Appendix 3B-1: Evaluation of Factors Influencing Methylmercury Accumulation in South Florida Marshes

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Introduction

The biogeochemistry of mercury (Hg) methylation and demethylation in the Everglades environment is multifarious. It is believed that the primary source of methylmercury (MeHg) in most wetlands is *in situ* methylation of inorganic Hg by sulfate-reducing bacteria (SRB) (Gilmour et al. 1998; Gilmour 2011). Because this process is biotically mediated, a suite of interactive environmental conditions and biogeochemical processes are highly influential on Hg methylation and demethylation (Hsu-Kim et al. 2013). Net Hg methylation is significantly affected by pH and oxidation-reduction potential (Hg methylation is an anaerobic process), as well as concentrations of sulfate (SO_4^{2-}), sulfide, and dissolved organic carbon (DOC) in surface and pore water (Aiken et al. 2011). Other biogeochemical factors are important as well, such as the composition of the methylating/demethylating microbial community and the availability of suitable electron donors (Hsu-Kim et al. 2013). Nutrients, such as phosphorus (P) concentrations, may also limit methylation by bacteria. Any condition or process that impacts these variables has the potential to influence net Hg methylation.

Total Hg in the minnow *Gambusia holbrooki* has been monitored in many studies as a metric of potential Hg bioaccumulation, since this species is prey for a number of predatory fish and birds. During the past two decades, tissue Hg levels in *Gambusia* and other fish, including largemouth bass, have declined in the Everglades marshes (Scheidt and Kalla 2007). Most investigators agree that this decline was likely due to the measured reduction in atmospheric deposition of inorganic Hg in the 1990s (e.g. Atkeson and Axelrad 2004; Axelrad et al. 2005; Pollman 2012), although some have argued that declining levels of sulfate in some parts of the Everglades have contributed to reductions in fish tissue Hg (Axelrad et al. 2005). Temporal changes in *Gambusia* tissue Hg levels, along with changes in sulfate as characterized by USEPA in their REMAP monitoring efforts, are depicted for the major Everglades compartments in **Figure 1**.

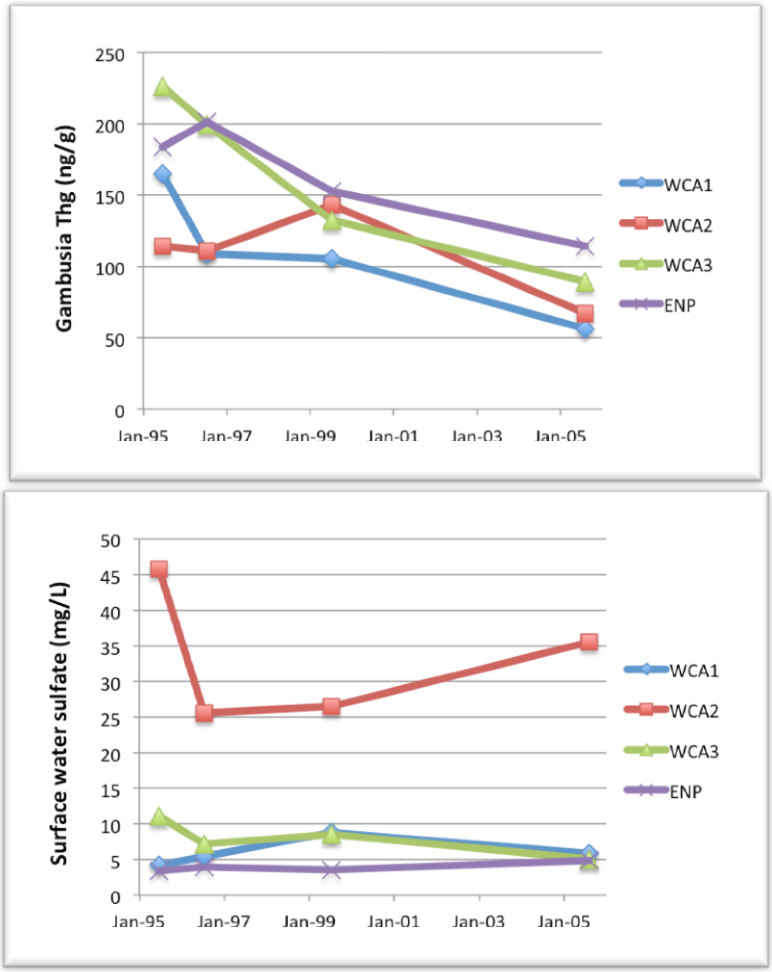

Figure 1. Temporal changes in *Gambusia* tissue THg (top) and surface water sulfate (bottom) for the main marsh compartments of the Everglades Protection Area, based on USEPA REMAP data. Each data point represents the mean value for combined wet and dry season samples from each compartment.

