Appendix 3B-2: Status Report on ACME Studies on the Control of Mercury Methylation and Bioaccumulation in the Everglades

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Status Report on ACME Studies on the Control of Mercury Methylation and Bioaccumulation in the Everglades

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OVERVIEW

The Aquatic Cycling of Mercury in the Everglades (ACME) project team, consisting of researchers from the US Geological Survey, the Smithsonian Environmental Research Center, the University of Wisconsin, Louisiana State University, and other instibtutions, has been investigating the factors leading to the formation and bioaccumulation of methylmercury (MeHg) in the Everglades since 1995. MeHg production from inorganic mercury (Hg) is a microbial process that occurs in soils, anoxic water, and decaying periphyton communities. It is the key reaction in the complex biogeochemical path from Hg deposition to MeHg accumulation in biota. Principal findings from the ACME study to date include the role of sulfate in MeHg production in the Everglades, and the high levels of sulfate that impact much of the northern Everglades Protection Area (EPA); the importance of newly deposited Hg to MeHg production; the importance of drying and rewetting cycles in MeHg production; and progress in understanding Hg complexation and bioavailability that have improved our models of the biogeochemical controls on net Hg methylation.

In the current phase of this research, the ACME team has focused on the response of MeHg production in the Everglades ecosystem to changes in the major factors that impact on net methylation — Hg, sulfur (S), dissolved organic matter (DOM), iron (Fe), and hydroperiod (drying and rewetting cycles). In the field, mesocosm studies have included Hg, sulfate, DOM and Fe additions to mesocosms, which are being used to provide a more precise estimate of the "effect levels" and timing of these factors on MeHg production. Laboratory soil incubations are being used to study how STA soil types, and drying and rewetting cycles quantitatively affect net mercury methylation. ACME researchers are also characterizing the bacterial communities that favor net mercury methylation, studying the interactions between Hg and DOM in order to understand Hg bioavailability for methylation, and developing numerical biogeochemical models for net MeHg production in Everglades soils.

In this appendix, overviews of the following recent research studies are presented:

- A mesocosm study in central Water Control Area 3A (WCA-3A) on the impact of Hg, sulfate, and organic carbon on mercury methylation and bioaccumulation.
- A mesocosm study to assess the impact of Fe(III) additions on mercury methylation, also in central WCA-3A.
- Research on the types of bacteria that produce MeHg in the Everglades.
- Research on the interactions between DOM and Hg in the anoxic water where MeHg is produced.

Research by the ACME team on sulfur sources and effects is described in Appendix 3B-3 of this volume.

SUMMARY OF FINDINGS

Sulfate, DOC, and Hg addition mesocosms in WCA-3A. A long-term mesocosm study was conducted at oligotrophic site 3A-15 in central WCA-3A during 2003–2004. This 18-mesocosm study was designed to provide a model for sulfate concentration versus MeHg production, as well to as examine the interactions between dissolved organic carbon (DOC), sulfate and Hg. MeHg production in the mesocosms was generally a linear function of surface water sulfate concentration, up to 20 mg/L, the highest dose used. Sulfide concentrations in pore waters during the experiment were insufficient to significantly inhibit net MeHg production. However, the zones of the Everglades where surface water sulfate is currently in the 20 mg/L range (WCA-2A and areas around canals in WCA-3A) have much higher average pore water sulfide concentrations. This experiment suggests that a model for net MeHg production based on both sulfate and sulfide concentrations will more accurately predict MeHg concentrations than surface water sulfate concentration alone. However, it confirms and extends previous smaller-scale mesocosm studies, suggesting that net MeHg production is proportional to sulfate at sulfate concentration found across most of the EPA. Only the most impacted areas of WCA-2A and 3A have average surface water sulfate concentrations above 10–20 mg/L, concentrations at which sulfide production may begin to limit methylation in Everglades soils. These data suggest that broad areas of the EPA currently exhibit sulfate concentrations at which increased sulfate levels would enhance, and decreased sulfate concentrations would reduce net MeHg accumulation in soils, and hence MeHg accumulation in biota.

