing methylcobalimins, as well as a methyl transferase enzyme (Choi et al., 1994). The microbial reduction of sulfate to sulfide involves an eight electron reduction process.

During this process, electrons are transported through an electron transport chain that contains proteins such as cytochrome C$_3$ (Brock et al., 1994). Conceivably, inefficiencies in the transport process could allow for the release of electrons that are, in turn, scavenged by other co-factors such as tetrahydrofolate or NAD. In addition, it can not be ruled out that SRB exposed to a mercury species are directly affected by mercury which disrupts the electron transport process resulting in a greater flux of electrons being
shunted or scavenged by other molecules. These molecules that have a unique specificity for other proteins in the methylation process are "activated" and available, provided a mercury species is available for methylation. Inefficiencies in a respiration process are not a new concept. Such inefficiencies have been documented in eukaryotic systems. The production of reactive oxygen species in the mitochondria of eukaryotic systems has been attributed to the release or escape of electrons from the electron transport chain (Flitter, 1993). In addition, enzymes such as exogenous NADH dehydrogenase have been shown to shuttle reducing equivalence from complex I of the respiratory chain to cytosolic NADH (Nohl, 1987). Thus, one could speculate that novel proteins exist in SRB that shuttle reducing equivalence from the sulfate respiratory chain to transferase enzymes in an effort to reduce the toxicity of the existing mercury pool. However, it can not be ruled out that mercury species disrupt the normal respiratory function of SRB that causes a greater flux of reducing equivalence to cytosolic proteins resulting in an increased potential for mercury methylation.

**Mercury Methylation Rates on a Per Cell Basis**

Mercury methylation rates (MMR), based on linear regression analysis of methylmercury concentrations observed over the 24-96hr period for all cultures of SRB were included in Figures 5.13, 5.14, 5.15, 5.16, and 5.17. These were normalized based on the average number of cells observed in pure cultures (Table 5.1) and are reported in Table 5.3.
Table 5.3

<table>
<thead>
<tr>
<th></th>
<th>Mercury methylation rates (MMR) in pure culture pg/cell-hr (± % std. error)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DSV</td>
</tr>
<tr>
<td></td>
<td>4.2x10^-8</td>
</tr>
<tr>
<td></td>
<td>(± 12.2%)</td>
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</tbody>
</table>

In general, the data indicate that all groups tested had different MMR. DBACTER and DSBM had MMR that were similar and had the highest observed MMR when compared to the other three phylogenetic groups.

The DSBM group had the highest observed MMR when normalized to the remaining SRB phylogenetic groups, and they were approximately 3.9, 23.0, 148, and 1441 times higher than DBACTER, DSC, DSV, and DSB, respectively. DSB had a lower MMR than other phylogenetic groups tested when normalized on a per cell basis (Table 5.3).

**Determination of f for SRB Phylogenetic Groups**

Unlike sediment slurry reactors which demonstrated a continued loss of total mercury within slurry water (see Chapter 4 and Chapter 6), pure culture analysis of total mercury indicated that no net loss depletion in total soluble mercury concentrations occurred from time 0 to 96 hr. This was due to the exceptionally high levels of soluble mercury
complexes in the media. Since a decrease in aqueous phase mercury was not detected over time in the pure culture, it could be argued that that the bacteria were not limited with respect to mercury available for methylation. However, the soluble mercury species that were available for methylation were not known. Thus, the concentration of mercury species available for methylation is not known and the relationship between that concentration and the value of the half-saturation constant remains undefined. Based on Equation 4.11, the net incidence term \( f \) can be calculated for the individual phylogenetic groups.

\[
\frac{r_{CH, Hg}}{-r_{SO_4}} = f = \hat{f} \frac{[Hg]}{K_{Hg} + [Hg]}
\]

Equation 4.11

The \( f \) term, which relates the MMR to SRR, can be used as an indicator of a potential to generate methylmercury. This value normalizes the observed MMR in terms of the observed SRR of a specific phylogenetic group. The units associated with the \( f \) term are n mole methylmercury per n mole sulfate reduced. The actual MMR reported on a pg/cell-hr basis was converted to n moles based on a molecular weight of approximately 215.2 g/mole for methylmercury. It should be noted that both SRR and MMR used in this calculation are reported on a per cell basis. Figure 5.19 illustrates the survey of the calculated \( f \) term for each of the phylogenetic groups.
Figure 5.19 Values of $\dot{f}$ for representatives of the SRB phylogenetic groups analyzed in pure culture.
Table 5.4 reports the $f$ values (nmol/nmol) for the phylogenetic groups tested.

<table>
<thead>
<tr>
<th></th>
<th>DSV</th>
<th>DSB</th>
<th>DSC</th>
<th>DBACTER</th>
<th>DSBM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Value</td>
<td>0.137 ± 0.018</td>
<td>0.028 ± 0.009</td>
<td>0.46 ± 0.13</td>
<td>0.41 ± 0.095</td>
<td>2.58 ± 0.28</td>
</tr>
</tbody>
</table>

Calculations of the $f$ term for different phylogenetic groups suggest that the $f$ term value varied among the phylogenetic groups (Figure 5.19). Comparing calculated $f$ terms between phylogenetic groups suggests that the *Desulfo bacterium* group generated the most methylmercury when normalized to SRR with $f$ of $2.58 \times 10^{-5}$ nmol per nmol. Both DBACTER and DSC had similar values that were significantly lower than DSBM. However, values for both DBACTER and DSC were significantly higher that DSV and DSB (Figure 5.19). The $f$ term results from the survey of all phylogenetic groups tested suggests that the potential to methylate mercury relative to the SRR from least to greatest is:
Desulfobulbus < Desulfovibrio < Desulfococcus = Desulfo bacter < Desulfo bacterium

In Figure 5.20, the lines connecting the SRB phylogenetic groups indicate the homology (genetic similarities) of the various species that are representative of the SRB phylogenetic groups tested in this study. (Devereux et al., 1992). Rooney-Varga et al. (1998) published a more detailed phylogenetic tree that indicates more species of bacteria belonging to each of the SRB phylogenetic group. The shorter the line distance between two species implies the more closely the two species are genetically similar. In Figure 5.20, it can be seen that the Desulfo bacterium species is most genetically similar to Desulfo bacter. Collectively, both Desulfo bacterium and Desulfo bacter are more closely related to Desulfococcus than Desulfo bulbus and Desulfo vibrio. Clearly this does argue that the potential to methylate mercury is genetically defined. Presumably, some species of SRB have evolved more efficient means of methylating mercury than other members of the same SRB family. The reason that SRB methylate mercury is still unclear. It has been hypothesized that bacteria methylate mercury as a means of reducing the toxicity of mercury (Robinson and Tuovinen, 1984). The inorganic, divalent forms of mercury have a very strong affinity for sulfur containing molecules such as enzymes and proteins. The addition of a methyl group to divalent mercury would make the metal more lipophilic. Thus, the methylated form of mercury would have an increased affinity for membranes and a decreased affinity for enzymes that have a direct role in cellular processes. In summary, the data presented in this chapter suggests that SRB phylogenetic groups have varying incidences of mercury methylation relative to SRR.
Figure 5.20 Phylogenetic Groups of SRB

After: Devereux et al., 1992
The data presented in this chapter suggests that DSBM has the greatest potential to methylate mercury relative to SRR. However, it should be noted that the bacteria used in this study are specific species representing a phylogenetic group. Thus, the possibility exists that other species that comprise a phylogenetic group may have an increased or decreased "net" incidence of methylating mercury relative to SRR. Future studies presented in this thesis will determine the ability of unique SRB consortia to methylate mercury in sediment slurry systems. These types of experiments, in conjunction with pure culture experiments, were conducted in order to substantiate the observed differences in the incidence of mercury methylation relative to sulfate reduction among SRB groups.
CHAPTER VI

PRODUCTION OF METHYLMERCURY IN SEDIMENT SLURRIES PRECONDITIONED WITH ACETATE AND LACTATE

In this chapter, the hypothesis that mercury methylation rates vary among the phylogenetic groups of sulfate-reducing bacteria was explored using sediment slurries that were preconditioned with organic acids. Results discussed in Chapter 5 indicated that cultures representing phylogenetic groups had variable incidences of methylmercury production relative to their SRR. In pure culture studies the incidence or methylmercury production normalized to the SRR is as follows from highest to lowest:

*Desulfo bacterium* > *Desulfo bacter = Desulfococcus* > *Desulfovibrio* > *Desulfo bulbus*

However, the conclusions derived from the pure culture study are limited since a multitude of individual species comprise a phylogenetic group, and these individual species within a phylogenetic group could have different potentials to methylate mercury. In order to establish differences in mercury methylation relative to existing SRB present in sediment, organic acids were introduced into sediment slurries. The SRB family of bacteria has been
grouped based on an ability to utilize acetic acid as an electron donor source (Brock et al., 1995). Thus, acetic acid was selected as an organic component since Desulfobacterium, Desulfobacter, and Desulfococcus can utilize it as an electron donor while Desulfovibrio and Desulfobulbus can not (Rooney-Varga, 1998). Lactic acid was chosen as another organic acid to run in parallel to the acetic acid because lactic acid or its metabolites can be utilized by all species of SRB (Rooney-Varga, 1998; Parkes et al., 1993). By operating the reactors for a period of 20 d in the presence of acetic acid, microbial populations capable of utilizing acetate as an electron donor would be the major population present (Parkes et al., 1993). This argument is additionally supported by experiments which demonstrated a termination in the measured SRR at times > 96 hr in prototype reactors that were not amended with substrate. Thus, after 96 hr the bacteria that were present in slurry reactors would rely solely on the substrate that was added. This, in turn, would dictate the population that was there by day 20. In this study, the reactors which were preconditioned with 10 mM acetic acid or 20 mM lactic acid were analyzed for SRR, methylmercury concentrations, total cell counts, total mercury in pore water, and SRB 16S rRNA 20 d after reactor construction. The experimental protocol required harvesting sediments for use in a series of controlled reactors to compare sediments exposed to organic acid for 20 d to unconditioned sediments. Thus, slurries that were conditioned to organic acids were collected 19 d prior to the collection of unconditioned sediments (See Materials and Methods). Twenty-four hours after control reactors were constructed, the existing preconditioned reactor and control reactor were inoculated with inorganic mercury. Quantified values obtained for each treatment indicated values obtained from
different reactors operated approximately 20 d apart (see Material and Methods). In other words, approximately 20 days elapsed before the collection of fresh sediment from coastal marshes. Table 6.1 demonstrates the sampling protocol for the various sediments.

<table>
<thead>
<tr>
<th>Table 6.1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sampling protocol followed for conditioned and control sediment slurries</strong></td>
</tr>
<tr>
<td><strong>Treatment</strong></td>
</tr>
<tr>
<td>Lactate 1</td>
</tr>
<tr>
<td>Control 1</td>
</tr>
<tr>
<td>Acetate 1</td>
</tr>
<tr>
<td>Control 2</td>
</tr>
<tr>
<td>Lactate 2</td>
</tr>
<tr>
<td>Acetate 2</td>
</tr>
<tr>
<td>Control 3</td>
</tr>
<tr>
<td>Lactate 3</td>
</tr>
<tr>
<td>Acetate 3</td>
</tr>
<tr>
<td>Control 4</td>
</tr>
</tbody>
</table>

In brief, the information in Table 6.1 denotes the sediment collection times in addition to the times inorganic mercury was added. For example, Lactate 1 sediment was collected
and 20 mM lactic acid added to that sediment 19 days prior to the collection of the control sediment. Then, 24 hr after the control sediment collection, both Lactate 1 and control 1 reactors received a dose of 950 ng/g of inorganic mercury. The amount of sulfate reduced, total soluble mercury, methylmercury concentrations were determined in the subsequent 36 hr (See Materials and Methods).

**Organic Acid Analysis of Preconditioned Sediment Slurries**

The organic acid concentrations that were present in the preconditioned slurry reactors were monitored for 20 d after initial construction in order to determine if either lactate (20 mM) or acetate (10 mM) would be in limited supply prior to inorganic mercury addition. Figure 6.1 illustrates the organic acid concentrations observed in the reactors for the 20 d following initial construction. In the 10 mM acetic acid treated reactor, concentrations of acetic acid were maximum at the zero hour timepoint with 96.6 % of the initial concentrations of acetic acid being recovered. Concentrations of acetic acid continued to decrease linearly for the duration of the experiment with 3.63mM acetic acid present in the reactor by the 20th day (Figure 6.1A). Figure 6.1B illustrates lactate concentrations in a reactor that was preconditioned with 20 mM lactate. Lactate concentrations quantified at the zero hour timepoint demonstrated an 87.4 % recovery in the initial lactate addition. Concentrations of lactate continued to decrease in a linear fashion for the duration of the experiment with concentrations approaching 3.5 mM by day 20.
Figure 6.1 Organic acid concentrations in slurry reactors over 20 d conditioning period in sediment. (A) Reactor received 10 mM acetate (acetate 3). (B) Reactor received 20 mM lactate (lactate 3). Both reactors contained sediment B (See Materials and Methods).
In the lactate pre-conditioned reactor, acetate was produced through lactate oxidation. Concentrations of acetate were minimum at the zero hour timepoint (0.35 mM). However, concentrations of acetic acid reached a relatively consistent value of approximately 2.50 mM by the 10\textsuperscript{th} day (Figure 6.1B).