Because of the complexities related to MeHg production and bioaccumulation, DB Environmental, Inc. (DBE) has initiated a multi-year project, funded by EAA-EPD, FDEP and FDACS, to better define the critical processes responsible for MeHg accumulation, and to identify management opportunities for minimizing tissue Hg levels in marsh fauna. Our first year's effort has focused on *in situ* and *ex situ* studies in WCA-3A, near a site (3A-15) that was a Hg "hot-spot" with respect to fish tissue Hg levels (Scheidt and Kalla 2007).

Methyl and total Hg have been monitored in surface water at site WCA3A-15 by the United States Geological Survey (USGS) under the Aquatic Cycling of Mercury in the Everglades (ACME) project, although the most recent data available in the ACME database is from October 2007. The ACME MeHg data suggest a general decreasing trend during the monitoring period, although shorter-term variability masks this progression to some extent. Concurrent data from the ACME database indicate that spikes in MeHg during this period were associated with elevated concentrations of both DOC and sulfate (1996 and 1998) or elevated DOC only (2000 and 2001) (Figure 2).

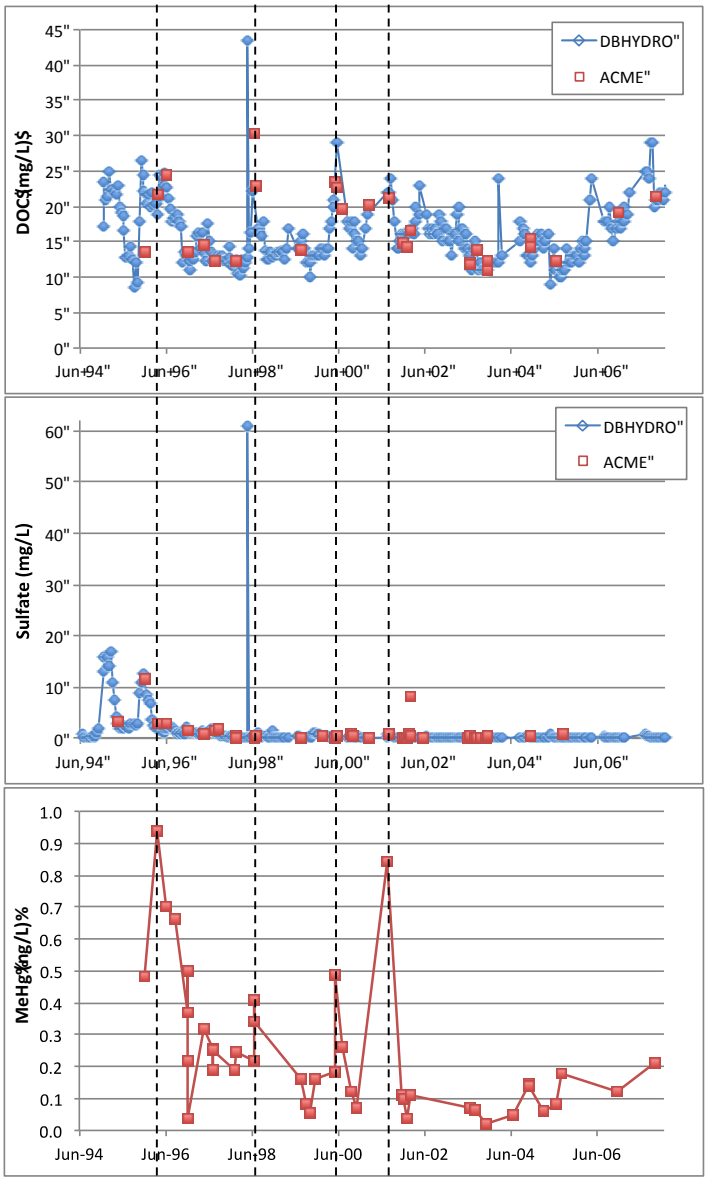

Figure 2. Concentration of DOC (top), sulfate (middle) and MeHg (bottom) in surface water at Everglades site WCA3A-15. Data were obtained from the DBHYDRO (SFWMD) and ACME (USGS) databases (plotted separately). Dashed line indicate temporal association between MeHg spikes and increased concentrations of either DOC, sulfate or both.

Key questions that will be addressed by DBE during this project include the following.

- **Are microbial assemblages, other than SRB, responsible for Hg methylation in the Everglades? If so, do these communities methlyate Hg at environmentally significant rates?**

- **Are *in situ* sources of sulfate (rainfall, groundwater, internal recycling of reduced sulfur species) in the Everglades adequate to support environmentally detrimental levels of methylation by SRB?**
- **Does the nature and concentration of dissolved organic matter (DOM) affect Hg methylation in the Everglades independent of sulfate concentration?**
- **Do seasonal drydown and reflood affect the MeHg concentration in the Everglades, and is this influenced by legacy soil sulfate and P enrichment?**
- **Does demethylation play a prominent role in the MeHg cycle in the Everglades?**
- **Which factors link MeHg production to Hg bioaccumulation?**

