Microbial Genomic Responses to Chronic Additions of Sewage Sludge Containing Mercury Residues to Experimental Plots in the Great Sippewissett Salt Marsh

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Abstract:

Mercury cycling plays an important role in ecosystems. Ionic mercury has the capacity to be methylated by sulfate reducing microorganisms, forming a potent neurotoxin that can be bioaccumulated up the food chain. Recent studies have shown that there are other microorganisms that transform ionic mercury as well. These microbes are deemed mercury resistant as they convert ionic mercury into its elemental form, a form that cannot be methylated. There are two genes responsible for these two activities, HgcA, which codes for mercury methylation and mercuric ion reductase (merA), which codes for conversion to elemental mercury. The goal of this study was to determine which pathway; either methylation or reduction to elemental mercury was more prevalent in Great Sippewisset Marsh. Great Sippewisset Marsh has been receiving sewage sludge fertilizer that contains heavy metals in varying concentrations since 1970. 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 presence/absence of dissimilatory sulfite reductase (dsr), HgcA and merA was observed by polymerase chain reactions. Total sulfur was higher in less fertilized plots and mercury methylation was coupled with sulfate reduction, as most sulfate reducing bacteria are mercury methylators. Future research is needed to amplify HgcA and merA in order to observe their relative abundance. Observing their relative abundance will provide insight into which pathway is more dominate than the other in Great Sippewisset Marsh.

Key Words and Phrases: Great Sippewisset Salt Marsh, fertilization, methyl mercury, sulfate reduction, merA, HgcA
Introduction:

There are different forms of mercury present in ecosystems. Biogeochemical cycling plays a role in determining mercury toxicity as mercury is primarily deposited in the environment as ionic mercury (Chadhain et al. 2006). Mercury enters the environment from both natural and anthropogenic sources. Coal mining is major release of mercury into the environment and recent estimates indicate that anthropogenic sources of mercury have exceeded natural inputs since the onset of the industrial period (Schutser, 2002).

When mercury is methylated it turns into a potent neurotoxin, (MeHg) and this has the potential to bioaccumulate up the food chain. Consumption of contaminated fish and shellfish is the most common mode of contamination for humans. Microorganisms have the capacity to transform ionic mercury. Most methylating microbes are sulfate-reducing bacteria (Chadhain et al. 2006). Many studies have found that mercury methylation occurs most readily in zones of microbial sulfate reduction (King 2011). These sulfate-reducing bacteria take in ionic mercury and methylate it, forming the most toxic form of mercury. The gene HgcA is required for mercury methylation, but the reason for why sulfate-reducing bacteria methylate the mercury is unclear.

Other microbes reduce ionic mercury to elemental mercury by the mercuric reductase enzyme (Chadhain et al. 2006). These microbes have been deemed mercury resistant because they convert ionic mercury to elemental mercury, a form that cannot be methylated. Bacterial mercury resistance is mediated by the mer operons, a set of genes that encode for enzymes that facilitate the uptake of ionic mercury and reduction to elemental mercury (Johs et al.). The activity of merA has the potential to impact microbial methylators by competing for ionic mercury (Chadhain et al. 2006). The conversion of ionic mercury to its elemental form has positive impacts on the environment as elemental mercury less likely to be methylated (Barkay 2006).

Great Sippewisset Marsh is an excellent location to study mercury methylation as it has been receiving sewage sludge fertilizer containing heavy metals since 1970. The focus of
my study is to see which pathway, either the conversion of ionic mercury to methyl mercury or the reduction of ionic mercury to elemental mercury is more dominate and what physical parameters support either pathway in Great Sippewisset Marsh.

Methods:

**Site Description:** This study was performed in Great Sippewisset Salt Marsh, which is located along the lower eastern Buzzards Bay shoreline in Falmouth, Massachusetts (Map 1). Circular plots (10 m diameter) have been fertilized every two weeks during the growing season since the early 1970s (Peng 2013). The plots are set up in replicate with three different levels of NPK fertilizer. Control, no fertilizer, high (HF; 25 g/m2/week), and extra high (XF; 75 g/m2/week). The fertilizer is a commercially available sewage sludge fertilizer (10% N, 6% P, 4% K by weight) (Peng 2013). 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 (Peng 2013).

**Sample Collection:** Duplicate sediment cores (1 inch diameter) were taken in extra high fertilization, high fertilization, and control plots on November 11, 2013. An additional set of duplicate cores were taken from replicate extra high fertilization, high fertilization, and control plots for a total of twelve sediment cores. All cores were taken from high marsh habitats. Cores were stored for twenty-four hours at four degrees Celsius and all cores were sectioned November 12, 2013 into two parts (0-5 cm) and (5-10cm). Six cores were used for genetic material, while the remaining six were used for mercury and total sulfur analysis.

