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Mercury Speciation, Retention, and Abundance of Genes Involved with Mercury Methylation in Fertilized Salt Marsh Sediments

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MERCURY SPECIATION, RETENTION AND ABUNDANCE OF GENES INVLOVED WITH MERCURY METHYLATION IN FERTILIZED SALT MARSH SEDIMENTS

by

Caroline Collins

A thesis submitted for partial fulfillment of the requirements for the degree of

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ABSTRACT

Mercury (Hg) is a global pollutant which exists in both aquatic and terrestrial systems in three main forms including elemental Hg, ionic mercury, and methyl mercury, (MeHg), and Hg cycling plays an important role in ecosystems. Great Sippewisset Marsh, (GSM) in Falmouth MA has been chronically treated with fertilizer that contains heavy metals such as Hg in varying concentrations since the early 1970s and provides for an excellent location to study Hg contamination. The overall goal of this study is to analyze the key geochemical and microbial conditions that lead to MeHg production in the presence of the applied fertilizer. Sediment cores were taken in three experimental plots, control, high, and extra high fertilization. Mercury concentrations were determined using cold vapor atomic fluorescence spectroscopy and direct mercury analysis. Total sulfur was determined and quantitative polymerase chain reaction (qPCR) data was generated for the bacterial 16S gene, and dissimilatory sulfite reductase (dsrA), as most sulfate reducing bacteria methylate Hg. Total Hg was higher in the fertilized plots, while total sulfur decreased with increasing fertilization. The average concentration of MeHg was highest in the high fertilized plot while the ratio of MeHg to total Hg was highest in the control plot despite the fact that this plot is only receiving mercury through atmospheric deposition. Bacterial 16S copy number per ng DNA extracted was lowest in the control and fluctuated with depth for control, low, and high fertilized conditions. Higher nitrate concentrations within fertilized treatment plots may play a role in decreasing MeHg production, but more research is needed to determine why MeHg concentrations are decreasing with increased fertilization and to develop a greater sense of the response of the bacterial communities to the applied fertilizer.
INTRODUCTION

Mercury as a Global Pollutant

Mercury (Hg) is a global pollutant that has the capacity to travel over long distances and its concentration has increased in the biosphere post the Industrial Revolution (Parks et al. 2013). Hg is a toxic trace metal that is present in different forms in both aquatic and terrestrial systems. The three main forms include elemental mercury, which is highly volatile, ionic mercury, and MeHg. This toxic metal enters ecosystems in its inorganic form, where it can be methylated due to microbial activity forming MeHg, a potent neurotoxin that poses significant risks during prenatal neurological development and when consumed in high quantities during adulthood (Myers et. al 1998; Parks et al. 2013). Exposure to this neurotoxin occurs primarily through the consumption of seafood, as MeHg accumulates in organismal tissue, and biomagnifies up food chains (Figure 1). For there to be substantial biomagnification within a particular trophic structure, MeHg must be taken up efficiently by the bacteria at the bottom of the food chain, retained in these organisms, and passed on to their predators (Morel et al. 1998).

Salt marshes are critical areas of Hg methylation due to geochemical cycling and certain physical parameters within marsh sediments (Chen et al. 2009). It is important to understand MeHg production and cycling within coastal sediments such as salt marshes as the MeHg produced in these environments has the capacity to be transported to deeper waters where it can accumulate in the tissue of top predatory marine organisms, such as swordfish and tuna, popular consumer fish.

Salt Marsh Dynamics

Salt marshes represent one of the world’s major ecosystems and provide many benefits to human society. Due to the structure of salt marshes, they have the capacity to trap and collect both nutrients and pollutants from the open ocean while serving as a buffer between the land and sea, protecting coastal areas during intense storms (Booman et. al 1999). The vertical portion of the front of the salt marsh can dissipate the high wave energy experienced during storm events (Booman et. al 1999). Also, these habitats provide spawning sites and nursery areas for many fish species of fishery value (Booman et. al1999). The high production of vascular plant detritus and protection from predation offered by shallow, spatially complex habitats benefit fish during early developmental stages (Boesch et. al 1984). Salt marshes additionally provide feeding,
roosting, and nesting areas for a wide range of bird species (Booman et. al 1999). These systems have been viewed as dynamic and capable of recovering from human and natural disturbances quickly, however with increasing global temperature and threats of substantial sea level rise, the health of these systems is at stake, and the notion of marshes as being resilient to ecological and anthropogenic disturbances is declining (Gedan et al. 2011).

Human disturbances represent a major source of influence on the overall health of salt marshes. Human impacts are concentrated in coastal ecosystems due to proximity of near shore human activities, significant number of human settlements along coastlines, and subsequent terrestrial inputs to adjacent watersheds (Gedan et al. 2011). Eutrophication (nutrient loading) is correlated with population density and is driven by sewage inputs into groundwater, which enters marsh systems most often in the form of runoff water (Gedan et al. 2011). Anthropogenic nutrient loading can dramatically alter community structure and function of marshes (Gedan et al. 2011). Nitrogen is a principal constituent of sewage wastes and often limits plant growth in coastal ecosystems. Nitrogen serves a critical role in determining the structure and function of salt marsh ecosystems as standing crops of salt marsh plants respond to nitrogen enrichment (Valiela et. al 1975). Nitrogen enters systems through rainfall, groundwater flow and fixation, while nitrogen losses are mainly through tidal exchanges and denitrification. These exchanges influence the overall above and belowground vegetation (Valiela and Teal, 1979). It is important to develop an understanding of how nitrogen can influence vegetation structure in a salt marsh system as salt marsh plants can act as natural sinks for trace metals such as Hg (Válega et. al 2008). The extent and uptake of how metals are distributed within plants can have important effects on the residence time of metals in plants in wetlands, as well as their release to the adjacent environment (Válega et. al 2008).