Herein we report the results of two laboratory incubations. The first investigation focused on defining the role of SRB and other microbial consortia on methylation and demethylation in WCA-3A soils. The second study was performed to better define the interrelationships between SO_4 and inorganic mercury (Hg(II)) loadings on MeHg accumulation in low SO_4 environments. Specifically, we were interested in determining whether: 1) "background" marsh sulfate levels at 3A-15 are adequate to promote significant Hg methylation in the presence of bioavailable Hg; 2) modest increases in SO_4 can sharply increase MeHg accumulation; and 3) even higher water column SO_4 levels will curtail MeHg accumulation. The hypothesis captured in items 2 and 3, that modest increases in SO_4 will enhance MeHg accumulation, whereas high levels of SO_4 will inhibit MeHg accumulation through the accumulation of sulfide, which can render Hg non-bioavailable, has been reported previously in the Hg methylation literature (e.g. Benoit et al. 2003; Orem et al. 2011; Pollman 2012).

Site Location and Description

The soil and surface water used in the lab incubations were sourced from the same slough location in WCA-3A, which is approximately 10 km northwest of site 3A-15 (**Figure 3**). For the past three years, the surface water at this experimental site consistently has exhibited SO_4 levels of less than 1.0 mg L^{-1}.

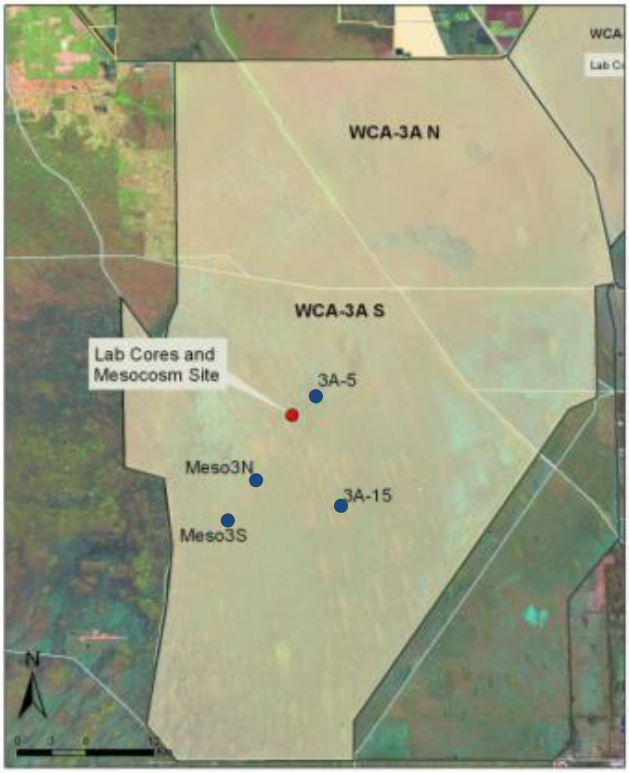

Figure 3. Location of the slough that served as the source of soil and surface water for laboratory incubations. Also depicted are locations of other USGS and SFWMD sites in WCA-3A.

Laboratory Methodology

The design and experimental procedure were similar for each of the two investigations. Controls and treatments were performed in triplicate. Each borosilicate incubation vessel received 100 mL (wet volume) of surficial soil (0-5 cm soil depth) and 900 mL of surface water from WCA-3A. After adding the measured amounts of experimental amendments (including Hg^{2+} [as $HgCl_2$], SO_4, or microbial inhibitors, depending on the experimental treatment) to the surface water, the contents of each incubation vessel were vigorously shaken for 30 seconds to render homogeneous the added water with the soil, which marked T=0. No further soil resuspension was performed during each 14-day incubation period. Each vessel was exposed to gentle bubbling with 0.03% CO_2 (balance N_2) for 1.5-4.0 hr either every day (first experiment) or every-other-day excluding weekends (second experiment) to promote anoxia and maintain a consistent pH. The two-week incubation occurred in the dark and at room temperature (22.5-25.5 °C).

During each incubation, the water column within vessels was subsampled on days 0 (immediately after mixing of soil, water and amendments), 7 and 14 for dissolved MeHg and SO_4. Dissolved total mercury (THg) concentrations were also measured in one of the three replicate vessels for each SO_4 treatment and control group at T=0 and T=14 days. All samples for MeHg and THg analysis were filtered through a 0.5-L Thermo-Scientific Nalgene MF 75 series sterile disposal filter apparatus. The filter diameter was 7.5 cm; the filter material was cellulose nitrate with a 0.45 µm pore size.

Sampling removed approximately 0.1 L of water from each vessel. After sample collection on days 0 and 7, that volume was replaced by reserve site surface water containing the same SO_4^{2-},

Hg^{2+}, or inhibitor concentrations as were initially present at the beginning of the experiment. This represented a replacement volume of 10% for each 1 L incubation vessel.

Experiment 1: The Roles of SRB and Methanogens in Net Mercury Methylation in WCA-3A Soils

Background

The addition of inhibitors specific to targeted groups of Hg methylating bacteria has proved to be a valuable technique in identifying which groups, such as SRB, iron-reducing bacteria (FeRB), and methanogens, are primarily responsible for Hg methylation and demethylation (Gilmour et al. 1998; Pak and Bartha 1998). In our study, we subjected the microflora in the soil of WCA-3A to two different inhibitors, separately and in combination, during a 14-day lab incubation.