Equation 6.1 illustrates a balanced equation for the complete oxidation of acetate to CO\textsubscript{2} utilizing sulfate as a terminal electron acceptor.

\[
\text{SO}_4^{2-} + \text{H}^+ + \text{CH}_3\text{COO}^- \rightarrow 2\text{CO}_2 + \text{S}^{2-} + 2\text{H}_2\text{O}
\]  
Equation 6.1

The equation indicates that every mole of acetate oxidized requires the reduction of 1 mole of sulfate. Thus, a 1:1 relationship exists between the oxidation of acetate and the reduction of sulfate. The data presented in Figure 6.1A indicates that the acetate oxidation rate was approximately 94.0 nmoles/g\textsubscript{dry}-hr. Thus, the 1:1 relationship between acetate oxidation and sulfate utilization suggests that the predicted SRR for the system would average approximately, 94.0 nmoles/ g\textsubscript{dry}-hr in the 20 d after initial acetate addition to the system. It should be noted that the SRR was not determined for the reactors durring the 20 d following acetate addition, so no comparison could be made between the calculated SRR and observed SRR. However, Parkes \textit{et al.} (1993) reported results similar to the calculated results in slurry reactors with a ratio of 1.26 nmoles acetate oxidized per nmol sulfate reduced. In addition, Dicker and Smith (1985a,b) reported an SRR of 295.6 nmoles/g\textsubscript{dry}-hr and an acetate oxidation rate of 337.4 nmoles/g\textsubscript{dry}-hr when 50-60mM acetate was added to sediment systems. This implies that approximately
1.14 nmoles of acetate where oxidized for every n mole sulfate reduced (Dicker and Smith, 1985a&b). Equation 6.2 illustrates the oxidation of lactate to acetate utilizing sulfate as an electron acceptor.

\[ 2 \text{CH}_3\text{HCOHCOO}^- + 2 \text{SO}_4^{2-} \rightarrow 2 \text{CH}_3\text{COO}^- + 2 \text{CO}_2 + 2 \text{S}^{2-} + 2 \text{H}_2\text{O} \quad \text{Equation 6.2} \]

Equation 6.2 suggests that for the oxidation of lactate to acetate requires the reduction of 1 mole of sulfate per 2 moles of lactate. Thus, approximately 2.0 moles of lactate are oxidized to acetate for every mole of sulfate reduced to sulfide. In the lactate conditioned treated reactor, the production of acetate implies that some fraction of lactate was oxidized to acetate (Figure 6.1). The steady state concentration of acetate in the lactate treated reactor after 15 d implies that the rate of production equals the rate of consumption. Combining Equations 4.1 and 4.2 to demonstrate a two-step oxidation of lactate to acetate to CO\(_2\) results in Equation 6.3.

\[ 1 \text{CH}_3\text{HCOHCOO}^- + 1.5 \text{SO}_4^{2-} + \text{H}^+ \rightarrow 3 \text{CO}_2 + 1.5 \text{S}^{2-} + 3 \text{H}_2\text{O} \quad \text{Equation 6.3} \]

Equation 6.3 indicates that 1.5 moles of sulfate are required for the oxidation of 1 mole of lactate. This suggests that 0.67 moles of lactate are metabolized to CO\(_2\) for every mole of sulfate reduced to sulfide. An average lactate oxidation rate was calculated based on results in Figure 6.1 and is reported as 0.704 mM/d which at a sediment concentration of approximately 140 g/L is 210.0 nmoles/g\(_{\text{dry}}\)-hr. Thus, the calculated SRR observed in
lactate reactors would be approximately 323.4 nmoles/g$\text{dry}$-hr for the complete oxidation of lactate to acetate. However, based on the incomplete oxidation of lactate to acetate (See Figure 6.1B Equation 6.2) the calculated SRR would be approximately 108.2 nmoles/g$\text{dry}$-hr. It should be noted that the SRR for the initial 20 d period was not quantified for lactate, so a comparison of predicted and calculated values cannot be conducted. However, amendments of 60 mM lactate to sediments obtained from Canary Creek in Lewes, Delaware resulted in SRR values of 278.0 nmoles/g$\text{dry}$-hr and lactate oxidation rates of 123.3 nmoles/g$\text{dry}$-hr (Dicker and Smith, 1985a,b). Thus, the ratio of these sediments was reported at 0.44 moles lactate oxidized to CO$_2$ for every mole sulfate reduced to sulfide. Other results of Parkes et al. (1993) suggest that 3.3 nmoles lactate are oxidized for every nmole sulfate reduced in the initial 5 d of sediment incubation. It should also be noted that results published by Parkes et al. (1993) also demonstrated that acetate was observed in reactors treated with lactate. The variability in results published by other groups suggests that the ratio of lactate metabolism to sulfate-reduction is specific to the consortia that are present in sediment. Dicker and Smith (1985a&b) published results that were similar to calculated values reported in Equation 6.3 for the complete oxidation of lactate. Other results published by Parkes et al. (1993) suggest that the presence of the metabolite acetate that is derived from the incomplete oxidation of lactate to acetate results in higher ratios of lactate metabolism to acetate with respect to SRR. The results of Parkes et al. (1993) are further validated with the calculated ratio of Equation 6.2. From the data presented in Figure 6.1 and the above analysis, it is clear that the SRR for the sediments were similar (e.g. 94.0 and 108 nmol/g$\text{dry}$-hr). This would also
indicate similar respiration rates for enhanced growth of in situ microbial SRB within the sediments.

**Sulfate-Reduction Rates in Pre-conditioned Sediment Slurries**

Preconditioned and control sediments were harvested, placed in replicate slurry reactors (concentration of approximately 140 g/L), and monitored over a 36 hr period. These experiments were conducted over a 4 month period using similar sediments as summarized in Table 6.1. The amount of sulfate reduced in control, lactic, and acetic acid amended slurries was determined over time (12 hr prior to the addition of inorganic mercury and continuing for the next 48 hr). Figure 6.2 and 6.3 illustrates the amount of sulfate reduced over time in reactors with lactate and acetate conditioned sediments. The amount of sulfate reduced over time in control sediments is illustrated in Figure 6.4. It is important to note that the control reactor was constructed 24 hr prior to inorganic mercury addition. Data points at each timepoint represent samples acquired from three reactors of the same treatment. The lines represented in Figure 6.2, 6.3, and 6.4 are the linear regression analysis based on the data points over the experimental period. The linear regression slope of each reactor generated an average SRR observed over the duration of the experiment.

In general, the average SRR value for the lactic acid and acetic acid amended cultures were 7.0 and 8.3 fold greater than the non-amended control, respectively. Average SRR values for the control, acetate, and lactate slurries were 11.04, 91.70, and 77.67nmol/g_{dry}-hr, respectively.
Figure 6.2 Sulfate reduced in lactate-conditioned slurry reactors. $^{35}$SO$_4$ was added at 0 hr and 950 ng/g$_{\text{dry}}$ of inorganic mercury was added at 12 hr timepoint (Sediment B).
Figure 6.3 Sulfate reduced in acetate-conditioned sediment slurry reactors. $\text{SO}_4^{2-}$ was added at 0hr and 950ng/g$_{dry}$ of inorganic mercury added at the 12 hr timepoint (Sediment B).
Figure 6.4 Sulfate reduced in control sediment slurry reactors. 
$^{35}$SO$_4$ was added at 0 hr and 950 ng/g$_{dry}$ of inorganic mercury added at the 12 hr timepoint (Sediment B).
Methylmercury Concentrations Observed in Preconditioned Reactors

Concentrations of methylmercury were determined periodically at specific timepoints in reactors conditioned with lactate and acetate. In addition, a control slurry reactor was constructed 24 hr prior to inorganic mercury addition and used for comparison purposes with the preconditioned reactors. The concentrations of methylmercury observed at various time points in the preconditioned and control reactors are illustrated in Figure 6.5, 6.6, and 6.7. It should be emphasized that the 0 hr timepoint depicted in these figures is analogous to the 12 hr timepoint in the sulfate reduced data (Figures 6.2-6.4). Data points at each time point represent samples acquired from three different reactors of the same treatment. The lines represented in the figure represent a non-linear statistical fit that was applied to the data. In general, all reactors demonstrated an increase in methylmercury concentrations over time. The greatest concentrations of methylmercury were observed in the acetate conditioned slurries. In the acetate conditioned slurries, maximum concentrations of methylmercury were observed at the later time points with an average concentration of 162.4 ng/g<sub>dry</sub>-hr observed at the 36 hr time point (Figure 6.5). Similarly, lactate conditioned slurries had maximum concentrations of methylmercury at the 36 hr timepoint with average concentrations of 100.8 ng/g<sub>dry</sub> (Figure 6.6). Comparing average values of methylmercury in lactate and acetate conditioned slurries at various time points, the acetate conditioned slurries had significantly higher concentrations of methylmercury beyond the 4 hr time point (P < 0.05) (Figures 6.5 and 6.6).
Figure 6.5 Methylmercury concentrations in acetate-conditioned sediment slurry reactors (Sediment B).
Figure 6.6 Methylmercury concentrations in lactate-conditioned sediment slurry reactors (Sediment B).
Figure 6.7 Methylmercury concentrations in control sediment slurries (Sediment B).
At all time points excluding 0 hr, acetate and lactate preconditioned slurries were significantly higher than the control values observed at the same time (P < 0.05) (Figures 6.5, 6.6, and 6.7). In general, the concentrations of methylmercury in the control reactor continue to increase for the duration of the experiment. An average maximum concentration of 9.6ng/g dry is observed at the 36hr time point (Figure 6.7).

**Soluble Mercury Concentrations Observed in Reactor Slurry Water**

Total mercury concentrations found in the aqueous phase of the slurries were assessed in lactate and acetate conditioned reactors immediately following inorganic mercury addition. Figure 6.8 illustrates the total soluble mercury concentrations observed in the slurry water of control, lactate, and acetate treated reactors. Mercury concentrations and profiles were similar in all reactors that were sampled. Maximum concentrations of mercury are observed at the beginning of the experiment. At the 0 hr timepoint, total soluble mercury concentrations range from 263 to 198 ng/L. Overall, the concentrations of mercury found in the slurry water of all reactors continue to decrease with time. The line in the data represents a non-linear statistical fit that was applied to the data. Concentrations of mercury found in the slurry water at the 36 hr time point range from 55.0 to 42.0 ng/L (Figure 6.8). In all reactors, the amount of inorganic mercury that was present in the slurry water decreased with respect to time.
Figure 6.8 Total soluble mercury observed in sediment slurry water for the 10 reactors (Table 6.1).
Non-linear regression analysis (Hyperbolic Decay, 2 parameters; Sigmaplot version 4.0 © 1997, SPSS inc.) conducted on the soluble mercury concentrations resulted in non-linear curve fits for control reactors, conditioned lactate reactors, and conditioned acetate reactors that were not significantly different based on calculated standard errors of the equation.

It is interesting to note that the concentrations decrease rapidly in the first 12hrs after inorganic mercury additions. Total mercury concentrations reported at the 36hr time point range from 68.4-52.3, 47.9-42.2, and 52.9-45.9 ng/L for lactate, acetate, and control reactors, respectively. At the 0hr timepoint, approximately 950 ng/g_{dry} of inorganic mercury was added to slurry reactors. Based on soluble inorganic mercury concentrations that ranged from 263-198 ng/L, approximately 99.8 % of the inorganic mercury introduced into the system was associated with the sediment-phase within the reactor. Although at the zero hour timepoint these systems are not considered at equilibrium in regard to mercury partitioning, Gagnon et al. (1997) found that less than 0.01 % of mercury added to sediment systems is associated with the bulk sediment. It is likely that a large concentration of the added inorganic mercury associated with the solid phase due to the presence of sulfide minerals already present in the sediment. Sulfide precipitates (e.g. FeS) have been reported as excellent scavengers of heavy metals, including mercury (Hyland et al., 1990). Several studies have reported the presence of acid volatile sulfides (AVS) as principal Hg sinks in sediments (Gagnon et al., 1997). AVS typically exist in sediments as FeS. Because mercury has the highest stability constants associated with sulfides of all trace metals (Dyrssen, 1988), mercury can form a more stable complex with
the sulfide ($S^2-$) that is bound to $Fe^{2+}$. Thus, the $Fe^{2+}$ is displaced over time and replaced with $Hg^{2+}$ to form solid $HgS_{(s)}$.