**Nutrient Loading and Methyl Mercury Production**

There is a pressing need to develop a greater understanding of how long-term nutrient loading will affect overall wetland ecological processes as nitrogen supplies have more than doubled over the last century since the invention of artificial fertilizer (Bertness et al. 2001). Great Sippewisset Marsh, (GSM) has been chronically treated with fertilizer since 1971 and serves as an experimental research location to observe how nutrient loading impacts New England salt marshes (Vailela et al. 1975). The applied fertilizer is in the form of sewage sludge from Milwaukee (Milorganite) (Vailela et al. 1975). Sewage sludge rather than effluent was used due to the ease of application, fewer health concerns, and also because the sludge contains heavy
metals, chlorinated hydrocarbons, and other pollutants, which would be of focus throughout the long-term ecological study (Vailela et al. 1975). The fertilizer is sprayed on the marsh in various loadings (see methods for details) twice a month during the growing season (April-October) (Vailela et al. 1975). The fertilizer has the capacity to influence geochemical processes within this habitat thus impacting the overall health and functioning of GSM. Turner et. al 2009 demonstrated that nutrient loading works to reduce organic matter belowground, that may result in significant loss in marsh elevation (Turner et. al 2009). Many studies have utilized GSM as a location to study the impacts of long-term nutrient loading.

The sewage sludge fertilizer contains heavy metals such as cadmium, copper, zinc, and Hg. All of these metals have the potential to negatively influence human health, but Hg is of particular significance because of its tendency to biomagnify up food chains and accumulate in organismal tissue. A recent study by Driscoll et al. (2012) has suggested that increased nutrient loading has the potential to impact Hg transformation by decreasing the bioavailability of mercury thus decreasing biomagnification in food webs. Nutrient loading can decrease bioavailability as ionic mercury has a high affinity for organic content and certain chemical intermediates such as sulfides. The Driscoll hypothesis assumes that changes in sulfide concentrations as a result of eutrophication can influence the production of MeHg, as most sulfate reducing bacteria (SRB) methyleate mercury in anoxic sediments (Shao 2012). In pure cultures in the absence of sulfate, no MeHg was generated from available inorganic Hg (Shao et al. 2012). Thus, it can be assumed that Hg methylation is linked to sulfate reduction catalyzed by SRB (Shao et al. 2012). Studies of how Hg cycling will be altered due to chronic nutrient loading in GSM are important because monomethyl mercury (MeHg) concentrations are a significant public health concern.

Biogeochemical cycling plays a role in determining Hg toxicity as Hg is primarily deposited in the environment as inorganic Hg, a form that is readily available to be methylated (Chadhain et al. 2006). Studies have focused on examining prominent zones of Hg methylation and have determined that these zones are mostly anoxic sediments inhabited by sulfate and iron reducing bacteria (Parks et al. 2013). Hg methylation by SRB is of significance because studies have found that mercury methylation occurs most readily in zones of microbial sulfate reduction (King 2011). Salt marsh sediments provide the geochemical components required for methylation. These sediments are anoxic near the surface and decomposition of organic matter occurs primarily through reduction pathways, such as the reduction of sulfate to hydrogen sulfide (Hines et al. 1989).
Bacterial Transformation of Mercury

Biogeochemical cycling plays a role in determining Hg toxicity as Hg is primarily deposited in the environment as inorganic Hg, a form that is readily available to be methylated (Chadhain et al. 2006). Many studies have focused on examining prominent zones of Hg methylation and have determined that these zones are mostly anoxic sediments inhabited by sulfate and iron reducing bacteria (Parks et al. 2013). Hg methylation by SRB is of significant focus as studies have found that mercury methylation occurs most readily in zones of microbial sulfate reduction (King 2011). Salt marsh sediments provide the geochemical components required for methylation. These sediments are anoxic near the surface and decomposition of organic matter occurs primarily through reduction pathways, such as the reduction of sulfate to hydrogen sulfide (Hines et al. 1989).

There are different experimental techniques used to measure MeHg concentration and cycling within ecosystems (Parks et al. 2013) including the use of stable isotopes. However methods are limited (Parks et al. 2013). A recent study (Parks et al. 2013 demonstrated that two genes are required for bacterial MeHg production. These genes include hgcA, which encodes for a corrinoid protein and hgcB, which encodes a ferredoxin protein). The study demonstrated that the C terminus portion of the hgcA protein is membrane embedded, potentially aiding in the export of MeHg across the cell wall (Parks et al. 2013). The adaptive significance of why iron and sulfur reducing bacteria methylate mercury is not clearly understood. It has been suggested that methylation may serve as a detoxification process. Studies have also shown that there is a tight coupling between Hg methylation and MeHg export from the cell; demonstrating that inorganic Hg intake and methylation may work to avoid build up of toxic Hg concentrations within the cell (Schaefer et al. 2011).