Experimental Design

All but one of the six groups of control and experimental vessels received Hg^{2+} as ($HgCl_2$), at a concentration of 139 ng L^{-1}, whereas four experimental vessel groups received 4.5 mg L^{-1} SO_4^{2-} amendments (**Figure 4**). Three of the experimental groups received surface water from WCA-3A that had been amended with one, or a combination, of two inhibitors, molybdate (MoO_4^{2-}) and bromoethanesulfate (BES). Molybdate (MoO_4^{2-}) was added as $Na_2MoO_4 \cdot 2H_2O$ to a concentration of 20 mM and BES was added as sodium 2-bromoethanesulfonate ($NaBrCH_2CH_2SO_3$) to a concentration of 50 mM. MoO_4^{2-} and BES inhibit methylation and demethylation by SRB and methanogens, respectively (Gilmour et al. 1998).

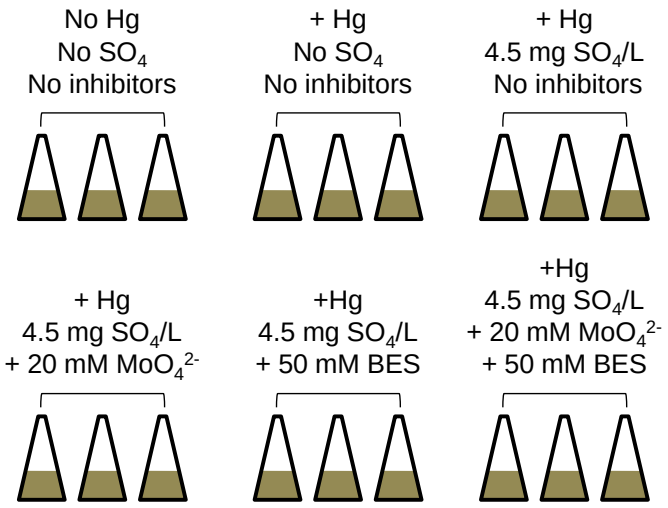

Figure 4. Experimental design for the lab incubation where WCA-3A water column and soil with and without added sulfate (SO_4^{2-}), inorganic mercury (Hg^{2+}) and inhibitors were incubated in the dark at room temperature for two weeks. MoO_4^{2-}= molybdate (20 mM); BES=bromoethanesulfonate (50 mM). See Table 1 for initial mercury (Hg) concentrations.

Results and Discussion

The initial dissolved MeHg and THg concentrations present in the site water used in this experiment, both before and after amendment with Hg^{2+}, and with and without soil, are found in Table 1. The initial dissolved THg concentration of 139 ng L^{-1} in the Hg-amended vessels is equivalent to 20.2 µg Hg^{2+} m^{-2}, or approximately 10.8 months of atmospheric Hg^{2+} deposition to

the Everglades (Liu et al. 2008). The addition of soil reduced the concentration of dissolved THg and MeHg in both Hg^{2+}-amended and unamended surface waters.

Table 1. Initial (T=0; n=1) dissolved methylmercury (MeHg) and dissolved total mercury (THg) concentrations for Hg^{2+}-amended and unamended WCA-3A surface water before and after mixing 900 mL of surface water with 100 mL of soil (0-5 cm depth layer). All values are in units of ng L^{-1}.

	MeHg Concentration (ng L^{-1})		THg Concentration (ng L^{-1})	
	No Hg^{2+} added	Hg^{2+} added	No Hg^{2+} added	Hg^{2+} added
Before mixing with soil	0.148	0.151	1.32	139
After mixing with soil	0.051	0.068	0.91	6.15

On days 7 and 14, the dissolved MeHg concentration in the unamended control was 0.11 ng L^{-1}. Amending with Hg^{2+} resulted in mean dissolved MeHg concentrations seven- to eight-fold higher ($P \leq 0.05$) on days 7 and 14, respectively, than in the unamended control (Figure 5). Adding 4.5 mg L^{-1} of SO_4^{2-} with the Hg^{2+} also significantly increased ($P \leq 0.05$) the mean MeHg concentration in the standing water on days 7 and 14 compared to both the unamended controls and the Hg^{2+}-only amended vessels. However, the incremental increase in MeHg observed when SO_4^{2-} was added with Hg^{2+} (0.38 ng L^{-1}) was less than the MeHg response to Hg^{2+} alone (**Figure 5**). The methylation response to Hg^{2+} and SO_4^{2+} additions was tested and discussed in more detail in Experiment 2, below.