The association of mercury with particulate organic matter may represent another contributing factor to the large fraction of mercury observed in the solid phase. Studies have demonstrated a correlation between the amounts of organic matter in sediments and the amount of mercury associated with sediments (Loring et al., 1983; Pelletier and Canuel, 1988; and Mucci and Edenborn, 1992). Loring et al. (1983) have suggested that terrigenous organic matter is the main scavenging agent and carrier of mercury to sediments in the Saguenay fjord. Results by Bono and Mucci (1995) have also demonstrated that 50-85 % of the mercury that was found in Saguenay fjord was associated with organic matter. Although 99.8 % percent of inorganic mercury was associated with inorganic mercury at the zero hour timepoint, soluble concentrations of inorganic mercury ranged from approximately 260.0 ng/L at zero hours to 44.0 ng/L at the 36 hr timepoint. The presence of soluble mercury in pore water has been attributed to the presence of dissolved organic matter (DOM) as well as polysulfides. Thermodynamic data available on the stability of mercury–DOM complexes in natural waters are scarce. However, recent observations demonstrated that large fractions of dissolved Hg may be associated with colloidal DOM (Guentzel et al., 1996; Stordal et al., 1996). Using tangential ultrafiltration, Guentzel et al. (1996) found the colloidal mercury represented 35-87 % of total dissolved mercury in estuaries and 10-50 % of the total dissolved mercury in the North Atlantic. Stordal et al. (1996) found that colloidal mercury co-varied with colloidal organic carbon concentrations in Galveston Bay waters, suggesting that most of
the mercury in filtrates was associated with large organic macromolecules. Together, these two studies demonstrate the influence DOM has on the dissolved mercury concentrations.

Mercury can also form stable, soluble complexes with sulfide and polysulfide species (HgS$_2$\textsuperscript{2-}, Hg(HS)$^2$-, Hg(HS)$_2$, etc.) which may dominate the soluble mercury species in sulfidic seawater (Gagnon et al., 1997). As mentioned in Chapter V regarding soluble mercury concentrations in pure cultures, it has been demonstrated that the presence of such polysulfides can increase the solubility of mercury as much as 1000 fold (Paquette et al., 1997). It should be noted that soluble mercury concentrations ranging from 17 to 500 ng/L were found in mercury-contaminated sediments located at Saguenay Fjord (Gagnon et al., 1997). In addition, soluble mercury concentrations reported in marine systems at Lavaca Bay, TX contaminated with mercury ranged between 20-60 ng/L (Bloom et al., 1999). It is interesting to note that these porewater values are in a concentration range equivalent to what was observed in sediment slurry reactors (Figure 6.8). Although the slurry reactors are not at an equilibrium during the initial 36 hr as evident by the decreasing soluble mercury concentrations, these concentrations are consistent with a range typical of contaminated sediments. Taking into account that 950 ng/g$_{dry}$ sediment was added to each reactor at the 0 hr time point, a mercury partitioning coefficient can be estimated for reactors when the slurry water mercury concentrations approach consistent minimum values. Estimated partitioning coefficient values range from 1.3-2.2 x 10$^4$ L/kg. Lyon et al. (1997) reported soil-water partitioning coefficients for inorganic mercury that ranged from 0.33-6.0x10$^4$ L/kg. In benthic sediments, partitioning coefficients for
inorganic mercury ranged from 5.7x10$^3$ to 9.9x10$^5$ L/kg (Lyon et al., 1998). Although the values for the partitioning coefficients have considerable variability within a given sediment type, the calculated partitioning coefficients for this study are within limits reported in other studies.

Cell Counts in Slurry Reactors

Cell counts at the 36 hr time point were performed for one experiment in which the lactate conditioned (lactate 3), acetate conditioned (acetate 3), and control (control 4) slurries were injected with inorganic mercury at the same time. Table 6.2 reports the range of cell numbers found within reactors as well as the overall average and percent standard deviation for cell counts.

<table>
<thead>
<tr>
<th>Reactor</th>
<th>Average Cell Counts 10$^9$ Cells/g$_{dry}$ (± % Std. Error)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate 3</td>
<td>9.70 (± 8.1%)</td>
</tr>
<tr>
<td>Lactate 3</td>
<td>9.45 (± 7.2%)</td>
</tr>
<tr>
<td>Control 4</td>
<td>6.40 (± 6.9%)</td>
</tr>
</tbody>
</table>
Data presented in Figure 6.2 indicate that a statistically significant difference exists between the cell populations in the control reactors and cells observed in acetate conditioned and lactate conditioned reactors. There was no statistical difference between cell numbers for lactate and acetate conditioned reactors. Since acetate and lactate conditioned reactors were not limited with the respective organic acid concentrations after 20d, a larger population of cells would be expected in conditioned reactors when compared to controls. Moreover, both acetate and lactate are in a molecular form that is readily available for metabolism. Control reactors were not amended with substrate, Thus, the cellular population in the reactors relied on an pre-existing amount of organic in the sediment as well as the hydrolysis of sediment organic matter by extracellular enzymes to provide available substrate.

**Determination of 16S rRNA in Organic Acid Preconditioned and Control Reactors**

The SRB phylogenetic groups and the percentage of specific 16S rRNA that was present in the reactors were quantified. Figure 6.9 illustrates the diversity of SRB present in the control, lactate conditioned, acetate conditioned reactors, and sediment cores (0-4cm). It should be noted that the sediment cores were harvested when sediments for the lactate conditioned reactor were collected. Results from the 16S rRNA profiles suggest that sediment samples collected for use in the organic acid preconditioned cultures had relatively the same concentrations and profiles as the control reactors (Figure 6.9).
Figure 6.9 Identification of SRB phylogenetic groups in slurry reactors (Sediment B) and sediment cores (0-4 cm; Sediment C). 16S rRNA was extracted from reactors 36 hr after 950 ng/g_{dry} of inorganic mercury addition.
In 20 mM lactate conditioned treated reactors, 16S rRNA concentrations significantly increased for *Desulfovibrio* (DSV), *Desulfobulbus* (DSB), and *Desulfooccus* (DSC) compared to controls (P<0.05). Concentrations of 16S rRNA for DSV, DSB, and DSC in lactate conditioned reactors were approximately 2.6, 2.9, 3.7 fold greater than control values, respectively. Reactors treated with 10 mM acetate had significant decreases in 16S rRNA for DSV and DSB groups when compared to controls (P < 0.05). Concentrations of 16S rRNA for DSV and DSB in control reactors were approximately 5.2 and 12.9 fold higher than acetate reactors, respectively. However, concentrations of 16S rRNA for *Desulfo bacter* (DBACTER) and *Desulfo bacterium* (DSBM) in acetate conditioned reactors were significantly higher than concentrations in control reactors (P<0.05). Concentrations of 16S rRNA for DBACTER and DSBM in acetate reactors were approximately 2.6 and 3.5 fold higher than control values, respectively. Comparing 16S rRNA profiles between lactate and acetate reactors indicated significant differences in concentrations existed in all phylogenetic groups identified (P<0.05) (Figure 6.9). DBACTER and DSBM 16S rRNA concentrations in acetate reactors were approximately 1.7 and 3.1 fold greater than lactate values, respectively. However, 16S rRNA concentrations of DSV, DSB, and DSC in lactate reactors were 13.5, 37.1, and 5.8 fold higher than concentrations determined in acetate reactors, respectively (Figure 6.9). Table 6.3 illustrates the total concentration of 16S rRNA quantified in the various reactors.
Table 6.3

<table>
<thead>
<tr>
<th>REACTOR</th>
<th>SEDIMENT (0-4cm)</th>
<th>CONTROL</th>
<th>LACTATE</th>
<th>ACETATE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total SRB 16S rRNA (ng/g&lt;sub&gt;dry&lt;/sub&gt;) ± % Std. Error</td>
<td>458.5 ± 2.0 %</td>
<td>601.7 ± 1.2 %</td>
<td>1208.3 ± 2.5 %</td>
<td>1176.1 ± 4.0%</td>
</tr>
</tbody>
</table>

Profiles of SRB in Preconditioned and Control Reactors

The introduction of 10mM acetic acid to sediment slurry systems altered the SRB composition in the sediment slurry system (Figure 6.10). Data collected from probe studies indicated that addition of acetate for a period of 20 d altered the SRB profiles such that *Desulfobacterium* and *Desulfo bacter* species represented 53.0 % and 41.7 % of the total population, respectively (Figure 6.10). Addition of lactate and incubation for 20 d resulted in bacterial profiles that were similar in percentage composition to control sediment slurries (Fig 6.10). Similar results have been observed in other slurry systems amended with lactate and acetate. Parkes et al. (1993) found that treatment of sediment slurries (comprised of sediment from Loch Etive, Scotland) with 20 mM acetate for 20 d increased the number of acetate-utilizing bacteria in sediment slurries by 225 % over controls.
Figure 6.10 Percentage of total SRB16S rRNA present in reactors. Figure derived from data in Figure 6.9.
There was no significant change in lactate-utilizing bacterial populations in reactors treated with 20mM acetate (Parkes et al., 1993). The number of lactate-utilizing bacteria increased by 224% in sediment slurries treated with 20 mM lactate (Parkes et al., 1993). The number of acetate-utilizing bacteria in the 20 mM lactate reactor increased by approximately 137%. In this experiment, the 16S rRNA of acetate-utilizing bacteria (DBACTER and DSBM) in reactors treated with 10mM acetate collectively increased 307% over controls (Figure 6.10). Lactate-utilizing bacteria (DSV, DSB, DSC, and DSBM) 16S rRNA in reactors treated with 20 mM Lactate collectively increased approximately 218%. The 16s rRNA of DBACTER and DSBM, which represent the acetate-utilizers, increased approximately 138% in lactate-treated reactors when compared to controls. It should be noted that Parkes et al. (1993) did not observe an increase in lactate-utilizing bacteria in acetate treated reactors. However, this experiment indicated that an approximate 26.0% decrease in 16S rRNA occurred in phylogenetic groups which are unable to utilize acetate (DSV, DSB, and DSC) (Figure 6.10). This difference in results can be explained based on metabolic activity. Pace et al. (1986) have argued that characterizing natural microbial populations using rRNA sequences has a potential for indicating metabolic activity of the bacteria present. Others have argued that the use of probes does not provide an indication of the actual number of cells present for a select phylogenetic group. However, the level of rRNA abundance should correlate to metabolic activity since rRNA increases with increased cellular activity (Deveruex et al., 1996, Pace et al., 1986). Thus, a decrease in 16S rRNA may not be indicative of a
reduced cell number, but rather a decrease/arrest of cellular activity by the phylogenetic groups in question.

**Calculated SRR on Per Cell Basis**

The SRR was determined both in amended and control slurries and was illustrated in Figure 6.2, 6.3, and 6.4. The average cell counts that are reported in Table 6.2 were used in the normalization of SRR for one experimental protocol. Table 6.4 illustrates the average SRR for each treatment reported in a nmole/cell-hr basis. The values obtained in the normalization of the SRR suggest that the sulfate-reduction rate is on the order of $10^{-9}$ nmol/cell-hr in sediment slurries. Kurtz et al. (1998) reported approximate SRR of $2.5 \times 10^{-9}$ nmole/cell-hr in sediment slurry systems that contained Florida sediments obtained from the Gulf of Mexico. These results suggest that the SRR reported on a per cell basis in this thesis are in good agreement with results published by Kurtz et al. (1998).

<table>
<thead>
<tr>
<th>Reactor Type</th>
<th>Average SRR nmol/g$_{dry}$-hr (± % std. error)</th>
<th>Average SRR nmol/cell-hr (± % std. error)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate 3</td>
<td>77.01 (± 5.0 %)</td>
<td>8.1x10$^{-9}$ (± 6.9 %)</td>
</tr>
<tr>
<td>Acetate 3</td>
<td>91.90 (± 7.0 %)</td>
<td>9.0x10$^{-9}$ (± 7.9 %)</td>
</tr>
<tr>
<td>Control 4</td>
<td>11.30 (± 3.8 %)</td>
<td>2.0x10$^{-9}$ (± 5.5 %)</td>
</tr>
</tbody>
</table>
Calculated MMR in Sediment Slurry Reactors

The concentrations of methylmercury were quantitated over a 36 hr time period in conditioned and control reactors that received 950 ng/g\textsubscript{dry} of inorganic mercury (Figures 6.5, 6.6, and 6.7). Mercury methylation rates (MMR) were calculated by assessing differences in methylmercury concentrations between time intervals. The MMR, which is in units of ng/g\textsubscript{dry}-hr, is illustrated in Figures 6.11. For lactate conditioned, acetate conditioned, and control reactors, maximum mercury methylation rates occur in the initial 0-4 and 4-8 hr range. In general, the mercury methylation rates decreased with respect to time (Figure 6.11). MMR values reported for acetate reactors at the 0-4 hr and 4-8 hr interval were significantly different from lactate conditioned reactors (P<0.05). Both lactate and acetate conditioned reactors had significantly higher calculated MMR when compared to controls for all time intervals excluding the 24-36 hr time interval (P<0.05). Maximum MMR values for acetate reactors ranged from 10.4-15.0 ng/g\textsubscript{dry}-hr. Similarly, the maximum MMR for lactate reactors ranged between 4.5-9.7 ng/g\textsubscript{dry}-hr. Finally, the maximum MMR calculated for control reactors ranged between 1.25-0.67 ng/g\textsubscript{dry}-hr. Values calculated for control reactors are similar to those published in the literature. In this study, it compares to MMR values observed between 8 and 24 hr for control reactors. Similarly, D’Planas (1994) observed a maximum MMR of 0.428 ng/g\textsubscript{dry}-hr when sediments were exposed to mercury at approximately 3000 ng/g\textsubscript{dry}. 