In addition to bacterial processes involved with methylation of inorganic Hg, there are also processes responsible for the reduction of inorganic Hg. The reduction of inorganic mercury (Hg\textsuperscript{II}) leads to the formation of elemental Hg (Hg\textsuperscript{0}), a less toxic, more volatile form of Hg. Microbes reduce ionic mercury to elemental mercury by the mercuric reductase, MerA enzyme (Chadhain et al. 2006). These microbes have been deemed mercury resistant because they convert ionic mercury to elemental mercury, a form that is less likely to be methylated. Bacterial mercury resistance is mediated by the mer operons, a set of genes that encode for enzymes that facilitate the uptake and transport of ionic mercury and organo-mercury compounds to the cytosol for degradation and reduction to elemental mercury (Johs et al. 2004; Chadhain et. al 2006). Transcriptional regulation of the mer operon is controlled by merR, a novel metal-
responsive regulator, that represses mer gene activation in the absence of inorganic mercury and
activates transcription of mer genes in the presence of inorganic mercury (Nazaret et al. 1994; Barkay et al. 2003). In vitro and in vivo experiments have shown that nanomolar concentrations of inorganic mercury are sufficient to induce substantial amounts of mer expression (Nazaret et al. 1994).

MerA has the potential to impact microbial methylators by competing for ionic mercury (Chadhain et al. 2006). The activity of MerA in anaerobic sediments has not been studied in great detail, despite the fact that in these environments the function of this gene can have the most significant impact, as MeHg is mostly produced in anoxic conditions (Chadhain et al. 2006). The conversion of ionic mercury to its elemental form has positive impacts on the environment, as elemental mercury is less likely to be methylated (Barkay 2006). Subsequently, microorganisms have the potential to provide a major role in ecosystem decontamination (Nascimento et al. 2003). The bacterial resistance systems for mercurials and organomercurials are of interest since they are a natural strategy for the detoxification of mercury-contaminated environments (Nascimento et al. 2003).

Mercury Methylation and Sulfate Reduction

To develop an understanding of how nutrient loading has impacted Hg cycling in GSM, the relationship between sulfate reduction by SRB and MeHg concentrations were examined in order to see if nutrient loading works to reduce MeHg production and if this is coupled with a decrease in SRB abundance. This question stems from a study by Driscoll et al. (2012) that examines the relationship between nutrient loading and Hg concentrations. Driscoll hypothesizes that eutrophication leads to increased organic matter and subsequent sulfate reduction, resulting in increased production of sulfide concentrations within sediments (Driscoll et al. 2012). Increased sulfide concentrations change the speciation of ionic mercury and may decrease its bioavailability to microorganisms. MeHg is produced predominantly by SRB, which reduce sulfate and release sulfide as a byproduct of their metabolism (Benoit et al. 1999). Substantial sulfide concentrations can inhibit MeHg production as ionic mercury can bind to sulfide forming mercury (II) sulfide, which thus impacts microbial uptake and methylation (Benoit et al. 1999).
Dissimilatory Sulfite Reductase

It is easy to detect the presence of gaseous hydrogen sulfide produced by SRB as it produces a rotten egg smell that is often associated with salt marshes. Identification and characterization of SRB has been facilitated by the use of dissimilatory sulfite reductase (DSR) as a target for phylogenetic analysis (Cook et. al 2008). The enzyme DSR catalyzes the six-electron reduction of sulfite to sulfide, which is a fundamental step in sulfate respiration and is therefore a highly conserved gene among sulfate reducers (Cook et. al 2008; Wagner et. al 1998). The ability to use sulfate as a terminal electron acceptor is characteristic of several bacterial lineages and one genus of Archaea (Wagner et. al 1998). The DSR gene consists of alpha and beta subunits, that most likely arose from an early gene duplication event (Oliveira et. al 2008). DSR belongs to a family of proteins that also includes assimilatory sulfite reductase and nitrite reductases (Oliveira et. al 2008). The genes encoding the two subunits are found adjacent to each other in their respective genomes and most likely arose from an early gene duplication event (Klein et al. 2001; Oliveira et. Al 2008).
**Goals of this Study**

The major goal of this study is to determine the effects of nutrient loading via fertilizer application on Hg transformation and concentrations in GSM. By correlating MeHg concentrations to \( dsrA \) copy number, a gene that codes for subunit A of DSR and total sulfur data we can see if there is a relationship among these three parameters. Additionally, we can determine how MeHg concentrations, \( dsrA \) copy number, and total sulfur data relate to varying levels of fertilizer concentration within each treatment plot. This study will provide greater insight into the Driscoll et. al (2012) hypothesis, that suggests that increased nutrient loading has the potential to impact Hg transformation by decreasing MeHg bioavailability. By comparing the gene abundances to the geochemical data patterns within each experimental plot we can develop a greater sense of how Hg cycling has changed as a result of fertilization and the overall response of GSM to long-term nutrient loading.
METHODS

This study includes methods and data presented in this project, but also data from a larger study that was conducted from summer 2013-present in the lab of Dr. Carl Lamborg (University of California, Santa Cruz). This larger data set is represented as unpublished data and is not included in this version of the thesis project. Data from Dr. Lamborg’s lab contains sediment cores that were sectioned in 3 cm intervals to a depth of 24 cm. These cores were from both high and low marsh settings, while the data I present is only from high marsh settings. I only present data on high marsh settings because of time and financial constraints and due to the fact that theoretically there should be greater Hg retention as there is less tidal fluctuation and inundation in this setting. The geochemical measurements in this larger data set include Total Hg, MeHg, percent MeHg, Loss of Ignition, and Total Hg inventories.