The addition of DOM also enhanced MeHg accumulation in the mesocosms, and was concentration dependent. The magnitude of MeHg response to sulfate and DOC additions was higher for the newly added Hg spikes than for ambient Hg in the mesocosms. The examination of Hg pools of different ages is possible through the use of Hg-stable isotope amendments. This study confirms prior mesocosm studies in the Everglades, and in other ecosystems, showing that Hg in sediments and soils becomes less available for methylation as it ages in place. Therefore, MeHg concentrations in fish reflect primarily the recent history of Hg deposition.

Iron addition mesocosms in WCA-3A. Recent research suggests that iron (Fe) may impact mercury methylation. If so, Fe concentrations in sediments and soils should be taken into account when predicting MeHg production and Fe amendments could be considered as a potential mitigation strategy for Hg. Fe may affect methylation by changing Hg solubility and bioavailability. It may also stimulate the activity of Fe-reducing bacteria, some of which are closely related to sulfate-reducing bacteria (SRB), and are capably of MeHg production (see below).

In 2005, a mesocosm study was conducted to asses the impact of Fe oxyhydroxide additions on net MeHg production in Everglades soils. In this study, mesocosms at 3A-15, an oligotrophic, low-sulfur site, were amended with three concentrations of Fe(III) oxyhydroxide (or a no addition control) plus a stable enriched stable Hg isotope spike. The Fe additions represented roughly one-half, one, and two times the amount of Fe accumulated in the soils at this site in one year. Hg and sulfur biogeochemistry in the mesocosms were examined after 3 days and after 2 months. The responses to Fe(III) were mixed. Fe inhibited MeHg production in soils at the highest dose, but enhanced MeHg concentrations in surface waters at low doses. The ^{200}Hg spike added to the mesocosms more strongly methylated in response to Fe dosing than was ambient Hg. Biogeochemical measurements suggest that Fe may have affected Hg availability for methylation through changes in filterable Hg concentrations. This study will improve our ability to model

MeHg production across different soil types in the Everglades, and to predict which soil types are most susceptible to net MeHg formation.

Microbial ecology of mercury methylation. Many studies in many freshwater ecosystems show that mercury methylation is tied to the activity of SRB. However, over the years, only about 10 strains of SRB have been shown to produce MeHg in pure culture. Recently, ACME researchers have been applying molecular phylogenetic techniques to better understand the distribution of mercury-methylating ability among the SRB, and to test whether closely related organisms may also have this ability. Previously cultured strains of SRB from many environments were tested for methylation ability, and the organisms were identified by sequencing the 16S rRNA gene. This information was used to construct the first phylogenetic tree for mercury methylation. It suggests that mercury methylation is widespread but relatively rare among the SRB. Fe-reducing bacteria were also examined. A number of *Geobacter* strains were capable of mercury methylation (Kerin et al. 2006). These bacteria gain energy from Fe reduction and are closely related to the SRB. ACME is also beginning to measure the rate of microbial Fe(III) reduction across the Everglades. These kinds of information are leading us closer to an understanding the still-unknown mechanism(s) of microbial mercury methylation. Phylogenetic and species-specific information will also help determine if microbial community structure is an important control on MeHg production, and if there are unique aspects of microbial communities in high methylation rate environments like the Everglades.