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Figure 6.11 Calculated MMR for (A) lactate and acetate conditioned reactors and (B) control reactors.
Comparison of MMR to Total Soluble Mercury Observed in Slurry Water

A decrease in the calculated MMR (Figure 6.11) occurred during the experiment and coincided with an observed decrease in slurry water mercury concentration (Figure 6.8). Thus, an effort was made to determine if the slurry water mercury concentrations were a function of the MMR observed in conditioned and control reactors. Average slurry water mercury concentrations for each interval of time were determined for each reactor and compared to the calculated MMR observed at the same time interval. For both the lactate, acetate, and control reactors, maximum MMR occur when slurry water mercury concentrations are highest (Figure 6.12, 6.13, and 6.14, respectively). Linear regression analysis was performed on all three data sets. The r² values for lactate conditioned, acetate conditioned, and control reactors were 0.71, 0.83, and 0.77, respectively. Linear regression analysis suggests that there is good agreement between the calculated MMR and the slurry water mercury concentrations, and the relationship is linear with respect to the inorganic mercury concentrations used in this experiment. The linear response (differential MMR calculated between time points compared to average slurry water Hg²⁺ concentrations during the same time) is analogous to the pseudo-first order or linear response at low levels of added mercury in Figure 4.8 (e.g., [Hg]_added levels of 95-955 ng/g) and is not indicative of those rates at higher mercury addition levels for the sediment in Figure 4.8. The kinetic data for the two experiments (i.e., saturation kinetics in Chapter 4 and conditioned and control reactor) MMR vs inorganic mercury are in conceptual agreement and indicate the critical role of mercury availability on methylation processes.
Figure 6.12 MMR plotted as a function of total soluble mercury in lactate conditioned reactors.

$r^2 = 0.71$

(slope = 0.041; std. error = 0.0072)
Figure 6.13 MMR plotted as a function of total soluble mercury in acetate conditioned reactors.

- MMR (ng/g dry-wt/hr)
- Total Soluble Hg (ng/L)

- Acetate 1
- Acetate 2
- Acetate 3

Linear Regression

$r^2 = 0.83$
(Slope = 0.084; std. error = 0.014)
Figure 6.14 MMR plotted as a function of total soluble mercury in control reactors
In all treatments, linear regression analysis indicated that a soluble mercury concentration of 31.6, 39.1, and 34.9 ng/L was still present in the slurry water of lactate conditioned, acetate conditioned, and control reactors, respectively, when MMR was zero. The existence of the x-intercept suggests that concentrations of soluble mercury still exist in the reactors even when the SRB are not methylating mercury. Since the SRB are still reducing sulfate at a sustained rate (Figures 6.2, 6.3, and 6.4), the decrease in MMR is not thought to be due to mercury toxicity. Thus, the data suggest that soluble species of mercury exist in slurry water that are not “bioavailable” for methylation. The soluble species of mercury that are “bioavailable” or “non-bioavailable” is not known in these experiments due to the dynamic nature of the results. Anderson et al. (1990) have argued that HgS(8) is a sink for mercury and is not available for methylation. However, mercury sulfide is not found as a soluble species in marine waters. Other species of mercury such as HgS(2), HgS(2)H, HgCl(4), and HgCl(3) are known to exist in anoxic marine waters (Stumm and Morgan, 1981). However, the degree that all or none of these species are bioavailable for methylation is not known. In addition, over 250 monomeric mercury (II) compounds have been identified (Holloway and Melnik, 1995). The degree that these compounds occur in nature and can be biologically methylated is not known. It should be noted that humic complexes with mercury are also possible in these anoxic environments and the degree they are available for methylation is not known. Conceivably, the methylation of humic-bound mercury would be a function of the degradation or digestion of the humic compound. Thus, MMR for humic bound mercury would be considerably slower than the MMR for the unbound inorganic mercury.
Normalization of MMR to Compare Preconditioned and Control Reactors

Chapter 4 of this thesis defined a relationship between the MMR, SRR, mercury availability, and cell biomass. Equation 4.10 defined the MMR as a function of SRR and mercury availability.

\[
MMR = \hat{f}(SRR) \left[ \frac{[Hg]}{K_{Hg} + [Hg]} \right]
\]

Equation 4.10

MMR is the mercury methylation rate and is to be converted from ng/g_dry-hr to nmol/g_dry-hr. SRR is the sulfate-reduction rate and is expressed in units of nmol/g_dry-hr. The term, [Hg], is the total soluble mercury and is in units of ng/L. The \( \hat{f} \) term is a constant term that relates the SRR to MMR and is expressed in terms of mole methylmercury produced per mole of sulfate reduced. Dividing both sides of the equation by the SRR results in Equation 4.11

\[
\frac{[MMR]}{[SRR]} = \frac{\hat{f} [Hg]}{K_{Hg} + [Hg]} = \hat{f}
\]

Equation 4.11

Since the relationship between MMR and slurry water mercury concentration is linear, it implies that [Hg] is significantly less than \( K_{Hg} \) or that \( (K_{Hg} + [Hg]) = K_{Hg} \). Thus, Equation 4.12 can be written as illustrated in Equation 6.1.
\[ f = \frac{\hat{f}}{K_{\text{Hg}}} \left[ \text{Hg} \right] \quad \text{Equation 6.1} \]

Figures 6.15 illustrates the mercury methylation rate (MMR) of each control reactor normalized by the respective SRR and plotted as a function of soluble slurry water mercury concentrations. Linear regression analysis of the data indicated that the slope is 2.3x10^{-6} \text{nmol/nmol} for the control reactors. Likewise, Figure 6.16 illustrates the MMR for each lactate conditioned reactor normalized to the respective SRR and plotted as a function of the total soluble slurry water mercury concentrations. Linear regression analysis of the data indicated that the slope for lactate was 2.5x10^{-6} \text{nmol/nmol}. Finally, Figure 6.17 illustrates the MMR/SRR plotted as a function of total soluble mercury observed for each reactor and linear regression analysis of the data indicates that the slope is 3.90x10^{-6} \text{nmol/nmol} for acetate conditioned reactors. Collectively, the linear aggression analysis in conjunction with the standard error of each graph suggests that the incidence of methylmercury production relative to SRR is the same in the lactate and control reactors. However, the incidence of methylmercury production is significantly greater in the acetate conditioned reactor.
Figure 6.15 MMR normalized by observed SRR in sediment reactors and plotted as a function of total soluble mercury.
Figure 6.16  MMR normalized by observed SRR in sediment reactors conditioned with lactate and plotted as a function of total soluble mercury.
$r^2 = 0.84$

Slope = $3.90 \times 10^{-6}$ L/ng (std.error = $5.2 \times 10^{-7}$ L/ng)

Figure 6.17 MMR normalized by observed SRR in sediment reactors conditioned with acetate and plotted as a function of total soluble mercury.
These results must be addressed in terms of the percentage composition of 16S rRNA present in the preconditioned slurry reactors (Figure 6.11). Reactors treated with 10mM acetate had a significant shift in 16S rRNA composition. Approximately 95% of the 16S rRNA in acetate treated reactors was DBACTER and DSBM. This coincides with a 1.70 fold increase in the \( \frac{f}{K_{Hg}} \) ratio observed in the acetate conditioned reactors when compared to controls (Figure 6.15 and 6.17). In contrast to the acetate conditioned reactors, the lactate treated reactors had 16S rRNA profiles that were similar in percentage composition to the control reactors (Figure 6.11). This correlated very well with nearly identical \( \frac{f}{K_{Hg}} \) ratios observed in both control and lactate treated reactors (Figure 6.16 and 6.17). By definition, the \( \frac{f}{K_{Hg}} \) is a unique term which defines the nmol of methylmercury formed relative to the nmol sulfate reduced \( f \) and the half-saturation constant of slurry water mercury that can be metabolically converted to methylmercury by a consortium of SRB (\( K_{[Hg]} \)). Although actual values for \( f \) and \( K_{[Hg]} \) cannot be determined in this data set, hypothetically both terms are unique to specific consortiums. That is, different SRB consortiums would have different \( \frac{f}{K_{Hg}} \) values as well as different concentration dependencies (i.e. \( K_{[Hg]} \)). Concentration dependencies for a specific consortium would include: internalization of the mercury species into the cell, rate of mercury conversion to methylmercury, cell-cell interactions, and tolerance to mercury toxicity. Collectively, the amended and control reactor data suggest that active
consortiums of SRB have distinctive properties that define their potential to methylate mercury relative to the SRR. These distinctive cellular properties, although not known, have been grouped collectively into the $\frac{f}{K_{Hg}}$ term to illustrate differences in consortiums.

Comparing values obtained for the lactate and control reactors in Figures 6.15 and 6.16, it can be suggested that SRR does significantly influence the rate of mercury methylation. Figure 6.10 suggested that the percentages of phylogenetic groups present in the lactate and control reactors are similar. This implies that the $\frac{f}{K_{Hg}}$ term is similar between the two reactors as well and is indicated in Figures 6.15 and 6.16. It should also be noted that slurry water mercury trends in both reactors are not significantly different (P>0.05). Based on Equation 4.11, this suggests that the increase in mercury methylation rates in the lactate reactor are directly influenced by the respiration rate of the SRR.

A review of Figures 6.12-6.17 illustrates that the MMR reached a value of zero prior to the complete loss of soluble mercury from slurry water. These data suggest that there are aqueous species of mercury that are not bioavailable for methylation. In these slurry systems, the MMR reached near zero value for the lactate conditioned, acetate conditioned, and control reactors when soluble mercury concentrations reached 31.6, 39.1, and 34.9 ng/L, respectively. The actual soluble species of mercury that are bioavailable for methylation are not known. However, the previous discussion of the soluble species of mercury “unavailable” for methylation centered on the complexation of inorganic mercury species with humic materials or other anions that prevented methylation.
of mercury from occurring. Thus, environmental conditions such as pH, redox conditions, and water chemistry dictate the "bioavailability" of inorganic mercury at later time intervals as opposed to the early time intervals where presumably inorganic mercury is more "biavailable." In conclusion, this study has demonstrated that mercury availability, consortium diversity, and SRR are the primary variables that determine the potential methylmercury production.
CHAPTER VII

SURVEY OF SRR, MMR AND 16S rRNA IN MARINE SEDIMENTS AND MODEL ASSESSMENT

In this chapter, a survey of marine sediment at the Skidaway Institute of Oceanography was conducted in August 1999. SRR, 16S rRNA determinations, and MMR potentials were determined with respect to depth in the sediment to link MMR to SRR and SRB populations. In addition, all three analytical methods were conducted in triplicate to understand spatial variations that exist in sediments. Using these data with data obtained from Chapter 5, an effort was made to define MMR sediment cores based on SRR and 16S rRNA profiles.

Sulfate-Reduction Rates in Sediment Cores

The SRR was determined in non-contaminated sediments. Figure 7.1 illustrates the SRR taken for three cores collected at the same time.
Figure 7.1 SRR in sediment cores collected in tidal marshes located at the Skidaway Institute of Oceanography (Sediment C).
In general, maximum mercury methylation rates occur in the upper 2 cm of each core. The most variability in SRR occurred at the 1-cm depth where values ranged from 22.15 to 49.6 nmol/g\(_{\text{dry}}\)-hr (Figure 7.1). Table 7.1 indicates the average SRR as well as the standard error observed for each centimeter interval. In general, SRR values remained < 13.0 nmol/g\(_{\text{dry}}\)-hr (excluding one point at 4 cm) at depths greater than 3 cm (Figure 7.1).

<table>
<thead>
<tr>
<th>Depth Interval (cm)</th>
<th>SRR ± std. error (nmol/g(_{\text{dry}})-hr)</th>
<th>MMR ± std. error (pg/g(_{\text{dry}})-hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-1</td>
<td>34.42 ± 8.05</td>
<td>92.02 ± 24.52</td>
</tr>
<tr>
<td>1-2</td>
<td>15.69 ± 2.12</td>
<td>119.19 ± 18.41</td>
</tr>
<tr>
<td>2-3</td>
<td>10.95 ± 1.33</td>
<td>58.86 ± 17.94</td>
</tr>
<tr>
<td>3-4</td>
<td>17.02 ± 5.45</td>
<td>72.42 ± 5.62</td>
</tr>
<tr>
<td>4-5</td>
<td>9.76 ± 1.13</td>
<td>57.89 ± 26.08</td>
</tr>
<tr>
<td>5-6</td>
<td>8.76 ± 0.63</td>
<td>31.47 ± 11.61</td>
</tr>
<tr>
<td>6-7</td>
<td>5.40 ± 1.64</td>
<td>6.84 ± 2.66</td>
</tr>
<tr>
<td>7-8</td>
<td>4.58 ± 1.52</td>
<td>7.70 ± 1.60</td>
</tr>
<tr>
<td>8-9</td>
<td>6.58 ± 2.14</td>
<td>20.25 ± 7.88</td>
</tr>
<tr>
<td>9-10</td>
<td>6.59 ± 1.36</td>
<td>9.92 ± 2.79</td>
</tr>
</tbody>
</table>
MMR in Sediment Cores

Relative mercury methylation rates were determined in sediment cores by injecting 1 μg of inorganic mercury at 1 cm intervals and analyzing core segments for methylmercury concentrations over time. Figure 7.2 illustrates the relative mercury methylation rates observed in three separate cores collected at the same time. The data suggest that maximum mercury methylation rates were located in the upper 4 cm of all cores (Figure 7.2). In addition, the largest degree of variability in MMR occurred in the upper 4 cm as well (Figure 7.2). Table 7.1 indicates the average MMR as well as the standard error observed for each centimeter interval. Minimum values of MMR were depths below 6 cm. In general, MMR values from 6 to 10 cm ranged between 2 and 14 pg/g dry-hr. In addition, the MMR values reported between 6 and 10 cm demonstrated the least variability among calculated data at a specific depth (Figure 7.2).