Site Description: This study was performed in Great Sippewisset Salt Marsh (41° 35’ 3.1” N, 70° 38’ 17.0” W), which is located along the lower eastern Buzzards Bay shoreline in Falmouth, Massachusetts (Figure 2). Circular plots (10 m diameter) have been fertilized every two weeks during the growing season since the early 1970s (Valiela et al. 1975). The plots are set up in duplicate with three different levels of NPK fertilizer. Control, no fertilizer, low: 0.85, high: 2.52, and extra high 7.56 g N m⁻² wk⁻¹ (Kinney and Valiela, 2013). The fertilizer is a commercially available sewage sludge fertilizer (10% N, 6% P, 4% K by weight) (Valiela et. al 1975). Two additional plots were not fertilized and served as controls. These plots were only receiving heavy metals through atmospheric deposition. The plots are located within an area of 10 hectares (Valiela et al. 1975).

Sample Collection: Sediment cores (1 inch in diameter) were collected from the control (C) (plot 3 and 7), extra high (XF) (plot 8 and 6), and high fertilization (HF) (plot 9 and 2) for a total of 12 cores. The recovered cores were wrapped in tin foil and transported back to the lab. The wet cores were weighed and sectioned at 5 cm resolution to a depth of 10 cm. The geochemical data presented in this study is derived from cores that were collected in fall of 2013, the molecular results pertain to cores that were collected in summer of 2013 by members of the Lamborg lab.
Molecular Methods:

**DNA Extraction and Quantification:** After sectioning, cores used for genetic material were stored at -80°C. Subsamples (approximately 10 g) were taken from each of the depth intervals from all of the 10 cores for a total of 80 environmental DNA extracts. DNA extraction was performed using PowerMax DNA Isolation Kit (MoBio, Carlsbad CA) according to manufacture’s guidelines. Extracted DNA was further concentrated using 30 kDa centrifugal filters (Amicon Ultra, Billerica MA) following manufacture’s guidelines. Extracted DNA was quantified spectrophotometrically pre and post centrifugal concentrating using Nanodrop (Thermo Scientific, Waltham MA). Only concentrated DNA was used for later downstream applications. Post extraction, DNA was stored at -80°C.

**Conventional PCR:** PCR reactions on dsrAB were performed using BioRad MyCycler Thermal Cycler (BioRad, Hercules CA) to assess the amplificability of DNA template and to determine optimal DNA template dilutions. Commercial enzyme AmpliTaq Gold 360 polymerase with AmpliTaq Gold 360 GC enhancer (Life Technologies, Grand Island NY) was used to generate PCR amplicons. dsrAB was amplified under the following conditions: 3 minutes of initial denaturation at 95°C, followed by 35 cycles of 95°C for 30 seconds, 54°C for 30 seconds, 72°C for 2 minutes, and a final extension for 10 minutes at 72°C. Observation of clean and distinct bands were used to ensure specificity and quality prior to later quantification measurements (qPCR). All PCR reactions were assessed by gel electrophoresis (1% agarose) with 5 microliters of GelRed and recorded digitally with Quantity One (Chemi Doc gel imaging system, Bio-Rad Laboratories, Hercules CA).

**DSR Clone Libraries and Development of qPCR standards:** Dissimilatory sulfite reductase (dsrAB) was amplified using dsrF (0.5 µM) and dsrR (Table 1) (0.5 µM) with 1:10 and 1:100 dilution factors of environmental samples. A positive control aliquot of dsrAB genomic DNA was used to ensure success of the PCR reaction. The 20 microliter reaction contained 4 µL Ultra-Pure DEPC-treated water, 10 µL 2X buffer, 1 µL (0.5 µM), dsrF1 µL, (0.5 µM) 1 µL dsrR, AmpliTaq Gold 360 enhancer with 3 µL template DNA. The average of the DNA template concentrations were 75 ng/µL. Table 1 contains primer sequences. dsrAB was
amplified under the following conditions: 3 minutes of initial denaturation at 95 °C, followed by 35 cycles of 95 °C for 30 seconds, 56 °C for 30 seconds, 72 °C for 2 minutes, and a final extension for 10 minutes at 72 °C. Four 1900 bp fragments were excised out of the agarose gel and amplicons were purified using QIAquick Gel Extraction Kit (Qiagen, Venlo Limburg). These four bands were selected over the others in the reaction as they were clean, bright, and distinct. The brightest, most distinct band was cloned using StrataClone PCR cloning kit (StratClone PCR Cloning Kit) as per manufacturer’s instructions along with a portion of the dsr positive control aliquot. This step was performed to see whether the positive control or the environmental sample produced more accurate qPCR standards. Two DNA libraries of 96 clones each were generated; one for the dsr positive control and one for environmental sample. Six clones were prepared for sequencing from each for a total of 12 overall samples to be sequenced. Two and a half microliters of EXOSAP-IT (USB) and 7.5 microliters of PCR product were added to each well and the reactions incubated for 15 minutes at 37°C, followed by 15 minutes at 80°C. The 12 clones were sequenced at Harvard Medical School (Dept. BCMP, C1-214, 240 Longwood Avenue, Boston MA 02115). Plasmid DNA was purified using QIAprep Spin Miniprep Kit (Qiagen, Venlo Limburg) following manufacturer’s guidelines. Purified plasmid DNA concentrations were measured spectrophotometrically using NanodropLITE (Thermo Scientific, Waltham MA).