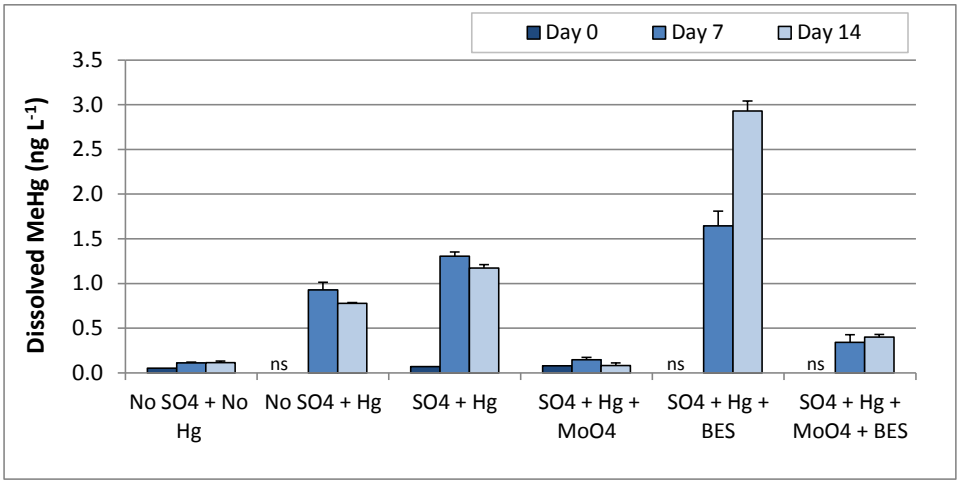

Figure 5. Mean (±1 S.E.; n=3) for days 7 and 14, and initial (n=1), standing water dissolved methylmercury (MeHg) concentrations in SO_4^{2-}-, mercuric (Hg^{2+})- and inhibitor-amended and unamended WCA-3A water-soil vessels. Initial MeHg concentration = 0.068 ng L^{-1} in all vessels with Hg^{2+} added and 0.051 ng L^{-1} in vessels with no Hg^{2+} added. MoO_4^{2-}= molybdate (20 mM); BES=bromoethanesulfonate (50 mM). ns = not sampled on Day 0 after the start of the incubation.

The net MeHg response to selective microbial inhibitors varied widely (Figure 5). The MoO_4^{2-} + SO_4^{2-} + Hg^{2+} treatment (SRB inhibited) yielded significantly lower ($P \leq 0.05$) mean MeHg concentration compared to the SO_4^{2-} + Hg^{2+} treatment without inhibitors. In fact, the net

methylation response in the presence of MoO_4^{2+} was similar to that of the unamended control. Conversely, the BES + SO_4^{2-} + Hg^{2+} treatment (methanogens inhibited) resulted in a significantly higher MeHg concentration on day 14 versus the SO_4^{2-} + Hg^{2+} treatment. When both inhibitors (MoO_4^{2-} + BES) were added (SRB and methanogens inhibited), along with SO_4^{2-} + Hg^{2+}, significantly more ($P \leq 0.05$) MeHg was produced than in the MoO_4^{2-} + SO_4^{2-} + Hg^{2+} treatment, but significantly less than in the SO_4^{2-} + Hg^{2+} treatment without inhibitors. Pak and Bartha (1998) reported that MoO_4^{2-} can completely inhibit both methylation and demethylation activities of sufidogens, whereas BES can completely terminate demethylation in methanogens. That, in conjunction with the net methylation response in our experiment, support three hypotheses related to the methylating microbial consortium. First, the low net methylation in the presence of MoO_4^{2+} indicates that Hg methylation in sediment and water from central WCA-3A is mediated primarily by SRB. This is in agreement with earlier ACME work, including Gilmour et al. (1998) and Cleckner et al. (1999). Second, the large MeHg accumulation in the presence of BES suggests that MeHg demethylation by methanogens in sediment and water from central WCA-3A actively governs net methylation at an environmentally significant rate. Finally, the non-zero rate of MeHg accumulation in the presence of both MoO_4^{2+} and BES suggests that non-SRB microbial guilds may contribute to net methylation, though at a lesser rate than SRB. For example, FeRB have been shown to methylate Hg (Fleming et al. 2006), and the gram-positive fermenters of the phylum Firmicutes have been reported to posses the two-gene cluster that has been found to methylate Hg in other bacterial groups (Parks et al. 2013). Both groups are found in the Everglades.

Experiment 2: Effects of Hg and Low-Level SO_4 Amendments on MeHg Accumulation

Background

Previous investigators have indicated that reductions in marsh MeHg levels can be achieved by controlling sulfate inputs from northern drainage waters (Orem et al. 2011). However, largemouth bass in Everglades National Park continue to exhibit unacceptably high tissue Hg levels (Gu et al. 2012) despite generally low water column sulfate levels (Scheidt and Kalla, 2007; Gilmour et al. 2007). This suggests that very low, and perhaps "background" levels of sulfate can promote environmentally significant levels of MeHg. This experiment was performed to characterize this response, using water and soils collected from a low-sulfate environment in WCA-3A.