**Molecular Methods:** After sectioning, cores used for genetic material were stored at
negative 80 ° C. Subsamples (0.7 g) were taken from each of the six cores for both sectioned depths, (0-5cm and 5-10cm). DNA was extracted using UltraClean Soil DNA extraction kit (MO BIO). DNA concentrations were measured spectrophotometrically using Nanodrop (Thermo Scientific). Based on Nanodrop concentrations in nanograms per microliter, samples with greater than 5 ng/µL were used for analysis. Post extraction, DNA was stored at negative 20 degree Celsius.

Bacterial 16S ribosomal RNA was amplified with EuB27F (10 µM) and U1492R (10 µM) with three dilution factors, undiluted, 1:10 microliter dilution, and 1:100 microliter dilution on nine samples. The 50 µL reaction contained 27.8 µL Ultra-Pure DEPC-treated water, 10 µL 5X buffer, 5 µL EuB27F (10 µM), 5 µL U1492R (10 µM), 1 µL dNTP mix and 0.2 µL GoTaq with 1 µL template DNA. Reaction was adjusted for the number of samples. Bacterial 16S ribosomal RNA genes were amplified under the following conditions: 3 minutes of initial denaturation at 94° C, followed by 35 cycles of 94° C for 40 seconds, 55 ° C for 1:30 seconds, 72 ° C for 2 minutes, and a final extension for 10 minutes at 72 ° C. A positive control from Eel Pond, Woods Hole, MA was used.

Dissimilatory sulfite reductase (dsr) was amplified using dsr1F (20 µM) and dsr4R (20 µM) with 1:10 and 1:100 dilution factors. The 50 µL reaction contained 26.8 µL Ultra-Pure DEPC-treated water, 10 µL 5X buffer, 5 µL (20 µM) dsr1F, 5 µL (20 µM) dsr4R, 1 µL dNTP mix and 0.2 µL GoTaq with 2 µL template DNA. Reaction was adjusted for the number of samples. Dsr was amplified under the following conditions: 3 minutes of initial denaturation at 95 ° C, followed by 35 cycles of 94° C for 35 seconds, 54 ° C for 1 minute, 72 ° C for 2 minutes, and a final extension for 10 minutes at 72 ° C. A positive control aliquot was received.

Mercuric ion reductase (merA) was amplified using A1snF (10 µM) and AsnR (10 µM) with a 1:10 dilution factor. The 50 µL reaction contained 34.0 µL Ultra-Pure DEPC-treated water, 10 µL 5X buffer, 1 A1snF (10 µM), 1µL µL AsnR (10 µM), 0.5 µL dNTP mix and 0.5 µL GoTaq with 3 µL template DNA. Mercuric ion reductase was amplified under the following condition: 2 minutes of initial denaturation at 95 ° C, followed by 45 cycles of 95° C for 30 seconds, 54 ° C for 30 seconds, 72 ° C for 1.5 minutes, and a final
extension for 5 minutes at 72 °C. A positive control aliquot (merAPJZ full) from Woods Hole Oceanographic Institution was used.

HgcA was amplified using aliquots of forward (10 µM) and reverse primer (10 µM) as the primers had not been published yet and the primer sequences could not be released. The 50 µL reaction contained 33.5 µL Ultra-Pure DEPC-treated water, 10 µL 5X buffer, 1 µL HgcAF (10 µM), 1 µL HgcAR (10 µM), 1.0 µL dNTP mix and 0.5 µL GoTaq with 2 µL template DNA. HgcA was amplified under the following conditions: 2 minutes of initial denaturation at 95 °C, followed by 35 cycles of 95° C for 10 seconds, 60° C for 30 seconds, 72 °C for 45 seconds, and a final extension for 7 minutes at 72 °C. Geobacter sulfurreducens PCA genomic DNA was used as a positive control.

All PCR reactions were assed by gel electrophoresis on (1% agarose) with 1.75 µL ethidium bromide.

**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 femtmoles MeHg/mL, standard 3: 0.5 mL 268 femtmoles 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 a 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.

The amount of total mercury loaded in 1970 through fertilization was compared to the measured total mercury concentrations determined in this study in order to calculate total mercury percent retention. A model was designed that considered accumulation and application rates and additional atmospheric deposition based off of the work of 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.

Total sulfur was measured using a LECO SC-32 sulfur analyzer against commercial coal standards (Giblin, 1990). Approximately 50 mg of sample was combusted.