Sequence Analyses: Sequences were analyzed using FinchTV software and compared to known DSR sequences from the NCBI database using BLAST. Percent identity compared to known sequences was recorded for all of the samples.

Quantitative PCR (qPCR): qPCR reactions were performed using the commercial enzyme IQ SYBR, SYBR Green Supermix (BioRad, Hercules, CA) according to manufacturer’s guidelines for both dsrA and bacterial 16S rRNA. For dsrA, the 20 microliter reaction contained 7 µL Ultra-Pure DEPC-treated water, 10 µL 2X buffer, 1 µL (0.5 µM) dsr1F+, 1 µL (0.5 µM) dsrR-R (Table 1), with 1 µL template DNA diluted 1:100. Reagents were adjusted for the number of reactions. Samples and associated assay standards were run using Bio-Rad CFX Manager 3.0 (BioRad, Hercules, CA). For bacterial 16S, the 20 microliter reaction contained 7 µL Ultra-Pure DEPC-treated water, 10 µL 2X buffer, 1 µL (0.5 µM) GM3, 1 µL (0.5 µM) EUB338R, with 1 µL template DNA diluted 1:10. Reagents were adjusted for the number of reactions. Samples and associated assay standards were run using Bio-Rad CFX Manager 3.0
qPCR standards were created using the plasmid DNA generated from environmental sample as described above. Ten fold dilution series of the standard was used to create a dilution curve for the qPCR standards for both $dsrA$ and bacterial 16S. Standard quantities used in the generation of the curve ranged from 10 ng/µL to 1 pg/µL for $dsrA$. A bacterial 16S standard was provided. Standard quantities used in the generation of the curve ranged from 1000 pg/µL to 1 pg/µL for bacterial 16S.

**Chemical Methods:**

Post sectioning, six cores were lyophilized for five days and ground into a fine powder for methyl mercury (MeHg), total mercury and total sulfur analysis. All glassware and tools used were acid washed with hydrochloric acid. MeHg was measured using cold vapor atomic fluorescence spectroscopy (CVAFS). Approximately 500 mg of ground sample was distilled in a Teflon vial to which 15 mL of distilled water, 0.4 mL 9 M sulfuric acid, and 0.4 mL copper sulfate was added. The samples were heated at 250°C and sparged with nitrogen gas until 60-80% of the sample solution was collected in a similar Teflon vial held in an ice bath. Two samples were spiked with 1 mL MeHg for comparison to unspiked sediments. After distillation, 25 µL ascorbic acid and 275 µL citrate buffer was added. pH was adjusted to 5.0 for each sample with potassium hydroxide. Sodium tetraethylborate was added to the final solution and allowed to react for 20 minutes prior to CVAFS detection. Five MeHg standards were used, standard 1: deionized water blank, standard 2: 0.1 mL 268 femtomoles MeHg/mL, standard 3: 0.5 mL 268 femtomoles MeHg/mL, standard 4: 0.5 mL 4.53 picomoles MeHg/mL, and standard 5: 1.0 mL 4.53 picomoles MeHg/mL.

Total mercury was measured using oxygen combustion-gold amalgamation using direct mercury analyzer (DMA-80). 500 mg of dried sample was combusted and the concentrations were recorded. Known concentrations of Mess 3 (0.091 PPM Hg) and Pac 2 (3.04 PPM Hg) were used as standards. Total sulfur was measured using a LECO SC-32 sulfur analyzer against commercial coal standards (Giblin, 1990). Approximately 50 mg of sample was combusted.
Milorganite fertilizer contains heavy metals such as cadmium, copper, nickel, zinc, and mercury. It is difficult to measure Hg concentrations (due to lack of recording, highly volatile) so zinc can be used to model Hg loading as zinc and Hg have similar physical properties (Figure A1).

The amount of total mercury loaded in 1970 through fertilization was compared to the measured total mercury concentrations determined in this study to calculate total mercury percent retention. A model was designed that considered accumulation and application rates and additional atmospheric deposition based Kinney and Valiela (2013). Bulk density for sectioned cores was used to determine total mercury concentration per sectioned slice and this was compared to predicted loaded total mercury. It was assumed that all mercury loaded in 1970 remained in sediments of each of the studied plots. From loaded total mercury concentrations and measured total mercury concentrations, the percent retention for each plot was calculated.

Statistical Methods: ANOVA Post Hoc Tests (Tukey HSD) and Univariate Analysis of Variance were performed in SPSS Statistics (IBM Corporation, Armonk New York). Data from duplicate cores in each plot were averaged prior to statistical analyses.

RESULTS
Chemical Results:

For all of the physical parameters analyzed, the two depth intervals of 0-5cm and 5-10cm were not statistically different from one another. Since there was not a lot of variability due to increasing depth, the two depth intervals were averaged for the replicate plots.

There was not substantial variation in loss of ignition among the treatment plots (Figure 1A). The loss of ignition versus depth profile for the control plot looked similar to the treatment plot receiving the greatest concentration of fertilizer. Additionally, loss of ignition was relatively uniform with increasing depth across all experimental conditions.

Total Hg was greater in the fertilized plots compared to the control plot. The averaged control cores contained 0.00030 umol/g compared to 0.0043 umol/g for the averaged cores in the extra high plot (Figure 3). The control plot contained significantly less total Hg compared to the extra high fertilized plot (P=0.021). The high fertilized plot also contained significantly more total Hg than control plot (P=0.031). The high fertilized and extra high fertilized plot were not statistically different from one another.