Research on Hg complexation and bioavailability. Lastly, a series of studies was conducted to examine the complexation of Hg in natural sulfidic conditions and laboratory solutions (Miller 2006; Miller et al. 2006). The dissolved phase complexation of Hg is a controlling factor in the bacterial production of MeHg. While both laboratory and field studies have shown that Hg complexed with dissolved organic matter (DOM) dominates the speciation of Hg under oxygenated conditions, the complexation of Hg under natural anaerobic conditions has not been investigated. Because mercury methylation occurs predominantly under anaerobic conditions, the complexation of Hg under these conditions is important. Current thermodynamic models predict that Hg-sulfide complexes should be the dominant Hg complexes in sulfidic waters, as a result of the stronger affinity of Hg to reduced sulfur relative to DOM. However, laboratory measurements of the formation of the complexes have been done without DOM present (Benoit et al. 1999b).

Carrie Miller examined the interactions between Hg, sulfide and DOM in anoxic waters for her Ph.D. thesis from the University of Maryland. Octanol-water partitioning extractions and centrifuge ultrafiltration techniques were used to separate complexes by size and charge. This work demonstrated the existence of a previously unknown complex between Hg, sulfide, and DOM. Dr. Miller proposed a ternary structure for this complex, proposed as DOM-S-Hg-S-DOM, although the structure is not known. The discovery of this complex means that thermodynamic models for Hg complexation — required to construct biogeochemical models for mercury methylation — will need to be updated to consider this finding. Estimation of a formation constant for this complex, required for modeling, is under way. Importantly, the interactions between Hg, DOC, and sulfide are likely to be highly dependent on the chemical characteristics of the DOC, and therefore additional research on those interactions will be needed to construct useful models.

I. INTRODUCTION

The ACME project team has been studying the biogeochemistry of the mercury (Hg) cycle in the Everglades since 1995. During the first years of the study, the team made detailed biogeochemical measurements at a suite of sites from Arthur R. Marshall Loxahatchee National Wildlife Refuge to Everglades National Park . The team found generally high rates of net MeHg production and accumulation across the system, relative to other ecosystems, and a zone of particularly high methylation and bioaccumulation in the central Everglades (Cleckner et al. 1998, 1999; Hurley et al. 1998: Krabbenhoft et al. 1998, 2000; Gilmour et al. 1998, 2000; Marvin-DiPasquale et al. 2000). Based on field measurements of methylation and partitioning of MeHg into food webs, the team concluded that soil surface flocs are the main location of MeHg production in the ecosystem (Gilmour et al. 1998, 2000; Krabbenhoft et al. 1998; Cleckner et al. 1999; Benoit et al. 2003), and that variability in net MeHg production, rather than variability if food web accumulation, accounts for most of the variability in MeHg in biota (Cleckner et al. 1998; Krabbenhoft et al. 2000). Further, based on field distributions, laboratory experiments, and experience in other ecosystems, the team concluded that Hg, sulfate, and dissolved organic matter (DOM) were the primary controls on net mercury methylation (see references above and Benoit et al. 1999a,b; Benoit et al. 2001a,b,c; Ravichandran et al. 1998; Drexel et al. 2002; 2003; Haitzer et al. 2002; Aiken et al. 2003). The team also documented sulfate contamination of the ecosystem, leading to increases levels of reduced sulfur compounds in soils, especially in WCA-2A and northern WCA-3A (Bates et al. 1998, 2002; Orem et al. 1997).

During the mid to late 1990s, the highest MeHg concentrations in surface soils and flocs (Gilmour et al. 1998; Benoit et al. 2003), fish (Stober et al. 1996, 2001), and wading birds (Frederick et al. 2002) in the Everglades were observed near the center of WCA-3A. Sulfate concentrations in surface water in central WCA-3A during this time were somewhat higher (2–10 mg/l) than in more pristine sites further south, while pore water sulfide concentrations were low enough (5 to 150 ug/L) to prevent significant inhibition of mercury methylation (Orem et al. 1997; Stober et al. 1996 and 2001). **Figure 1** shows the average distribution of sulfate in surface waters, sulfide in soil pore waters, and the rate of methylmercury (MeHg) production across the eight main ACME sites during 1995–1998.