Phylogenetic Quantification of SRB Groups in Intact Cores

The phylogenetic groups of SRB were quantitated with respect to depth in three sediment cores. The five probes that were used to identify specific SRB phylogenetic groups are reported in chapter 3 (Table 3.2). The identification of 16S rRNA that hybridized to a probe has been designated according to a specific genus that was used to calibrate the oligonucleotides (Table 3.2). Figure 7.3 illustrates the 16S rRNA profiles for all populations of SRB and is reported in terms of relative abundance.
Figure 7.2 MMR in sediment cores collected in tidal marshes located at the Skidaway Institute of Oceanography (Sediment C).
Figure 7.3 Relative Abundance of total SRB 16S rRNA in sediment cores collected in tidal marshes located at the Skidaway Institute Oceanography (Sediment C).
In cores 2 and 3, a maximum relative abundance value of 1 was determined in the upper 2 cm of the sediment core (Figure 7.3). In cores 2 and 3, the relative abundance value continues to decrease with respect to depth in the sediment. The lowest observed relative abundance value for cores 2 and 3 occurred in the 8-10 cm range. Relative abundance values in core 1 generated no observable trend in the SRB populations. The maximum relative abundance value occurred in the 2-4 cm interval. However, the minimum value occurred in the 4-6 cm interval that was followed by another high relative abundance value in the 6-8 cm interval (Figure 7.3).

Figure 7.4 illustrates the quantities of 16S rRNA extracted for five SRB phylogenetic groups in the same three cores. Compared to other SRB 16S rRNA quantitated in these cores, 16S rRNA concentrations were lowest for DSC (Figure 7.4). For DSC, maximum values of 20.2 and 38.1 ng/g_dry were reported at the 0-2 cm depth in cores 2 and 3, respectively (Table 7.2). Excluding core 1, 16S rRNA concentrations of DSV generally decreased relative to the 0-2 cm value as depth increased in the cores (Table 7.2). For DSB, there are no clear trends with respect to DSB 16S rRNA concentrations and depth (Table 7.2). Standard errors reported in Figure 7.4 for DSV, DSB, and DSC suggest that the amount of 16S rRNA quantitated for a group did not vary significantly with respect to depth in the cores (Figure 7.4). In contrast to DSV, DSB, and DSC profiles, both DBACTER and DSBM demonstrate a significant subsurface maximum in 16S rRNA concentrations at the 2-4 cm interval (Figure 7.4). Maximum concentrations of 238.6 and 266.8ng/g_dry were quantified at the 0-2 cm interval for DBACTER in cores 1 and 3, respectively (Table 7.2).
Figure 7.4 16S rRNA Profiles for *Desulfovibrio* (DSV), *Desulfobulbus* (DSB), *Desulfococcus* (DSC), *Desulfobacter* (DBACTER), and *Desulfobacterium* (DSBM). Cores are Sediment C (See materials and methods).
<table>
<thead>
<tr>
<th>Depth Interval (cm)</th>
<th>Core 1</th>
<th>Core 2</th>
<th>Core 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSB (Desulfobulbus)</td>
<td>35.2</td>
<td>69.9</td>
<td>89.7</td>
</tr>
<tr>
<td>DSV (Desulfovibrio)</td>
<td>0.2</td>
<td>2.4</td>
<td>4.6</td>
</tr>
<tr>
<td>DSC (Desulfococcus)</td>
<td>58.1</td>
<td>553.7</td>
<td>586</td>
</tr>
<tr>
<td>DSBM (Desulfobacterium)</td>
<td>11.6</td>
<td>20.2</td>
<td>34.7</td>
</tr>
</tbody>
</table>
| 16S rRNA for Sulfate-Reducing Bacteria Phylogenetic Groups in Sediment Cores (ng/gw)
<table>
<thead>
<tr>
<th>Core 1</th>
<th>Core 2</th>
<th>Core 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-2</td>
<td>32.9</td>
<td>34.5</td>
</tr>
<tr>
<td>2-4</td>
<td>32.9</td>
<td>34.5</td>
</tr>
<tr>
<td>4-6</td>
<td>34.7</td>
<td>34.7</td>
</tr>
<tr>
<td>6-8</td>
<td>42.3</td>
<td>29.3</td>
</tr>
<tr>
<td>8-10</td>
<td>37.1</td>
<td>27.4</td>
</tr>
</tbody>
</table>
Excluding the average maximum reported in the 0-2cm interval, the amount of DBACTER 16S rRNA remained relatively constant with values reported between 123.5 and 155.0 ng/g_{dry} (Figure 7.4). The 16S rRNA profiles for DSBM demonstrated a pronounced increase in 16S rRNA concentration at the 2-4 cm interval when compared to the 0-2 cm interval (Figure 7.4). Maximum concentrations of 194.1, 221.8, and 284.1 ng/g_{dry} are reported for DSBM in cores 1, 2, and 3, respectively (Table 7.2). Following the 0-2 cm interval, concentrations of DSBM 16S rRNA continue to decrease to minimum values which are observed at the 8-10 cm interval (Figure 7.4).

Application of Equations to Intact Cores

In this thesis, the equations proposed in Chapter 4 demonstrated that biological variables influence mercury methylation potential. Equation 4.11 illustrates the model that will be used for analysis of sediment core data.

\[
\frac{MMR}{SRR} = \hat{f} \left[ \frac{[Hg]}{K_{1[Hg]} + [Hg]} \right] = \hat{f} 
\]

Equation 4.11

MMR and SRR data obtained from sediment cores were utilized to determine the \( \hat{f} \) term that exists with respect to sediment depth in the core.
Figure 7.5 illustrates the \( f \) terms that were calculated by taking each MRR value observed at a given depth and dividing it by each SRR value observed at the same depth. Thus, nine terms were calculated for each 1 cm interval. The data indicate that the greatest range of values in \( f \) term occurred in the 2-5 cm interval (Figure 7.5). Moreover, this is also the depth interval where maximum values occurred (Figure 7.5). A maximum value implies that a region exist where the incidence of methylmercury production is highest relative to SRR (i.e. increase in \( f \)).

**The Use of \( f_i \) to Ascertain MMR in Intact Sediment Cores**

Table 7.3 reports the average \( f \) and standard error observed at each depth in Figure 7.5. As illustrated in Table 7.3, average \( f \) values for the sediment cores ranged from a maximum of 11.7 pg/nmol at 2 cm to 1.6 pg/nmol at 10cm. Table 7.4 illustrates the \( f_i \) term reported in Chapter 5 for sulfate-reducing bacteria grown in pure culture.

Comparing \( f \) values observed in sediment cores (Table 7.3) to \( f_i \) values observed in pure culture (Table 7.4) indicates that \( f_i \) values for DSV, DSB, DSC, and DBACTERE were significantly below those measured in the sediment cores. The \( f_i \) value reported for DSBM compared the best with respect to \( f \) values reported in sediments.
Figure 7.5 Calculated values of $f^*$ in sediment cores (Sediment C).
Although the individual \( f_i \) were significantly lower than core \( f \), summing all \( f_i \) observed in pure culture results in similar values for sediment intervals excluding 1-3 cm. Collectively, these results suggest that it is reasonable to make comparisons between pure culture and sediment core observations.

<table>
<thead>
<tr>
<th>Depth Interval (cm)</th>
<th>( f^* ) value (pg/nmol)</th>
<th>Standard error (pg/nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-1</td>
<td>3.0</td>
<td>0.5</td>
</tr>
<tr>
<td>1-2</td>
<td>11.7</td>
<td>2.1</td>
</tr>
<tr>
<td>2-3</td>
<td>9.7</td>
<td>1.6</td>
</tr>
<tr>
<td>3-4</td>
<td>5.0</td>
<td>0.6</td>
</tr>
<tr>
<td>4-5</td>
<td>6.1</td>
<td>1.4</td>
</tr>
<tr>
<td>5-6</td>
<td>3.6</td>
<td>0.6</td>
</tr>
<tr>
<td>6-7</td>
<td>1.6</td>
<td>0.5</td>
</tr>
<tr>
<td>7-8</td>
<td>2.1</td>
<td>0.4</td>
</tr>
<tr>
<td>8-9</td>
<td>3.7</td>
<td>0.8</td>
</tr>
<tr>
<td>9-10</td>
<td>1.6</td>
<td>0.3</td>
</tr>
</tbody>
</table>
It should be noted that the reason for observed differences in $f$ terms (sediment compared to pure culture) is unclear. Since the pure culture and sediment cores represent two distinct environments, a difference in the magnitude of $f$ terms can be expected.

| Table 7.4 | $f_i \ * Term$  \\ | $f_i \ * Term \pm$ std. error (nmol/nmol) | $f_i \ * Term \pm$ std. error (pg/nmol) |
|-----------|-----------------|
| SRB       | 1.4x10$^6 \pm$ 1.8x10$^{-7}$ | 0.30 ± 0.04 |
| DSV  \\ *Desulfovibrio* |                     |                     |
| DSB  \\ *Desulfobulbus* | 2.9x10$^{-7} \pm$ 9.9x10$^{-8}$ | 0.06 ± 0.02 |
| DSC  \\ *Desulfooccus* | 4.6x10$^{-6} \pm$ 1.3x10$^{-6}$ | 1.0 ± 0.27 |
| DBACTER  \\ *Desulfobacter* | 4.1x10$^{-6} \pm$ 9.6x10$^{-7}$ | 0.88 ± 0.20 |
| DSBM  \\ *Desulfobacterium* | 2.58x10$^{-5} \pm$ 2.88 x 10$^{-6}$ | 5.54 ± 0.62 |
Equation 4.11 indicated that mercury availability was a critical component in the production of methylmercury. The difference in pure culture and sediment environments could have resulted in a variation in mercury speciation. Presumably, the mercury in the pure culture was less available for methylation. This would result in lower \( f_i \) values.

Calculations of MMR based on Observed SRR and Pure Culture \( f_i \).

A calculated MMR was determined based on the pure culture \( f_i \) for each phylogenetic group and the fraction of an observed phylogenetic group in sediment. In addition to the \( f_i \) value, the metabolic activity each phylogenetic group contributes to methylation of mercury had to be considered. Table 7.5 indicates that the SRR observed in pure culture varies between phylogenetic groups on a per cell basis (See Chapter 4).

<table>
<thead>
<tr>
<th>Table 7.5</th>
<th>Calculated SRR of Pure Cultures (nmol/cell-hr ± % std. err.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSV</td>
<td>DSB</td>
</tr>
<tr>
<td>1.41x10^{-7} (± 7.9%)</td>
<td>6.96x10^{-8} (± 14.6%)</td>
</tr>
</tbody>
</table>

The fact that the magnitude of SRR varied among individual phylogenetic groups in pure culture suggested that the calculated SRR in the natural sediments cannot be equally allocated to the individual SRB phylogenetic groups. Moreover, each SRB phylogenetic
group would then have a different unit activity in terms of methylmercury production since the SRR is a component that determines MMR. Since sulfate reduction by SRB is coupled to the methylation of mercury, the rate of respiration by individual groups must be taken into account. Thus, Table 7.5 provides an indication of the relative activity of sulfate-reduction by individual groups in natural systems.