Average percent sulfur was highest in the control plot compared to the two fertilized plots. The control plot contained more than twice as much sulfur as in the extra high plots. The extra high fertilization plot contained the least amount of total sulfur with an average of 0.77 % (Figure 4). None of the treatment conditions were statistically different from one another.

Despite the fact that the high fertilization and extra fertilization plots have relatively similar values for total mercury, there was a greater amount of average methyl mercury in the high fertilization plot compared to the extra high treatment (Figure 5). The high fertilization plot contained significantly more MeHg compared to the control (P=0.013) and the extra high fertilization plot (P=0.038).

Although total MeHg concentrations were lowest in the control plots, the relative percent of MeHg to total Hg was highest in control plots. Percent MeHg is defined as the portion of MeHg compared to the concentration of total Hg. The control plot contained significantly more percent MeHg compared to the extra high fertilization plot (P=0.005). The high fertilization plot contained significantly more percent MeHg compared to the extra high as well (P=0.019). The
control plot however, does not contain significantly more percent MeHg compared to the high fertilized treatment (Figure 6).

The control plot had a substantially higher average percent total Hg retention (189 %) compared to less than 1 % for both the fertilized plots. The high and extra high fertilization plot had similar percent retentions, however the high plot was slightly higher with 0.26 % compared to 0.13 % (Figure 7).

**Molecular Results:**

The amount of DNA extracted in micrograms per gram of sediment from the environmental samples decreased with increasing depth among all treatment plots. Generally across all treatment plots there was an increase in DNA concentration from 5-10 cm. The extra high fertilization plot contained the greatest fluctuation in DNA concentration (Figure 8).

Furthermore, bacterial 16S copy number per nanogram of DNA was lowest in the control plot with a starting copy number value (0-3cm) around 1.00E+05. The high fertilization condition had the highest starting copy number value starting which was slightly greater than 1.00E+06. For all three treatment plots, copy number per nanogram of DNA fluctuated with depth. All plots had an increase in 16S abundance from the 5-10 cm depth interval followed by variation in abundance with increasing depth. Bacterial 16S copy number per nanogram of DNA for cores collected in the extra high fertilized treatments are not present due to technical problems in some of the qPCR runs leading to a lack of copy number data. Unfortunately, there was not enough time to re-run these samples. Due to time constraints, I also just looked at copy number data from high marsh cores as the geochemical data I generated comes from high marsh settings.

dsrA copy number per nanogram DNA extracted is not included in this study as the values were orders of magnitude higher than expected values. Careful consideration was given to experimental technique, standard readings, machine technicalities, and copy number calculation errors, and we are fairly confident that this increase in dsrA abundance is due to an issue with the primer set that was used. More time is needed to re-run experiments with new primers or potentially design new primers as sequence data indicates that there is substantial variability within the samples. Despite the probable primer issue, it was interesting to see that the trend in
dsrA copy number per nanogram DNA extracted was similar to the trends seen in bacterial 16s abundance. There was the highest starting concentration in the high fertilized treatment plot, however all plots were fairly similar in concentration to one another and fluctuated with depth.
Many studies have examined the impact of long-term fertilization on the aboveground health of GSM, but less research has been conducted on the effect of nutrient loading on MeHg production and the influence on the microbial communities involved with Hg methylation. Increased fertilizer application has the potential to influence many microbial and geochemical processes (Morris et. al 1991). Similar to trends seen in total Hg concentrations in this study, Bretler and Teal (1980) have shown that increased fertilization leads to greater amounts of total Hg within the treatment plots. Therefore, it is important to develop an understanding of how GSM will respond to nutrient loading and how this will impact bacterial populations.

**Impact of Fertilizer on Geochemical Processes**

It was expected that the applied fertilizer would lead to varying geochemical conditions within the different treatment plots. The addition of sewage sludge fertilizer has increased the amount of total Hg in the experimental conditions. In 1970, the initial sewage sludge contained an increased amount of heavy metals compared to the more recently applied fertilizer, as industrial inputs were connected to the sewer system (Hamlett, 1986). The control plots are only receiving Hg through atmospheric deposition and the values for total Hg are much lower than the fertilized plots. The fertilizer is adding much larger amounts of heavy metals than what would be received through atmospheric deposition.

Since the plots are receiving increasing intervals of fertilizer, one would expect to see an increase in MeHg across the treatment plots, however a different pattern was observed. The high fertilized plot had the highest average MeHg concentration, rather than the extra high fertilized condition. Also, it was interesting to see that in the control, where there was the greatest amount of total sulfur, the ratio of MeHg to total Hg was also the highest. This indicates that the efficiency of Hg methylation is high considering that the control plot is only receiving Hg through atmospheric deposition. Total sulfur was highest in the control condition suggesting that SRB abundance might be influencing MeHg concentrations as studies have shown that SRB methylate mercury (King 2011).