Results:

**Chemical Results:** Total mercury was greater in the fertilized plots compared to the control plot. The averaged control cores contained 0.00030 umol/g compared to 0.0040 umol/g for the averaged high fertilization cores and 0.0043 umol/g for the averaged cores in the extra high plot (Fig 1).

Average percent sulfur was highest in the control plot compared to the two fertilized plots. The control plot contained 1.83 % sulfur, while the high fertilization plot contained 1.54 % sulfur. The extra high fertilization plot contained the least amount of total sulfur with an average of 0.77 % (Fig 2).

Despite the fact that the high fertilization and extra fertilization plots have similar values for total mercury, there was a greater amount of average methyl mercury in the high fertilization plot compared to the extra high. The high fertilization plot contained 5.11
pmol/g MeHg while the extra high plot contained 1.12 pmol/g MeHg. The control plot contained the least amount of MeHg with 0.39 pmol/g (Fig 3).

The percent of MeHg was highest in the control plot with 0.16 % MeHg. The high fertilization plot consisted of 0.13 % MeHg, while the extra high plot had the smallest percentage, 0.037 % MeHg (Fig. 4). The percent MeHg follows the same trend as total sulfur (Fig 2). In the control, where there was the greatest amount of total sulfur, the percentage of MeHg was the largest (Fig 4).

The control plot had a substantially higher average percent total mercury 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 % (Fig. 5).

**Molecular Results:** Bacterial 16S ribosomal RNA amplified under 1:10 and 1:100 dilution factors for all samples (Fig. 6).

Dsr amplified in all of the study plots with the greatest relative abundance in the control and high fertilization plot. The extra high fertilization plot had the smallest, faintest band (Fig. 7). The amplification of dsr in these plots reflects the trends for total sulfur (Fig. 2).

Several dilution factors were tried for merA, but only the positive control amplified. Amplification of the positive control was unsuccessful for HgcA. More time is needed to eliminate the possibility of inhibitors in samples and to fine tune thermal profiles.

**Discussion:**

Many studies have examined the impact of long-term fertilization on the aboveground health of Great Sippewisset Marsh, but less research has been conducted on the effect on microbial communities. Barkey et al. 2003 studied mer genes and their importance in bioremediation. Understanding merA activity could provide insight for decreasing the
amount of toxic methyl mercury in ecosystems as this gene converts ionic mercury to elemental mercury.

The addition of sewage sludge fertilizer has increased the amount of mercury in the high and extra high fertilization plots. 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 mercury through atmospheric deposition and the values for total mercury are much lower than the fertilized plots (Fig. 1). The fertilizer is adding much larger amounts of heavy metals than what would be received through atmospheric deposition.

The addition of fertilizer is not only adding heavy metals to Great Sippewisset Marsh, but also changing other physical parameters such as total sulfur and overall vegetation structure. Total sulfur decreases with increasing fertilization (Fig 2). Fertilization leads to increased vegetation, resulting in greater evapotranspiration leading to more oxygenated sediments (Turner et al 2009). More oxygenated sediments leads to less sulfate reduction as sulfate reducers live in anaerobic environments. Therefore, the extra high fertilization plot contained less sulfate reducing bacteria and less total sulfur. Gel electrophoresis indicated less sulfate reduction in the extra high fertilization plot as the samples from this plot had the thinnest, least bright bands (Fig 7).

Differences in the population of sulfate reducing bacteria have the potential to alter the concentration of methyl mercury as most sulfate reducing bacteria methylate mercury. Unlike inorganic forms of mercury, which originate from atmospheric deposition, methyl mercury is generated in the environment predominately by anaerobic, sulfate reducing bacteria (Parks et al. 2013). The high fertilization plot had the highest amount of methyl mercury and an intermediate amount of total sulfur compared to the control and extra high plot (Fig 2) (Fig 3). Mercury methylation rates are sensitive to concentrations of sulfur as both low and high concentrations decrease rates of methylation (Shao et al. 2013). Gel electrophoresis indicated that there was a greater relative abundance of sulfate
reducing bacteria in the high fertilization plot compared to the extra high (Fig. 7). It was interesting to see that in the control where there was the greatest amount of total sulfur, the ratio of methyl mercury to total mercury was also the highest (Fig 2) (Fig 4). This indicates that the efficiency of mercury methylation is high considering that the control plot is only receiving mercury through atmospheric deposition. I was unable to successfully amplify \textit{HgcA}, however since sulfate reduction and mercury methylation are linked and concentrations of methyl mercury were detected, I would expect to see this gene in my samples. More time is needed to adjust the PCR cocktail and thermal profile.