Equation 7.1 describes the MMR as the sum of respective rates for each individual phylogenetic group.

\[
MMR_{\text{Total}} = \sum MMR_i 
\]

Equation 7.1

From earlier equations it can be re-stated that the individual MMR\(_i\) is a function of the \(\hat{f}_i\), term as well as the individual SRR and available mercury. Equation 7.2 defined the MMR\(_i\) with respect to other variables on a phylogenetic group basis.

\[
MMR_i = \hat{f}_i \cdot SRR_i \cdot \frac{[Hg]}{K_{[Hg]} + [Hg]} = \hat{f}_i \cdot SRR_i 
\]

Equation 7.2

In this equation, \(\hat{f}_i\) defined the maximum quantity of methylmercury produced per sulfate reduced by that specified phylogenetic group. The \(K_{[Hg]}\) term represents a half-saturation constant for cellular internalization/transport of mercury for methylation. The \(\hat{f}_i\) term groups the \(\hat{f}_i\) term and mercury availability term together. The individual SRR\(_i\)
contribution of each phylogenetic group present in the sediment cores was not known. Thus, the SSR would be a function of individual phylogenetic groups present at depth and the unit SSR activity of that group. The fraction of a total group present \( \frac{16S\ rRNA_i}{16S\ rRNA_{tot}} \) represents a method to account for specific phylogenetic groups that compose a fraction of the total consortium with respect to depth, and the unit activity of the groups can be expressed in terms of the SRR observed in pure culture normalized to cell number \( \frac{SRR_{pc}}{Cell_{pc}} \). Thus, the individual SRR values can be calculated based on the fraction of 16S rRNA that was present with respect to depth and the observed activity of sulfate-reduction relative to other SRB in pure culture \( \frac{SRR_{pc}}{Cell_{pc}} \). Equation 7.3 illustrates the calculation of the individual contributions of SRR.

\[
SRR_i = \frac{SRR_{total}}{\sum \left( \frac{16S\ rRNA_i \cdot \frac{SRR_{pc}}{Cell_{pc_j}} \cdot \frac{Cell_{pc}}{16S\ rRNA_{pc_j}}}{16S\ rRNA_{pc}} \right)}
\]

Equation 7.3

From Equation 7.3, the \( \frac{Cell_{pc}}{16S\ rRNA_{pc}} \) indicates the amount of 16S rRNA per cell found in pure culture. It is assumed that the amount of 16S rRNA per cell is equal with respect
to all five pure cultures of SRB. Thus, this term is common to both the numerator and denominator, and it cancels out of the equation. This assumes that the amount of 16S rRNA present in each cell is constant among SRB phylogenetic groups. Thus, the SRR$_i$ can now be evaluated based on the observed SRR in the sediment (SRR$_{Total}$), fraction of 16S rRNA for each individual group (16S rRNA$_i$), and the fraction of SRR unit activity of each group expressed in terms of the pure culture SRR $\left(\frac{SRR_{pc}}{Cell_{pc}}\right)$. Table 7.6 indicates the sum total amount of 16S rRNA (ng/g$_{dry}$) for all five SRB phylogenetic groups present at each depth as well as the percentage of 16S rRNA of each group at that depth.

<table>
<thead>
<tr>
<th>Depth Interval (cm)</th>
<th>Sum of Total SRB 16S rRNA (ng/g$_{dry}$)</th>
<th>Percentage DSV (%)</th>
<th>Percentage DSB (%)</th>
<th>Percentage DSC (%)</th>
<th>Percentage DBACTER (%)</th>
<th>Percentage DSBM (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-2</td>
<td>314.1</td>
<td>20.7</td>
<td>19.9</td>
<td>7.4</td>
<td>39.3</td>
<td>12.7</td>
</tr>
<tr>
<td>2-4</td>
<td>601.0</td>
<td>7.0</td>
<td>13.0</td>
<td>3.7</td>
<td>37.5</td>
<td>38.8</td>
</tr>
<tr>
<td>4-6</td>
<td>306.4</td>
<td>7.8</td>
<td>10.7</td>
<td>3.7</td>
<td>46.9</td>
<td>30.9</td>
</tr>
<tr>
<td>6-8</td>
<td>356.4</td>
<td>9.9</td>
<td>15.3</td>
<td>4.1</td>
<td>42.1</td>
<td>28.6</td>
</tr>
<tr>
<td>8-10</td>
<td>261.4</td>
<td>10.5</td>
<td>13.6</td>
<td>5.0</td>
<td>59.6</td>
<td>11.3</td>
</tr>
</tbody>
</table>
Substituting Equation 7.3 into Equation 7.2 results in an expression (Equation 7.4) that allows for the calculation of MMR$_i$ as function of $f_i$, the SRR observed with respect to depth in the core, the fraction of a specific phylogenetic group that is present at a depth, and the SRR unit activity for each phylogenetic group.

$$
MMR_i = \frac{\sum 16S\ rRNA_i \cdot \left( \frac{SRR_{pc}}{Cell_{pc}} \right) \cdot f_i}{\sum 16S\ rRNA_i \cdot \left( \frac{SRR_{pc}}{Cell_{pc}} \right)}
$$

Equation 7.4

Substituting Equation 7.4 into 7.1 results in an equation that allows for the calculation of MMR in sediments. Equation 7.5 illustrates the final equation.

$$
MMR = \frac{\sum 16S\ rRNA_i \cdot \left( \frac{SRR_{pc}}{Cell_{pc}} \right) \cdot f_i}{\sum 16S\ rRNA_i \cdot \left( \frac{SRR_{pc}}{Cell_{pc}} \right)}
$$

Equation 7.5

Since the $f_i$ term is not known for the phylogenetic groups in the sediment cores, $f_i$ terms (nmol methylmercury produced/nmol sulfate reduced) defined in pure culture
experiments will be substituted into the equation. The \( f_i \) term is reported in Table 7.4. Calculated values generated by Equation 7.5 were reported in pg/g\(_{\text{dry}}\)-hr by converting calculated values using the molecular weight of methylmercury.

Figure 7.6 illustrates how the calculated MMR compares to the observed MMR for the same core region. Overall, the figure illustrates that a good correlation exists with respect to the location where maximum and minimum values are observed. Calculated MMR values indicate that maximum mercury methylation rates exist in the 2-4 cm region (Figure 7.6). This is followed by a decrease in calculated MMR as depth increases. The relative MMR that were observed in sediment cores support this general trend. The observed data suggest that maximum mercury methylation rates on the order of 80-140 pg/g\(_{\text{dry}}\)-hr typically occur in a 2-5 cm range (Figure 7.6). This is followed by a sustained decrease in MMR (generally 10-15 pg/g\(_{\text{dry}}\)-hr) at depths greater than 6 cm (Figure 7.6).

Figure 7.6 clearly indicates that discrepancies exist between actual MMR values and calculated MMR values. Table 7.7 illustrates the average observed and calculated MMR values in regions of the sediment cores where core values have been averaged. The largest disagreement between predicted and observed MMR occurs in the 0-2 cm region. In this section of the core, observed values are approximately 2.7 fold higher than predicted MMR values (Table 7.7). Overall, data compiled from sediment regions indicate that there is a significant difference between MMR observed and predicted values for depths less than 6 cm. However, a ratio of the observed values normalized to the calculated values indicated that the average ratio for the upper 6 cm was 2.3 (Table 7.7).
Figure 7.6 Observed and predicted MMR in sediment cores
Table 7.7

Calculated and observed MMR in sediment cores

<table>
<thead>
<tr>
<th>Depth (cm)</th>
<th>Calculated MMR ± Std. Error (pg/g_dry-hr)</th>
<th>Observed MMR ± Std. Error (pg/g_dry-hr)</th>
<th>Ratio Obs. / Calc. ± Std. Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-2</td>
<td>39.3 ± 6.9</td>
<td>105.6 ± 15.1</td>
<td>2.7 ± 0.5</td>
</tr>
<tr>
<td>2-4</td>
<td>37.1 ± 3.4</td>
<td>65.6 ± 8.9</td>
<td>1.8 ± 0.3</td>
</tr>
<tr>
<td>4-6</td>
<td>18.9 ± 1.0</td>
<td>44.7 ± 14.1</td>
<td>2.4 ± 0.8</td>
</tr>
<tr>
<td>6-8</td>
<td>10.9 ± 1.6</td>
<td>7.3 ± 1.4</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>8-10</td>
<td>8.7 ± 1.7</td>
<td>15.1 ± 4.4</td>
<td>1.7± 0.6</td>
</tr>
</tbody>
</table>

This implies that a 2.3 fold difference exists between observed MMR and the calculated MMR for the 0-6 cm region of sediment. It should be noted that there was better agreement between observed and predicted MMR for depths greater than 6 cm. In this region of sediment the observed MMR normalized to the calculated MMR was approximately 1.2. The variations between calculated and observed MMR can be attributed to the spatial variability both in the horizontal and vertical axis with respect to intact sediment cores.
Certainly the use of 6 distinctly different cores to determine SRR and the fraction of phylogenetic groups present would generate error with respect to predicting the MMR observed in another three distinct cores.

Another reason for the disagreement in the upper 6cm could be the assumption built into Equation 7.5 that the SRR activity of the SRB phylogenetic groups are identical to the SRR activity reported in the pure culture. As demonstrated in Equation 7.5, the predicted MMR is contingent upon the SRR unit activity for each of the SRB phylogenetic groups. Thus, the inability to correctly predict the MMR in the upper 6cm could be a result of variations in the SRR activity among the SRB phylogenetic groups with respect to depth. Since there is variation in the distribution of SRB phylogenetic groups with respect to depth, the variation in SRR activity among phylogenetic groups is entirely plausible.

Another possible explanation for the difference in observed and calculated MMR centers on the reduction in SRR per cell when cells are grown in the presence of particles. Fukui and Takii (1994) demonstrated that Desulfovibrio that were grown in pure culture had an average SRR of 2.62x10^-7 nmol/cell-hr. However, in the presence of resin particles the same Desulfovibrio group had an SRR of 9.1x10^-9 nmol/cell-hr. The reduction in SRR was attributed to particle attachment. This was also demonstrated in the data presented in this thesis which reported SRR in pure culture on the order of 10^-6-10^-7 nmol/cell-hr (Chapter 3) and SRR on the order of 10^-9 nmol/cell-hr in sediment slurry systems (Chapter 4). Since f_i is defined by the MMR normalized to the SRR, a higher observed SRR in
pure culture would result in lower calculated $f_i$ terms. Thus, the calculated MMR based on individual $f_i$ terms would be lower than the observed MMR values since the SRR observed in sediment cores is generally two orders of magnitude lower than pure culture SRR on a per cell basis (Fukii and Takii, 1994). This assumes that the rate of mercury methylation stays relatively the same between pure culture and sediment based systems provided sediment systems are not limited with respect to available mercury for methylation.

Another difference in the calculated MMR and actual MMR data in cores could be the species of mercury present. Given the large concentration of polysulfides observed in the pure culture, the mercury “available” for methylation might require more “processing” by the cell as oppose to the inorganic mercury injected into the sediment cores. Thus, MMR in pure culture would be slower than that observed in the intact cores. If the MMR was indeed slower than that observed in sediment cores, then the $f_i$ value calculated in pure culture would be lower than the $f_i$ in sediment cores since $f_i$ is defined as methylmercury produced normalized by the sulfate-reduction rate and “available mercury” (Equation 4.12). It is the $f_i$ value from the pure culture that is used in the calculation of the curves in the core.

In any event, utilizing the $f_i$ term calculated in pure culture, observed SRR values, and the fraction of active phylogenetic groups present at depth generated calculated MMR
values that had similar trends with observed MMR values. This result clearly demonstrates that the proportional assignment of $f_i$ obtained in pure culture to the various phylogenetic groups in cores is appropriate and further emphasizes the role SRB speciation and respiration have in the production of methylmercury.
CHAPTER VIII

CONCLUSIONS AND RECOMMENDATIONS
FOR FUTURE RESEARCH

The principal factors thought to influence biologically mediated MMR were defined. Through the use of equations and experiments in this thesis, it has been demonstrated that the SRR directly influences MMR. Experiments have demonstrated that a reduction in SRR proportionally reduces methylmercury production. Similarly, increases in the SRR resulted in proportional increases in mercury methylation. Other studies demonstrated how the inhibition of SRR using sodium molybdate (specific inhibitor to sulfate-reduction) effectively inhibits the production of methylmercury. In addition, using pure cultures of SRB phylogenetic groups grown in sulfate-depleted systems were unable to generate methylmercury.

It was demonstrated that the availability of mercury determines how much methylmercury is produced. It was demonstrated that mercury availability for methylation follows saturation kinetics when doses of inorganic mercury were added that exceeded maximum utilization rates. In addition, a pseudo-first order relationship exists when slurry
water mercury concentrations are significantly lower than $K_{\text{Hg}}$. That is, highest MMR occurred at the highest observed slurry water mercury concentrations, and lowest MMR occurred at the lowest observed slurry water mercury concentrations.