Nitrogen concentrations within the fertilized plots may also be playing a role in the decreased ratio of MeHg to total Hg in the fertilized plots, especially in the extra high-fertilized condition. Increased applied nitrogen through fertilization may result in an increase in nitrogen reducing bacteria and various intermediates associated with nitrification and dentrification resulting in changes in geochemical conditions and microbial processes. Hamersley et. al (2005) have reported that extra high fertilized treatment plots are receiving up to 11.2 mol organic N m-2 yr-1 for the past 16 to 23 years. Increased nitrate concentrations could signal a disruption of
nitrifiers, but also a change in the total composition of the microbial community (Philips et. al 2002). Additionally, sulfate reducing and nitrogen reducing bacteria may potentially be competing with one another more intensely for organic carbon in the extra high fertilized plot, where there is a greater concentration of nitrogen and therefore, theoretically a greater abundance of nitrogen reducing bacteria. This competition might result in a decrease in MeHg production by the sulfate reducers. Nitrogen and sulfate reducers may be in close proximity with one another as sulfate reduction and components of nitrogen reduction occur in anaerobic conditions. In oxygen-depleted soils, sediments, and water, nitrite is reduced to gaseous products, which are released into the atmosphere (Cole and Brown 1979). However, Bowen et. al (2011) and Peng et. al (2012) have demonstrated minimal responses of nitrifiers and denitrifiers to long-term fertilization, which suggests that factors other than nutrients may be playing a role in the abundance of these microorganisms within the treated plots. More research is needed to look at other parameters, such as redox conditions, which may be limiting the abundance of certain types of bacteria (Peng et. al 2013).

Ionic mercury partitioning is an explanation for why there might be less methyl mercury in the extra high fertilization plot, however loss of ignition tests show that organic content was fairly uniform across treatment plots. An increase in fertilization leads to greater vegetation and greater vegetation can lead to more organic content within sediments. Ionic mercury has the potential to bind with organic content and once it is bound, it less bioavailable to be methylated (Hammerschmidt et al. 2006; Breteler et. al 1980). Changes to nitrogen inputs into ecosystems has the potential to decrease bioavailability and mercury trophic transfer as ionic mercury has an affinity for organic content (Driscoll et al. 2012).

**Total Hg Percent Retention**

From total mercury inventories, I determined percent retention for each of the plots. Percent retention is the amount of Hg withheld in the sediment. Dr. Carl Lamborg and I designed a model based on data presented in Kinney and Valiela (2013) that enabled me to compare the amount of mercury loaded in 1970, assuming all mercury had remained in the sediment, to the measured total Hg concentrations I determined in this study. The control plot had a much larger percent retention compared to the two fertilized plots. The decrease in percent retention in the high and extra high plot is due to the loading of fertilizer. Since the two fertilized plots are receiving fertilizer they might reach a maximum saturation point. More research is needed to determine the saturation kinetics of salt marshes and their ability to retain heavy metals over a
long period of time. Additionally, the input of nutrients to these plots changes the sediment composition potentially influencing percent Hg retention as well (Gordon, 1980).

**Microbial Responses to Fertilizer**

Along with looking at the geochemical components of this study, it is also important to develop an understanding of the microbial responses to the applied fertilizer as microbial activity plays a critical role in production and cycling of MeHg (Gilmour et. al 1991). Bacterial 16s abundance data demonstrates that fertilizer does not lead to substantial differences in the overall abundance of bacteria within the treatment plots. In the high fertilization plot there was a greater abundance of bacterial 16s at the surface (0-3cm) compared to the two other treatment plots, but all the conditions were fairly similar in their abundances and fluctuated with depth. More research is needed to look at the abundance of certain types of bacteria in order to develop an understanding of how fertilizer potentially impacts the abundance of bacteria involved with methylation. For example, analyzing $dsrA$ copy number could be used as a proxy for observing the abundance of SRB. The issue with the primer set used in the qPCR runs for $dsrA$ prevents a more detailed and complete understanding of SRB abundance within the treatment plots, but when looking at the general trends for $dsrA$ generated in this experiment, it is evident that the abundance is fairly similar to the bacterial 16s further demonstrating that fertilizer does not substantially alter the abundance of bacterial communities. However, since total sulfur and the percent MeHg compared to total Hg was highest in the control plot, I would potentially expect to see a greater abundance of $dsrA$ copy number in the control condition. I was unable to successfully amplify $HgcA$, a gene required for MeHg production (Parks et. al 2013), however since sulfate reduction and mercury methylation are linked, and the rate of methylation in the control plot seems to be high considering this plot is only receiving Hg through atmospheric deposition, I would expect to see this gene in the control plot as well.

**MerA Bioremediation**

Barkey et al. (2003) studied mer genes and their importance in bioremediation. Understanding $merA$ activity could provide insight for decreasing the amount of toxic MeHg in ecosystems as this gene converts ionic mercury to elemental mercury (Chadhain et. al 2006). Additionally, Barkey et. al (2003) demonstrated that systems that are perpetually exposed to heavy metals select for microorganisms that are heavy metal resistant. Heavy metal ions such as Hg(II) are of nonbiological origin and are toxic to cells at high concentrations (Nies 2000). Microorganisms with $merA$ activity are deemed heavy metal resistant as they convert ionic
mercury to elemental mercury, an extremely volatile and less toxic form of Hg. More research is needed to develop an understanding of where merA is more abundant within the treatment plots and how the presence of this gene is related to MeHg production. It would be interesting to see if the extra high treatment plot, the condition that is receiving the greatest amount of nutrient loading and thus heavy metals, contained a greater abundance of the merA gene, and if the potential greater abundance provides an explanation for the decrease in MeHg in the extra high treatment plot as, merA codes for the reduction to elemental Hg, a form that is not readily available to be methylated (Chadhain et. al 2006).