We hypothesized that in the Everglades, areas at the downgrade edge of the sulfate contamination plume have sulfate and sulfide levels in the correct balance to promote maximum MeHg production. In pristine areas of the Everglades, MeHg production may be limited by low levels of sulfate (<1 mg/l) and low rates of microbial SR. In areas of the Everglades heavily contaminated with sulfur (e.g., northern WCA-2A), MeHg production may be limited by the inhibitory effects of high pore water sulfide concentrations (Gilmour et al., 1998; Benoit et al. 2003).

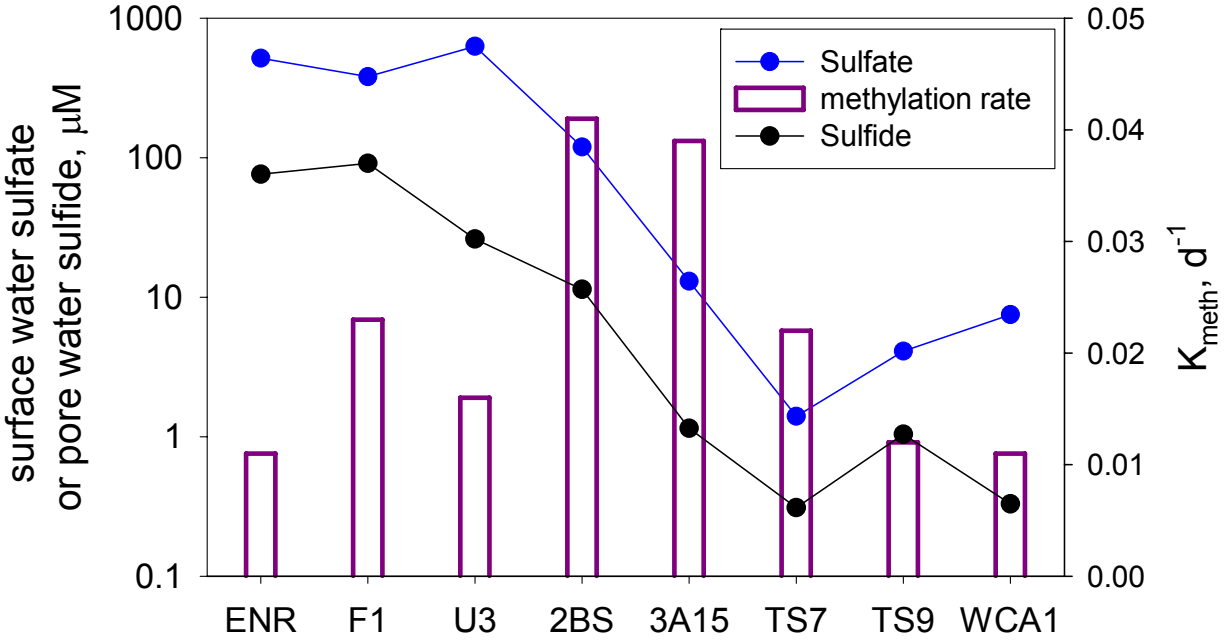


Figure 1. Average concentrations of surface water sulfate and pore water sulfide across the main ACME sites in the Everglades, 1995–1998. Bars show mercury methylation rates estimated from isotopic Hg additions to soil cores.