Pure culture experiments demonstrated that different species of SRB have the potential to methylate mercury at different rates on a per cell basis. These differences were made more evident when the mercury methylation rates were normalized to the SRR (Figure 6.8). The differences in bacterial consortiums to methylate mercury were also demonstrated. Changes in the composition of the consortium, in conjunction with statistically significant differences, studies conducted by other authors implicated the sulfate-reducing bacteria as the primary biological producers of methylmercury (Compeau and Bartha, 1985; Compeau and Bartha, 1984; Blum and Bartha, 1980; Gilmour and Capone, 1987). In the introduction, the rate of sulfate reduction by SRB in anoxic systems was mathematically defined by Equation 4.7.

$$-r_{SO_4} = \left( \frac{1-Y}{Y} \right) \mu \left( \frac{S_s}{K_s + S_s} \right) \left( \frac{[SO_4]}{K_{SO_4} + [SO_4]} \right) X_B$$  

Equation 4.7

Evidence presented in this thesis as well as by other authors suggests that the methylation of mercury is coupled to the microbial reduction of sulfate. The coupling of MMR to SRR was represented in Equation 4.10.
\[
\frac{d[CH_3Hg^+]}{dt} = \hat{f}(-r_{SO_4}) \frac{[Hg]}{K_{Hg} + [Hg]} = r_{CH_3Hg} \quad \text{Equation 4.10}
\]

Treatment of sediments with molybdate, a specific biological inhibitor of sulfate reduction, effectively reduced the methylation of mercury in sediments while inhibiting sulfate reduction. Similar results have been reported by Compeau and Bartha (1985). In pure culture studies presented in this thesis, SRB that were grown in sulfate-depleted cultures produced trace amounts of methylmercury compared to the same bacteria grown in sulfate-rich cultures. Similar results were also described by Pak and Bartha (1998b) when Desulfovibrio desulfuricans LS were grown in the absence of sulfate. The above mentioned results indicate sulfate reduction must occur in order for mercury to be methylated. Equation 4.10 mathematically states that the MMR is a function of the SRR and amount of mercury available for methylation. The \( \hat{f} \) term is operationally defined as the maximum amount of methylmercury formed per amount of sulfate reduced. Normalizing the \( r_{CH_3Hg} \) term by the sulfate reduction term \( (-r_{SO_4}) \) yields equation 4.11.

\[
\frac{r_{CH_3Hg}}{-r_{SO_4}} = \hat{f} = \hat{f} \frac{[Hg]}{K_{Hg} + [Hg]} \quad \text{Equation 4.11}
\]

The \( \hat{f} \) term is defined as the net incidence term reported as nmol methylmercury produced per nmol sulfate reduced. This term incorporates the maximum mercury methylation rate
per sulfate reduced \( f \) and mercury availability term. This term is used to compare

“net” incidence of methylmercury formed per unit sulfate reduced in systems that are subject to limitations in mercury availability.

**Mercury Availability**

Experiments conducted in slurry systems demonstrated that mercury availability was a critical component in establishing an MMR. This was made evident by an observation that sequential dosing of reactor systems with 955ng/g\_dry of inorganic mercury produced spikes in methylmercury production within a 12hr interval after inorganic mercury addition (Figure 4.9). Additions of different concentrations of inorganic mercury to slurry systems indicated that larger concentrations of inorganic mercury added to reactors resulted in larger concentrations of methylmercury produced in the initial 12hrs of reactor operation (Figure 4.10). Analysis of the MMR in the initial 12hrs of reactor operation indicated that saturation kinetics occurred (Figure 4.12). Thus, a considerable amount of evidence suggests that that the amount of inorganic mercury added to a system determined the amount the amount of methylmercury that was produced. Moreover, there is a maximum MMR that, when reached, is not affected by larger and larger added inorganic-mercury concentrations. In addition, experimental results indicated that a correlation between methylmercury production and slurry water mercury concentrations exist. Data presented in Figure 4.8 clearly demonstrate that the maximum MMR observed between data points occurred when slurry water mercury concentrations were at a maximum. The same type
correlation is observed in the sediment slurries preconditioned with organic acids (Chapter 6). Results from control reactors, acetate treated, and lactate treated reactors indicated that higher MMR occurred when slurry water mercury concentrations were the highest for each treatment (Figure 6.7).

Based on data acquired in Chapter 6, linear regression analysis indicated that the MMR values were approaching zero at soluble mercury concentrations that ranged from 30 to 40 ng/L for all reactor systems. This result suggests that species of soluble mercury exist that are not “bioavailable” for methylation. It has been demonstrated that insoluble HgS\(_{(s)}\) is unavailable for methylation. However, the species of mercury that are “bioavailable” for methylation are not known. Conceivably, the higher MMR observed in the initial periods of reactor operation (0-12hrs) are indicative of time intervals in which higher concentrations of available mercury exist. It is logical to assume the abiotic; physical/chemical processes are driving the reactors to an equilibrium state as time progresses. Thus, the higher MMR observed in the 0-12hr period reflect a period where concentrations of inorganic mercury that has not been sequestered or complexed is highest. For example, over time inorganic mercury may form complexes with soluble humic acids or other anions in the system. As a result, the inorganic mercury may not be “available” to enter the cell through simple diffusion. Thus, the availability of mercury to the cell decreases and the resulting MMR of the system decrease.

It should be noted that saturation kinetics was not observed in these sediment slurry based systems that received a 950 ng/g\(_{dry}\) dose of inorganic mercury. Data observed in Chapter 4 suggest that higher concentrations of inorganic mercury would have to be
added in order to observe the saturation kinetics phenomena, i.e. doses greater than 950ng/g_{dry}(Figure 4.8). Thus, the linear response for all treatments illustrated in Figures is analogous to a pseudo-first order response at low levels of added mercury (i.e. [Hg]_{added} levels of 95-955ng/g_{dry}). It should also be noted that MMR varied between each treatment at the higher slurry water mercury concentrations. However, the correlation between MMR and slurry water mercury concentrations is similar between treatments. The reason for the variations in MMR between reactor systems will be discussed later.

**Influence of SRR on MMR**

As illustrated in Equation 4.10, the MMR is also a function of the SRR. Experimental results reported in this thesis demonstrated that changes in sediment slurry SRR resulted in a similar response in MMR. In sediment slurry experiments reported in Chapter 4, the temperature was changed to assess variations in SRR and MMR. Results from that experiment suggest that temperature affected SRR and MMR similarly (Figure 4.2 and 4.3). Results from the acetate and pyruvate amended slurries reported in Chapter 4 indicated that there was a proportional response between SRR and observed MMR for reactors receiving the same treatment (Figure 4.6 and 4.7). In both the temperature and substrate treated reactors, an increase in MMR was observed with a simultaneous increase in SRR. Likewise, a decrease in SRR resulted in decreased MMR for the initial 12hr period. These results, in addition to the inhibition of both SRR and MMR using molybdate, indicate that SRB respiration is coupled to the mercury methylation process. Models used in Chapter 4 to predict MMR based on observed SRR and inorganic mercury
applied to the reactor system demonstrated the significance of the SRR in terms of estimating methylmercury production. The influence SRB respiration has on methylmercury production was also demonstrated in a comparison of sediment slurries preconditioned with lactate and control reactors of Chapter 6. In sediment reactors that operated for 20 days after an initial dose of 20 mM lactate, the observed SRR was approximately 7 fold higher than control SRR values (Figure 6.2 and 6.4). Figure 6.12 and Figure 6.14 demonstrated that the slope of MMR as a function of slurry water mercury concentrations was different for control and lactate treated reactors. However when the MMR for each treatment was normalized by the respective SRR, the two slopes were identical (Figure 6.15 and 6.16). Since both reactor SRB populations were similar in the percentage composition (Figure 6.10), it was concluded that the increase in MMR observed in lactate treated reactors was primarily due to increased SRB respiration (i.e. SRR). In addition, it was demonstrated that sulfate reduction was required for mercury methylation to occur. Two groups (DSV and DSBM) did not methylate mercury when sulfate was absent from the culture (Figure 5.12). This implies a need for microbial sulfate-reduction in order to methylate mercury, and it justifies comparing MMR when normalized to SRR as illustrated in Equation 4.11.

Influence of Microbial Diversity on Mercury Methylation

Other data sets collected in this thesis suggest that various SRB phylogenetic groups methylate mercury with a greater “incidence” than others relative to the SRR. MMR and SRR data was collected using pure culture representatives of the phylogenetic groups:
Desulfovibrio (DSV), Desulfobulbus (DSB), Desulfococcus (DSC), Desulfobacter (DBACTER), and Desulfobacterium (DSBM). Based on methylmercury production, it was determined that all groups methylated mercury at different rates (Table 5.3). MMR was normalized to the corresponding SRR and the resulting \( f \) term compared between phylogenetic groups. Results indicated that the calculated \( f \) was different for all pure cultures used to represent the different phylogenetic groups (Figure 5.12). The results suggest that DSBM had the highest incidence of methylating mercury when normalized to the SRR. It should also be noted that DSV and DSB have the lowest incidence of methylating mercury relative to SRR. From the data compiled in Chapter 5, it was concluded that the incidence profile (least to greatest) for methylmercury production relative to SRR was:

\[ \text{DSB} < \text{DSV} < \text{DSC} \approx \text{DBACTER} < \text{DSBM} \]

These results, in conjunction with Figure 5.12 suggest that an evolutionary scheme exist for the increased incidence of MMR relative to SRR (For more discussion on the genetic homology of the groups and the \( f \) value (Chapter 5). In addition to the pure culture results, the organic acid amended slurry data in Chapter 6 suggests that the species of SRB in a consortium can affect the rate of methylmercury production relative to the SRR. Reactors treated with a slug of lactate or acetate 20 days prior to inorganic mercury addition resulted in different percent compositions of SRB between the two reactor systems (Figure 6.10). It should be noted that the control reactor system had SRB profiles that most resembled the lactic acid treated reactors (Table 6.1). Methylmercury
production was then quantified in the control, acetate, and lactate reactors and normalized to the respective SRR (Figure 6.15, 6.16, and 6.17). Since the data were obtained at slurry water mercury concentrations that are limiting, the slope of the linear regression analysis is \( \frac{\hat{f}}{K_{Hg}} \) for each treatment. The graph clearly shows that the control and lactate treated reactors have nearly identical \( \frac{\hat{f}}{K_{Hg}} \) terms. However, the \( \frac{\hat{f}}{K_{Hg}} \) term is larger for the acetate treated reactors. This implies that the consortium in the acetic acid reactor had an increased incidence of methylating mercury relative to the SRR. This argument is further validated by the 16S rRNA data that showed a difference in consortia between the acetate reactor and both the lactate and control reactors. The data collected from the sediment slurry reactor coincides with data collected from pure culture that suggests DSBM and DBACTER have increased incidence of methylating mercury when normalized to the SRR.

**Electron Equivalence Associated with Methylmercury Production**

Once methylmercury has been biologically synthesized by SRB, the methylmercury compound represents material synthesized in the cellular matrix and released to environment. Now, the respiration and the methylation reaction represent a net movement of reducing power to the extra-cellular environment. It should be noted that the valence on the mercury atom in methylmercury remains +2. Thus, electrons were not
added to the mercury atom of methylmercury. However, the electrons associated with the carbon group represent a release of available energy to the environment by the sulfate-reducing bacteria. In this regard, $S^-$ and $CH_3Hg^+$ represent the net movement of electrons into the aquatic environment. Equation 7.1 and 7.2 define the electron equivalence associated with methylmercury and sulfide, respectively.

$$CH_3Hg^+ + 2H_2O \rightarrow CO_2 + Hg^{++} + 7H^+ + 8e^-$$ \hspace{1cm} \text{Equation 7.1}

$$S^- + 4H_2O \rightarrow SO_4^{2-} + 8H^+ + 8e^-$$ \hspace{1cm} \text{Equation 7.2}

Both reactions demonstrate a net flux of 8 electrons out of the cell. Although the incidence term ($f^*$) was defined as methylmercury produced per sulfate reduced, it remains appropriate to express the $f$ term in equivalents since the 8 electrons associated with methylmercury represented a source of electrons available to the cell for growth. However, the SRB group sacrifices these electrons to produce methylmercury. Since both reactions (Equations 7.1 and 7.2) are numerically equal in terms of equivalents, the $f^*$ term can be expressed on a molar basis as well. It should be noted that the use of equivalents to relate sulfate reduction to mercury methylation is a novel concept. However, continued research in this field will further demonstrate a need to relate the methylation of mercury to SRB cellular processes (i.e. respiration). The use of equivalents provides the means to "couple" such processes and quantitatively relate the two distinct biochemical pathways.
Environmental Engineering Significance

The data presented in this thesis has documented mercury availability, sulfate-reduction rates, and speciation of sulfate-reducing bacteria as key factors that determine the methylation of mercury. Since methylmercury is the species of mercury most readily available through the consumption of fish and shellfish, limiting exposures is a primary concern from a human health perspective. The research presented in this thesis represents an initial quantitative approach to assessing mercury methylation processes in natural and contaminated systems. From an engineering standpoint, understanding how mercury availability, sulfate reduction rates, and SRB microbial speciation affect the methylation of mercury \textit{in situ} represents the next critical step. Decreasing the bacterial synthesis of methylmercury represents the “first line-of-defense” in decreasing potential exposure. It is possible that knowledge of the above mentioned parameters could lead to a decrease in the production of methylmercury by sulfate-reducing bacteria. For example, data presented in this thesis suggests that sequestering of soluble mercury could reduce SRB mediated methylmercury synthesis. The addition of metal chelators or subsurface injections of sulfide (S$^2-$) to mercury contaminated sediments could reduce the soluble mercury “available” for methylation. In addition, data presented in this thesis also indicated that the sulfate-reduction rate (SRR) was coupled to the mercury methylation rate process. Thus, it is conceivable that decreasing the \textit{in situ} SRR of sediment could also decrease the potential to methylate mercury. Decreasing the SRR of a natural system would be extremely complex. This is coupled with the fact that sulfate reduction produces sulfide which serves as a natural sink for mercury, and HgS is thought to be “unavailable”
for methylation. However, the synergistic treatment of sediments with a SRR inhibitor and a mercury chelator (injection of sulfide) may provide a means to sequester soluble mercury while suppressing SRB respiration. Finally, this document has provided evidence that the SRB microbial consortia present in sediments has the potential to influence methylmercury production. From an engineering perspective, it should be possible to “select” for SRB phylogenetic groups that have a lower potential to methylate mercury relative to SRR. This technology could only exist provided a wealth of knowledge was available regarding factors that determine cell growth, cell-cell interactions, respiration, and cell death. It is this researchers opinion that the manipulation of in situ microbial consortia represents the most intriguing yet complex “preventive” strategy for methylmercury production. However, conceptually it does represent another method for the reduction in methylmercury production.