Experimental Design

The design and methods for Experiment 2 followed those described in the Laboratory Methodology section above. **Figure 6** provides an overview of the sulfate (SO_4^{2-}) and Hg^{2+} treatments specific to this experiment. All but two of the nine sets of control and treatment vessels received Hg^{2+} as $HgCl_2$, at a concentration of 115 ng L^{-1}. Increments of SO_4^{2-} amendments were 0.5, 1.0, 2.5, 4.5, 10, and 20 mg L^{-1}. All controls and treatment levels were conducted in triplicate.

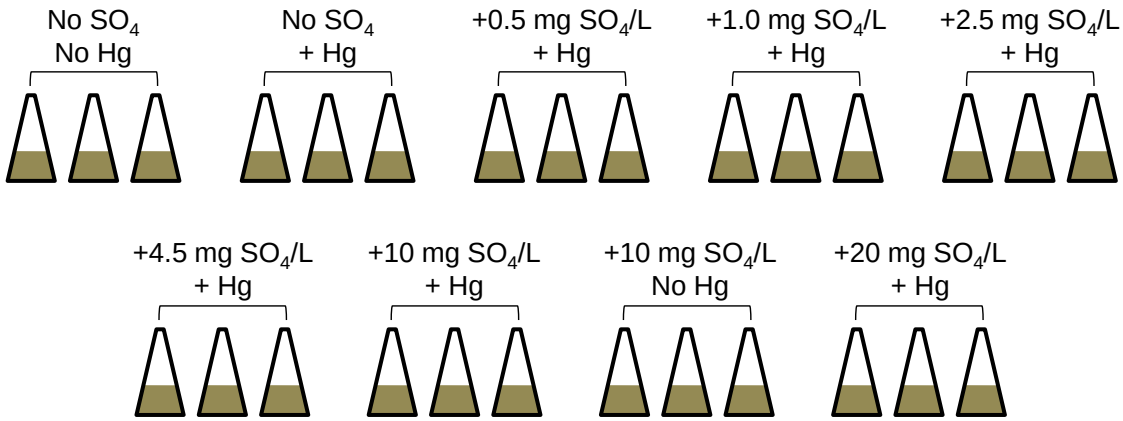

Figure 6. Experimental design for Experiment 2 where WCA-3A water column and soil with and without added sulfate (SO_4^{2-}) and inorganic mercury (Hg^{2+}) were incubated in the dark at room temperature for two weeks.

Results and Discussion

Initial dissolved MeHg and THg concentrations for the raw unamended WCA-3A water used in the experiment were 0.048 and 0.550 ng L^{-1}, respectively, one week after collection in the field (Table 2). Our initial dissolved MeHg concentration of 0.048 ng L^{-1} in the unamended WCA-3A water was at the mid-range of values reported for nearby 3A-15 site water (see Figure 3 for location) from 2010 to 2013 (Table 3).

After adding Hg^{2+} as $HgCl_2$ to the WCA-3A water prior to adding soil, the dissolved MeHg concentration increased to only 0.066 ng L^{-1}, whereas the dissolved THg concentration increased to 115 ng L^{-1} (Table 2). Upon adding soil to the water at a 1:9 (vol:vol) ratio and thoroughly agitating, the dissolved THg concentrations decreased from 115 to 5.06 ng L^{-1} in the Hg^{2+}-amended sacrificial vessel, indicating 95% of the added Hg^{2+} was bound to soil particles at T=0. This initial dissolved THg concentration of 115 ng L^{-1} is equivalent to 16.7 µg Hg^{2+} m^{-2}, or approximately 8.9 months of atmospheric Hg^{2+} deposition to the Everglades (Liu et al. 2008).

Table 2. Initial (T=0; n=1) dissolved methylmercury (MeHg) and dissolved total mercury (THg) concentrations for Hg^{2+}-amended and unamended WCA-3A surface water before and after mixing 900 mL of surface water with 100 mL of soil (0-5 cm depth layer). All values are in units of ng L^{-1}.

	MeHg Concentration (ng L^{-1})		**THg Concentration (ng L^{-1})**	
	No Hg^{2+} added	**Hg^{2+} added**	**No Hg^{2+} added**	**Hg^{2+} added**
Before mixing with soil	0.048	0.066	0.550	115
After mixing with soil	<0.021	0.029	0.560	5.06

Table 3. Methylmercury (MeHg) and total mercury (THg) concentrations in the surface water at 3A-15 from August 2010 to November 2012. Data from SFWMD Hg Hotspots study (unpublished). All values are in units of ng L^{-1}.