The Hg and sulfur cycles are intimately linked. The balance between sulfate and sulfide is a key control on Hg net methylation rate in many ecosystems (Munthe et al. 2006). Sulfate, along with pH and DOC, has been identified as a parameter that relates to Hg levels in fish among water bodies (Wiener et al. 2006). Sulfate stimulates mercury-methylating sulfate-reducing bacteria (SRB), while excess sulfide creates Hg complexes that are not bioavailable for uptake by methylating bacteria (Benoit et al. 1999a, b; Marvin-DiPasquale and Agee 2003). Sulfate stimulation of methylation has been demonstrated in studies that range from pure culture (King et al. 2000; Benoit et al. 1999a,b) to sediment and soil amendments (Compeau and Bartha 1985; Gilmour et al. 1992; Harmon et al. 2004; King et al. 2001; Benoit et al. 2003) to field amendments to lakes and wetlands (Watras et al. 1994; Branfireun et al. 1999; Benoit et al. 2003; Jeremiason et al. 2006). Among these studies, the optimal concentration for methylation ranges from 1 to about 30 mg/L sulfate, dependent on microbial activity and the accumulation of dissolved sulfide. At a fixed rate of sulfate reduction, increased concentrations of dissolved sulfide will decrease net MeHg production rates. Factors such as Fe and organic matter concentration that impact Hg and S complexation impact the optimum level of sulfate in a given environment. However, for most freshwater environments, sulfide concentrations are insufficient to significantly inhibit methylation, and MeHg production is a function of surface water sulfate concentration.

Since 2000, the ACME project has used *in situ* mesocosms to study the effects (individual and synergistic) of Hg, sulfate, organic carbon and Fe on MeHg production and accumulation in the Everglades. Data from the two most recent studies, conducted in 2003–2004 and 2005, are included in this report.

ACME mesocosm studies conducted between 2000 and 2003 showed that:

- At multiple locations across the ecosystem, net Hg methylation and bioaccumulation responded linearly to single-dose Hg loads up to twice the annual wet deposition rates.
- The slope of the methylation response to Hg loads varied substantially across the Everglades, reflecting differences in biogeochemistry.
- The net amount of MeHg produced from Hg spikes was maximal within days to weeks after the spike, and declined thereafter. The timing of the response varied among sites. Bioaccumulation of spike Hg into Gambusia followed the same pattern, with a longer delay.
- Sulfate and dissolved organic carbon (DOC) additions to mesocosms in central WCA-3A stimulated net mercury methylation and bioaccumulation over periods of weeks.
- Sulfate and DOC had stronger impacts on the methylation of new Hg spikes than of Hg stored in soils.

II. NEW RESEARCH

This report provides information on two major mesocosm studies, conducted in 2003–2005, and associated laboratory studies. During 2003–2005, mesocosm studies were used to evaluate the following objectives:

- Examine the response of net methylation and bioaccumulation to sulfate and DOC in more detail, examining:
  - A wider range of sulfate and DOC concentrations
  - Longer-term responses to sulfate loading
- Determine the quantitative relationship between sulfate and net MeHg production, including an assessment of the "optimal" sulfate concentration for MeHg production in the Everglades.
- Examine the interactions between Hg, sulfate, and DOC on net methylation and bioaccumulation.
- Perform an initial assessment of the role of Fe in net methylation in the Everglades.

A. MERCURY METHYLATION AND BIOACCUMULATION RESPONSE TO HG, SULFATE AND ORGANIC CARBON, SITE 3A-15, JUNE 2003–NOVEMBER 2004

This large mesocosm study was designed to provide a model for sulfate concentration versus MeHg production, as well to as examine the interactions between DOC, sulfate and Hg. The experiment was conducted at the 3A-15 site in the central Everglades, between June 2003 and November 2004. In this study, five separate levels of sulfate dosing were used, each in duplicate. This study targeted the critical range of sulfate concentrations between sulfate stimulation and sulfide inhibition, in order to provide information on the magnitude of response, the timing of the

response and the linearity of the response through time and with sulfate load. Target sulfate concentrations in the mesocosms encompassed a range of sulfate concentrations from those found in central WCA-3A up to those found in the least impacted areas of WCA-2A — at which concentration we anticipated some sulfide inhibition of net methylation. In addition, two levels of DOC were examined in duplicate, and the interaction between DOC and SO4 was examined in another pair of mesocosms.