In conclusion, the research presented in this thesis identifies factors that affect methylmercury production in marine sediment systems. Results presented here should provide groundwork for additional assessments of contaminated sediments that ultimately lead to engineering applications that prevent human exposure to methylmercury.

**Recommendations for Future Studies**

In conclusion, this thesis has focused on understanding of the fundamental processes or factors that influence the biological production of methylmercury. The data reported in this thesis clearly demonstrated that mercury availability significantly influences the biological synthesis of methylmercury. The experiments illustrated that amount of
methylmercury generated in sediment slurry systems was dependant on the amount of inorganic mercury added to the system. Evidence also indicates that a correlation between the slurry water total mercury concentration and MMR exists. Future research should be focused on elucidating the saturation kinetics that occur with respect to MMR and higher slurry water mercury concentrations. All data obtained in sediment slurry systems excluding the inorganic mercury applied data, was conducted at slurry water mercury concentrations that resulted in pseudo-first order kinetics with respect to MMR. Data obtained at slurry water mercury concentrations that exceeded the concentrations presented in this thesis would provide evidence for Michaelis-Menton type kinetics as it relates to MMR and slurry water mercury concentrations. In regard to mercury that is available for methylation, future research should be directed at understanding the speciation of mercury in pore waters of contaminated sediments. It can be assumed that the total amount of mercury that is present in contaminated sediments is not available for methylation due to complex interactions with humics or other materials. This suggests that the amount of mercury available for methylation is contingent upon the matrix and composition of the sediment. Hollowly and Melanie (1995) published more than 250 examples of monomer mercury (II) compounds that can exist. The extent to which these compounds can be internalized and biologically methylated is unknown. Thus, an understanding of mercury speciation in contaminated sediments would contribute greatly in understanding what forms are available for methylation.

This thesis also demonstrated a unique coupling between the SRR and the production of methylmercury. Although the biological production of methylmercury has been linked
to SRB, there is a very limited amount of information regarding the cellular processes that allow methylmercury production to occur. Choi et al. (1994) isolated a corrinoid protein from DSV that generated methylmercury from Hg$^{2+}$ and CH$_3$-tetrahydrofolate. However, the magnitude of corrinoid protein production is unknown in SRB that are fermenting substrates is unknown. To understand the cellular processes of methylmercury production and how it is linked to SRB respiration, future test should focus on comparative studies which investigate changes in cellular processes between SRB that are fermenting or using sulfate as a terminal electron acceptor. It is possible that SRB, which are not utilizing sulfate as a terminal electron acceptor, do not induce specific proteins or enzymes that are linked to methylmercury synthesis. In addition, the literature suggests that the SRB group of bacteria is unique in its ability to biologically produce methylmercury. Future biochemical or molecular studies should focus on identifying unique physiological properties of SRB that allow for the production of methylmercury.

The pure culture studies represented in this thesis suggest that there are differences between phylogenetic groups in the “net” incidence of methylmercury production relative to SRR. This study suggests that DSBM and DBACTER have the greatest rate of methylmercury formation relative to the SRR. However, it should be noted that in this study only one species of bacteria was used to represent an entire phylogenetic group. Thus, an argument can be made that the species selected was not representative of the entire population that comprises the phylogenetic group. Future research should focus on testing other species of SRB that constitute a phylogenetic group. By testing other SRB,
a better understanding of the inherent differences between phylogenetic groups can be established.

This thesis also demonstrated that organic acid amendments to sediment slurries for prolonged periods result in changes in the bacterial consortiums that are present. Results published in this thesis suggest that the changes that occurred due to organic acid addition resulted in consortiums that had different “net” incidences of methylmercury production relative to SRR. Future research should be directed at generating other consortiums through organic acid additions over prolonged periods of time. Once the consortium has been established, the incidence of methylmercury production can be determined and assessed with respect to SRR, cell populations, and 16S rRNA profiles. The literature suggests that other organic acids such as formic, fumaric, and malic acids can be utilized by some phylogenetic groups but not by others (Rooney-Varga et al., 1999). Using these organic acids would allow for specific consortiums to develop. Thus SRB bacterial consortiums, unique to the Georgia coast could be assessed for their potential to methylate mercury.
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Tanaka, K., Min, K.S., Onasaka, S., Fukuhara, C., and Ueda, M. (1985) The origin of

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pp.203-216.


APPENDIX A

Table A.1 Media for Sulfate-Reducing Bacteria Cultures

<table>
<thead>
<tr>
<th>Component I</th>
<th>Component VII</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>Sodium Citrate 1.5 g</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>dH₂O 50 mL</td>
</tr>
<tr>
<td>NaSO₄</td>
<td></td>
</tr>
<tr>
<td>dH₂O</td>
<td></td>
</tr>
<tr>
<td>1.0 g</td>
<td>0.1 g</td>
</tr>
<tr>
<td>4.0 g</td>
<td>550 mL</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Component II</th>
<th>Individual Additions*:</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgCl₂</td>
<td>Reazurin (100 µL of 0.3% w/v)</td>
</tr>
<tr>
<td>dH₂O</td>
<td>Trace Element Solution (1.0 mL)</td>
</tr>
<tr>
<td>0.4 g</td>
<td>Selenite/Tungstate Solution (1.0 mL)</td>
</tr>
<tr>
<td>50 mL</td>
<td>Vitamin Solution (1.0 mL)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Component III</th>
<th>Na₂S (7.5mL of 0.20 M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₄Cl</td>
<td>Thiosulfate Solution (3.0 mL of 1.0M)</td>
</tr>
<tr>
<td>dH₂O</td>
<td>Reductant Solution (1 mL)</td>
</tr>
<tr>
<td>0.25 g</td>
<td></td>
</tr>
<tr>
<td>50 mL</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Component IV</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Lactate</td>
<td>1.5 mL of 6.8M solution</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>0.1 g</td>
</tr>
<tr>
<td>dH₂O</td>
<td>200 mL</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Component V</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NaAcetate</td>
<td></td>
</tr>
<tr>
<td>dH₂O</td>
<td>50 mL</td>
</tr>
<tr>
<td>5.0 mL of 2.0M solution</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Component VI</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NaHCO₃</td>
<td></td>
</tr>
<tr>
<td>dH₂O</td>
<td>50 mL</td>
</tr>
<tr>
<td>2.5 g</td>
<td></td>
</tr>
</tbody>
</table>

*Individual Additions (total Volume indicated) were added to the total volume of pure culture media (1 L).
Table A.2  Molar Quantities of Components in Pure Culture Media

I. Inorganics

A. Cations

<table>
<thead>
<tr>
<th>Ion</th>
<th>Molarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺</td>
<td>145.6 mM</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>0.86 mM</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>4.2 mM</td>
</tr>
<tr>
<td>NH₄-N</td>
<td>4.6 mM</td>
</tr>
</tbody>
</table>

B. Anions

<table>
<thead>
<tr>
<th>Ion</th>
<th>Molarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl⁻</td>
<td>30.4 mM</td>
</tr>
<tr>
<td>SO₄²⁻</td>
<td>33.6 mM</td>
</tr>
<tr>
<td>S²⁻</td>
<td>1.5 mM</td>
</tr>
<tr>
<td>S₂O₅²⁻</td>
<td>3.3 mM</td>
</tr>
<tr>
<td>HCO₃⁻</td>
<td>29.7 mM</td>
</tr>
</tbody>
</table>

II. Organics

<table>
<thead>
<tr>
<th>Ion</th>
<th>Molarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate⁻</td>
<td>10.0 mM</td>
</tr>
<tr>
<td>Acetate⁻</td>
<td>10.0 mM</td>
</tr>
<tr>
<td>Citrate⁻</td>
<td>7.1 mM</td>
</tr>
</tbody>
</table>
APPENDIX B

Chromium reduction procedure

Reactive Cr$^{2+}$ solution was produced from the more stable Cr$^{3+}$ by percolating 1M CrCl$_3$·6H$_2$O in 0.5N HCl through reduction with "mossy zinc" granules (Aldrich chemicals). Reduction was verified by a color change from dark green (Cr$^{3+}$) to bright blue (Cr$^{2+}$). Prior to Cr reduction, the Zn granules were washed with 1N HCl and then twice with dH$_2$O. Mossy zinc is then placed in a bottle and the Cr$^{2+}$ solution poured into the bottle. Complete reduction of Cr$^{3+}$ was confirmed 30 minutes later with change in color.
APPENDIX C

Reagents for Organic Acid Detection

Reagent I.

Heptafluorobutyric acid (HFBA)

Aliquot 5.2 g of 0.077 M stock solution into 1 L double distilled H₂O.
(Degas 10 min with stirring under vacuum, < 15 mm Hg)
Flow rate for HFBA solution (1.0 ml/min)

Reagent II.

Tetrabutylammonium hydroxide (TBAOH)
5 mN TBAOH (10 ml of 2.06 M in 4 L dH₂O)
Flow rate for TBAOH solution (5 ml/min)
APPENDIX D

Artificial sea water (ASW)

<table>
<thead>
<tr>
<th>Component</th>
<th>Component Name</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>NaCl</td>
<td>22.2g</td>
</tr>
<tr>
<td></td>
<td>MgSO₄</td>
<td>9.8g</td>
</tr>
<tr>
<td></td>
<td>dH₂O</td>
<td>900ml</td>
</tr>
<tr>
<td>II</td>
<td>KCl</td>
<td>5.5g</td>
</tr>
<tr>
<td></td>
<td>NaHCO₃</td>
<td>1.6g</td>
</tr>
<tr>
<td></td>
<td>dH₂O</td>
<td>100ml</td>
</tr>
<tr>
<td>III</td>
<td>KBr</td>
<td>0.8g</td>
</tr>
<tr>
<td></td>
<td>SrCl₂</td>
<td>0.34g</td>
</tr>
<tr>
<td></td>
<td>dH₂O</td>
<td>100ml</td>
</tr>
<tr>
<td>IV</td>
<td>Na silicate</td>
<td>0.4g</td>
</tr>
<tr>
<td></td>
<td>dH₂O</td>
<td>100ml</td>
</tr>
<tr>
<td>V</td>
<td>NaF</td>
<td>0.24g</td>
</tr>
<tr>
<td></td>
<td>dH₂O</td>
<td>100ml</td>
</tr>
<tr>
<td>VI</td>
<td>NH₄NO₃</td>
<td>0.16g</td>
</tr>
<tr>
<td></td>
<td>dH₂O</td>
<td>100ml</td>
</tr>
<tr>
<td>VII</td>
<td>Na₂HPO₄</td>
<td>0.8g</td>
</tr>
<tr>
<td></td>
<td>dH₂O</td>
<td>100ml</td>
</tr>
<tr>
<td>VIII</td>
<td>CaCl₂•2H₂O</td>
<td>23.8g</td>
</tr>
<tr>
<td></td>
<td>dH₂O</td>
<td>100ml</td>
</tr>
<tr>
<td>IX</td>
<td>EDTA</td>
<td>300mg</td>
</tr>
<tr>
<td></td>
<td>FeCl₃•6H₂O</td>
<td>38.4mg</td>
</tr>
<tr>
<td></td>
<td>MnCl₂•7H₂O</td>
<td>43.2mg</td>
</tr>
<tr>
<td></td>
<td>CoCl₂•6H₂O</td>
<td>0.2mg</td>
</tr>
<tr>
<td></td>
<td>ZnCl</td>
<td>3.15mg</td>
</tr>
<tr>
<td></td>
<td>CuCl₂</td>
<td>0.025mg</td>
</tr>
<tr>
<td></td>
<td>H₃BO₃</td>
<td>34.2mg</td>
</tr>
<tr>
<td></td>
<td>dH₂O</td>
<td>100ml</td>
</tr>
</tbody>
</table>
For Component IX  Make sure each metal is dissolved prior to the next metal addition.

To Component I add Component II (10ml), Component III (10ml), Component IV (1.0ml), Component V (1.0ml), Component VI (1.0ml), Component VII (1.0ml), Component VIII (10ml), and Component IX (10ml).
APPENDIX E

Components Necessary for the Identification of 16S rRNA

Hybridization solution

The following solution components were combined and utilized for both pre-
hybridization and hybridization of oligonucleotides:

6x SSPE

0.1%(w/v) SDS

1x Denhardt solution

Wash solution

Wash solution consisted of the following:

6X SSPE

0.1%(w/v) SDS

Stock solutions

20x SSPE - 27.6g NaH₂PO₄•H₂O; 7.4g EDTA in 800mL of dH₂O. Adjust pH to 7.4 and
dilute to 1000mL.

50x Denhardt's - 5g Ficol (Type 400), 5g PVPP, 5g BSA. Adjust volume to 500mL with
dH₂O.
VITA

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