Conclusions

Overall, nutrient loading alters geochemical conditions in GSM by increasing the amount of total Hg within treatment plots. Interesting trends were observed that showed an increase in MeHg concentration in the high fertilized plot and a greater ratio of MeHg to total Hg in the control plot. The microbial data generated in this study demonstrates that fertilizer does not substantially impact bacterial abundance. The increase in MeHg in the high fertilized plot might be explained by a increase in nitrogen levels in the extra high treatment resulting in competition between nitrogen reducing bacteria and SRB for organic carbon. The greater ratio of MeHg to total Hg in the control plot may be explained by the fact that there was more total sulfur within the control plot, however total sulfur concentrations are not statistically different from one another across the treatment plots so more research is needed to develop a greater understanding of why the methylation rate is high in the control condition.
**Future Research**

There are many more experimental steps that could be taken in order to determine how fertilizer has changed the overall health of GSM and also to provide greater insight into the Driscoll hypothesis which states that increased fertilization leads to a decrease in MeHg production. More time is needed to amplify and analyze *dsrA, merA*, and *HgcA* in order to compare the presence of the three genes of interest to one another in the treatment plots. Additionally, nitrate concentrations and the concentration of the nitrite reductase genes (*nirS*) could be used to develop a greater understanding into whether nitrate levels and subsequent concentrations of nitrogen reducing bacteria are competing with sulfate reducers for organic carbon, resulting in less MeHg production within the extra high fertilization plot (Braker et. al 1998). Also, more research on the saturation kinetics of salt marsh sediments is needed in order to understand the sediments capacity to retain heavy metals over time and how this retention impacts the overall geochemical and microbial processes of the marsh.
REFERENCES


Ecol Prog Ser;434:229-37.


Johs 1994. The dynamic of mercuric ion reductase MerA. Environmental Sciences Division.


FIGURES
Figure 1: Biomagnification of methylmercury in aquatic ecosystems.
Figure 2: Map of study plots in Great Sippewisset Marsh, Falmouth MA.
Figure 3: Average concentration of total mercury in micromoles per gram across study plots.
Figure 4: Average percent total sulfur across study plots.
Figure 5: Average concentration of methyl mercury in picomoles per gram across study plots.
Figure 6: Average percent of methyl mercury compared to total mercury across study plots.
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Figure 8: DNA extracted from sediment cores in micrograms per gram versus depth for all experimental conditions.
Figure 9: Bacterial 16S copy number per nanogram of DNA extracted versus depth in control, low, and high fertilized plots from sediment cores collected in high marsh settings.
Figure 1: Biomagnification of methylmercury in aquatic ecosystems
(Environmental Health)

Mercury can be converted to MMHg, a potent neurotoxin through biogeochemical processes. MMHg enters the body and is absorbed much faster than inorganic Hg. MMHg has the capacity to be stored in tissues and thus can biomagnify up food chains.
Figure 2: Map of study plots in Great Sippewisset Marsh, Falmouth MA (Peng 2013). Study plot contains four treatment plots, control, low fertilization, high fertilization, and extra high fertilization located in both low and high marsh settings. Control plot is not receiving fertilizer; it only receives heavy metals through atmospheric deposition.
Figure 3: Average concentration of total mercury in micromoles per gram across study plots. Total mercury measurements include elemental, mono-methyl mercury, and dimethyl-mercury. Sediment core slices of 0-5cm and 5-10cm were averaged for the replicate plots. Greater variability in the fertilized plots is most likely due to the fact that these samples contain a greater amount of fibrous material leading to variability in sample preparation. These samples are harder to homogenize, which may result in variability in their Total Hg concentrations, as well as other physical parameters measured in this experiment.
Figure 4: Average percent total sulfur across study plots. Percent total sulfur values are composed of sulfate, sulfides, and sulfites within the plots. Sediment core slices of 0-5cm and 5-10cm were averaged for the replicate plots.
Figure 5: Average concentration of methyl mercury in picomoles per gram across study plots. MeHg concentrations were determined separately from total Hg concentrations through distillation and detection by CV AFS. Sediment core slices of 0-5cm and 5-10cm were averaged for the replicate plots. As stated above, variability may be related to the fibrous content of the high fertilized plot.
Figure 6: Average percent of methyl mercury compared to total mercury across study plots. Concentrations of methyl mercury detected by CV AFS were compared to total mercury concentrations determined by direct mercury analysis to calculate the percentage of methyl mercury. Sediment core slices of 0-5cm and 5-10cm were averaged for the replicate plots.
Figure 7: Average percent retention of total mercury in sediment across study plots. Amount of total mercury loaded in 1970 through fertilization was compared to the measured total mercury concentrations determined in this study based off work of Kinney and Valiela 2013. Percent retention values were averaged for the two sectioned depths for the replicate plots.
Figure 8: Concentration of DNA extracted in micrograms per gram of sediment versus depth for all treatment plots. For all treatment plots, extracted DNA concentration decreased with increasing depth.
Figure 9: Bacterial 16S copy number per nanogram of DNA extracted (log scale) versus depth in control, low, and high fertilized plots from sediment cores collected in high marsh settings. Bacterial 16S copy number per nanogram of DNA extracted was determined for cores collected in high marsh settings. Bacterial 16S abundance is smallest in the control and abundance of the gene fluctuates with depth across all experimental conditions.
### Table 1: DSR genes targeted in PCR assays and their associated PCR Primers.

<table>
<thead>
<tr>
<th>Primer Target</th>
<th>Primer Name</th>
<th>Primer Sequence (5’-3’)</th>
<th>Expected Amplicon Length</th>
<th>Primer Design Source</th>
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<td>dsrAB</td>
<td>DSR1F</td>
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<td>DSR-R</td>
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