Date	MeHg	THg
8/19/2010	0.033-0.129	1.21-1.60
2/23/2012	0.066	< 0.67
6/28/2012	0.085	4.49
11/29/2012	< 0.02	0.73
03/21/13	0.048	0.67

Within treatments that had received an inorganic Hg dose, concentrations of SO_4^{2-} had a negligible effect on the MeHg concentrations in the standing waters after 7 days of incubation ($P > 0.05$), but a MeHg-accumulation effect ($P \leq 0.05$) was distinct by day 14 (Figure 7, Figure 8). Day 14 MeHg concentrations ranged from 0.40 ng L^{-1} for the control (no SO_4^{2-} amendment) to 0.98 ng L^{-1} in the 20 mg SO_4^{2-} L^{-1} amendment with Hg^{2+} added, representing a 2.5-fold increase in MeHg concentration over the 0-20 mg SO_4^{2-} L^{-1} amendment range. MeHg concentrations in this experiment did not indicate a unimodal response to the amended SO_4^{2-} concentrations (0-20 mg L^{-1}) as has been observed by other investigators (e.g., Benoit et al. 2003; Gilmour et al. 2004a; Gilmour et al. 2007). This unimodal phenomenon is attributed to changing Hg bioavailability, as inorganic Hg can complex with accumulated sulfide in porewaters at high SO_4^{2-} concentrations (Benoit et al. 2001). By day 7, oxidation-reduction potentials in our incubation vessels were lower than redox levels observed in a group of *in situ* sulfate-dosed mesocosm enclosures that we have maintained near the 3A-15 site for three years. While of short duration, this suggests that our lab incubation provided suitable conditions for sulfide production at the higher SO_4^{2-} doses.

Interestingly, even very small SO_4^{2-} additions (0.5-1.0 mg L^{-1}) increased the water MeHg concentration, relative to controls, at day 14 of our incubation. This finding suggests that "non-abatable" sources of SO_4^{2-} to the Everglades (Pollman 2012) could support meaningful MeHg production, especially in the presence of bioavailable inorganic Hg. In contrast to our findings for MeHg in standing water, we found that sulfate additions to the Hg^{2+} spiked vessels did not increase MeHg accumulation in soils (**Figure 9**).

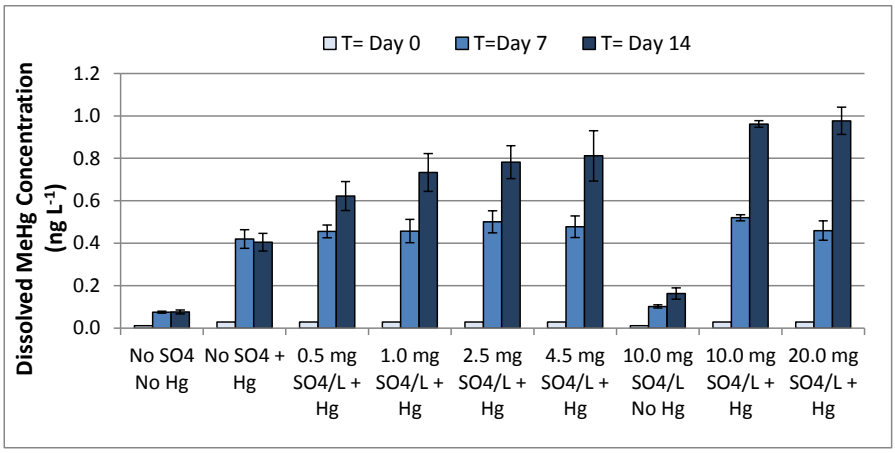

Figure 7. Mean (±1 S.E.; n=3) for days 7 and 14, and initial (n=1), dissolved methylmercury (MeHg) concentrations at T=0 and days 7 and 14 in sulfate (SO_4^{2-})- and mercury (Hg^{2+})-amended and unamended WCA-3A surface water and soil.

The effect of Hg^{2+} dosing on aqueous MeHg concentrations was even more marked ($P \leq 0.05$) than for SO_4^{2-}: between 4.5 and 5 times higher MeHg production was observed for the Hg^{2+}-spiked vessels compared to vessels of the same SO_4^{2-} dose with Hg^{2+} omitted (**Figure 7**). This result was anticipated from the literature (e.g., King et al. 1999) and is in agreement with early findings from the ACME program that found added, isotopically-labeled Hg^{2+} was methylated more readily than "native" Hg in *in situ* mesocosms in WCA-1 and WCA-3A (Gilmour et al. 2004). Notwithstanding that the MeHg response to Hg^{2+} addition is likely to depend on the Hg^{2+} concentration, the increase in net methylation to bioavailable Hg^{2+} in our experiment suggests that controlling the bioavailability of inorganic Hg in the Everglades through source reductions could play a crucial role in mitigating MeHg accumulation.

We also note that net MeHg production per unit of added sulfate is highest at the lowest sulfate addition rates. These data indicate that in areas of high Hg bioavailability (as yet unidentified in the Everglades, but perhaps indicated by MeHg "hotspots"), decreasing SO_4^{2-} inputs may constrain MeHg production, but only if residual SO_4^{2-} concentrations are sufficiently low, e.g., < 0.5 mg L^{-1}.