Ionic mercury partitioning is another explanation for why there is less methyl mercury in the extra high fertilization plot (Breteler et al. 1980). An increase in fertilization leads to greater vegetation and greater vegetation leads 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.). Sulfate reducing bacteria methylate mercury within sediment pore water. Changes to nitrogen inputs into ecosystems have the potential to decrease bioavailability and mercury trophic transfer as ionic mercury has an affinity for organic content (Driscoll et al. 2012).

From total mercury inventories, I determined percent retention for each of the plots. Percent retention is the amount of mercury withheld in the sediment. Dr. Carl Lamborg and I designed a model based off of 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 mercury concentrations I determined in this study. The control plot had a much larger percent retention compared to the two fertilized plots (Fig. 5). 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 mercury retention as well (Gordon, 1980).
I looked closely at the percent retention between the two fertilized plots in order to see if the extra high plot had a decreased percent retention. Barkey et al 2003 demonstrated that systems that are perpetually exposed to heavy metals select for microorganisms that are metal resistant. The slight decrease in percent retention in the extra high plot compared to the high could also be explained through microbial mercury resistance. The conversion of ionic mercury to elemental mercury, through merA activity is another potential explanation for the decreased retention in the extra high plot as elemental mercury is the most volatile type of mercury.

In order to determine which pathway, either formation of methyl mercury, or the formation of elemental mercury is more dominant than the other; I examined the presence/absence of the two genes responsible for these activities, merA and HgcA. For merA, only my positive control amplified, which demonstrated that there might be inhibitors in the sample preventing the amplification of merA or that there is potentially no merA in my samples. HgcA is a newly discovered gene and more time is needed to fine-tune my methods for amplification. Future research is needed to rid samples of inhibitors in order to accurately determine the presence/absence of each gene and to quantify each gene to observe the abundance.

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Figures and Map:

Map 1: Map of study plots in Great Sippewisset Marsh, Falmouth MA
Figure 1: Average concentration of total mercury in micromoles per gram across study plots.
Figure 2: Average percent total sulfur across study plots.
Figure 3: Average concentration of methyl mercury in picomoles per gram across study plots.
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Figure 5: Average percent retention of total mercury in sediment across study plots.
Figure 6: Gel electrophoresis showing the amplification of bacterial 16S ribosomal RNA.
Figure 7: Gel electrophoresis showing the amplification of dissimilatory sulfite reductase (dsr) with 1:10 and 1:100 dilution factors.
Figure 8: Gel electrophoresis showing the amplification of only the positive control for mercuric ion reductase, (merA) with a 1:10 dilution factor.
Figure 9: Gel electrophoresis showing no amplification of the positive control for HgcA.
Map 1: 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. I took sediment cores from control, high fertilization, and extra high fertilization. Control plot is not receiving fertilizer; it only receives heavy metals through atmospheric deposition.
Chemical Figures:

Figure 1: 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.
Figure 2: 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 3: 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 CVAFS. Sediment core slices of 0-5cm and 5-10cm were averaged for the replicate plots.
Figure 4: Average percent of methyl mercury compared to total mercury across study plots. Concentrations of methyl mercury detected by CVAFS 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 5: 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 6: Gel electrophoresis showing the amplification of bacterial 16S ribosomal RNA. Target gene size: 1465 base pair fragment of bacterial 16S ribosomal RNA, 1 KB DNA ladder was used.
Figure 7: Gel electrophoresis showing the amplification of dissimilatory sulfite reductase (dsr) with 1:10 and 1:100 dilution factors. Target gene size: 1900 base pair fragment, 1 KB DNA base pair ladder was used. Lane 1: 1 KB ladder, Lane 2: HF sample 1:10 dilution, Lane 3: HF sample 1:100 dilution, Lane 4: HF 1:10 dilution, Lane 5: HF sample 1:100 dilution, Lane 6: Control 1:10 dilution, Lane 7: Control 1:100 dilution, Lane 8: Control 1:10 dilution, Lane 9: Control 1:100 dilution, Lane 10: XF 1:10 dilution, Lane 11: XF 1:100, Lane 12: XF 1:10 dilution, Lane 13: XF 1:100, Lane 14: 1 KB ladder, Lane 15: HF 1:10, Lane 16: HF 1:100, Lane 17: HF 1:10, Lane 18: HF 1:100, Lane 19: XF 1:10, Lane 20: XF 1:100, Lane 21: positive control, Lane 22: negative control.
Figure 8: Gel electrophoresis showing the amplification of only the positive control for mercuric ion reductase, (merA) with a 1:10 dilution factor. Target gene size: 285 base pair fragment, 100 base pair ladder was used.
Figure 9: Gel electrophoresis showing no amplification of the positive control for HgcA. Target gene size: 650 base pair fragment, 100 base pair ladder was used.