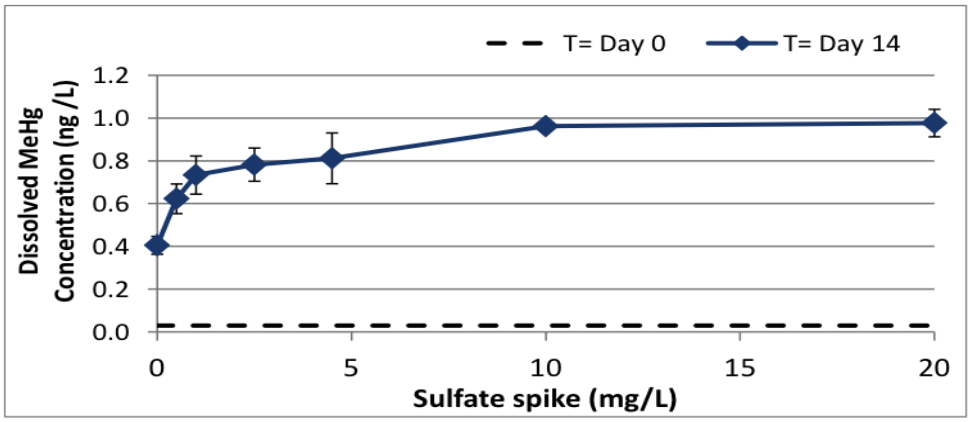

Figure 8. Accumulation of dissolved MeHg as a function of added sulfate concentration in surface water of laboratory reactors spiked with inorganic Hg. The solid line indicates MeHg concentrations at Day 14 of the incubation, while the dashed line indicates initial MeHg concentrations.

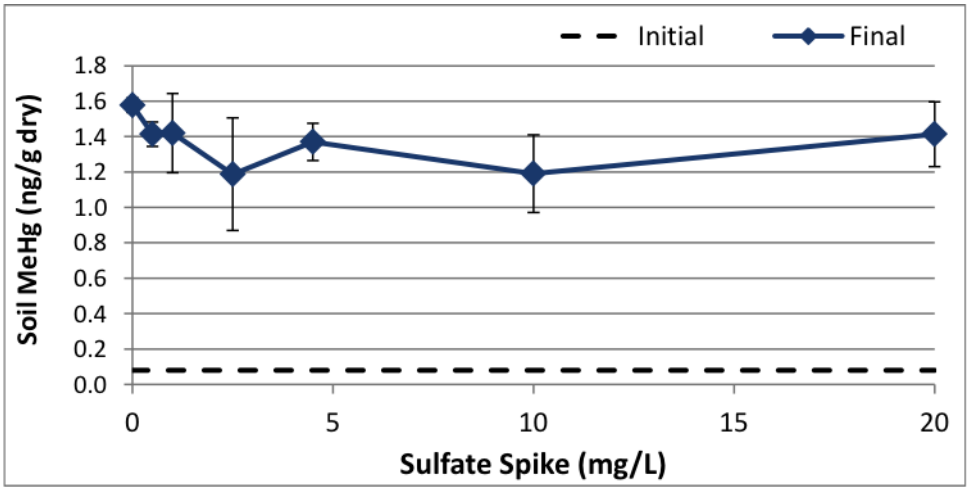

Figure 9. Accumulation of dissolved MeHg as a function of added sulfate concentration in soils of laboratory reactors spiked with inorganic Hg. The solid line indicates MeHg concentrations at Day 14 of the incubation, while the dashed line indicates initial MeHg concentrations.

Conclusions

For the surface water and soil collected within WCA-3A near site 3A-15, we found that SRB are the dominant Hg biomethylators. At this location, methanogens serve to demethylate Hg, but apparently at a lower rate than the Hg methylation rate of SRB. A third group of biomethylators, as yet unidentified, may also be contributing MeHg, but at a lower rate than the SRB. More experimentation needs to be undertaken before the identification and significance of microbial groups (other than SRB) in methylating and demethylating Hg in the Everglades can be confirmed.

Addition of bioavailable Hg (as Hg^{2+}) enhanced biomethylation, even in vessels containing only native levels (0.3 mg L^{-1}) of SO_4^{2-}. Whether this response was due to the activity of SRBs, or an unknown biomethylator group, is unknown. Supplemental low-level (0.5-1.0 mg L^{-1}) SO_4^{2-} amendments further increased MeHg accumulation in the presence of bioavailable Hg. The enhancement of MeHg accumulation in response to SO_4^{2-} with bioavailable Hg amendments exhibited a plateau at higher SO_4^{2-} concentrations (10-20 mg L^{-1}).

Our findings suggest that *in situ* sources of sulfate (rainfall, groundwater, internal recycling of reduced sulfur species) in the Everglades may be adequate to support environmentally detrimental levels of methylation by SRB since SO_4 levels as low as 0.3 mg L^{-1} without added Hg^{2+} supported modest MeHg accumulation in the laboratory incubation. Furthermore, the addition of bioavailable Hg markedly enhanced methylation, even in vessels containing only native levels (currently < 0.5 mg L^{-1} at site WCA-3A) of SO_4. Further research is needed to confirm whether soils and water from other south Florida marsh sites exhibit similar responses, and whether responses observed in these laboratory trials mimic those in the field.

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