Objectives: The specific objectives for this study were to:

1. Determine quantitatively the effects (individual and synergistic) of Hg, sulfate, and dissolved organic carbon loading on MeHg production at site 3A-15.
2. Determine the critical range of sulfate concentrations between sulfate stimulation of SRB activity, and sulfide diminution of SRB-bioavailable Hg, providing information on the magnitude of MeHg production response, the timing and duration of the response, and the linearity of response through time and with sulfate load for site 3A-15.
3. Determine how the net methylation efficiency for newly deposited Hg changes through time for site 3A-15.

Design: The experiment was conducted at the 3A-15 site in central WCA-3A, between June 2003 and November 2004. **Figure 2** shows the general design and timeline of the study. Eighteen separate mesocosms were used. Installation of the new mesocosms took place during mid-April 2003 to allow time for re-equilibration prior to initiating the experiment in June. The mesocosms were place in slough habitat at site 3A-15, a few hundred yards from where previous slough mesocosm experiments had been conducted at this site. Dominant vegetation in the slough at 3A-15 was lilypad, spike rush, and *Utricularia*.

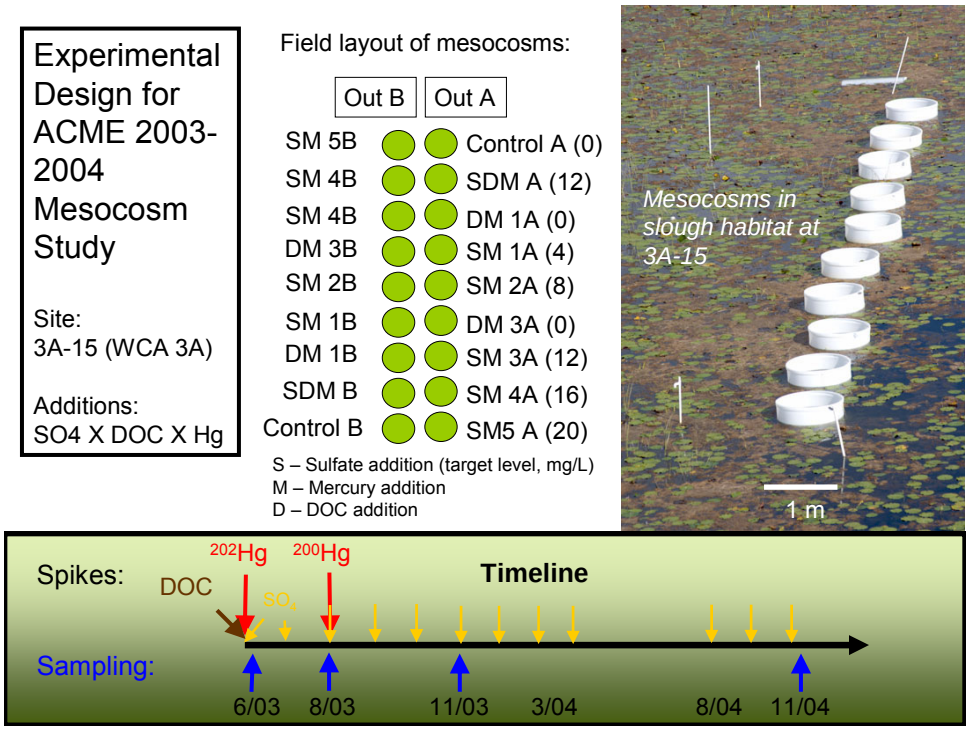


Figure 2. General design for the ACME 2003–2004 mesocosm study at 3A-15.

In total, 18 mesocosms were used in the study, plus designated control sites outside of the mesocosms. **Table 1** shows the dosing design. Ten mesocosms were used for sulfate plus Hg additions. There were five different target sulfate dosing levels (4, 8, 12, 16, and 20 mg/l), each in duplicate, and all with a single Hg level of 1X ambient atmospheric (22 μg/m^2; or 14.3 μg Hg per mesocosm). Each dosing level was done in duplicate because of our experience with natural variability among these experimental systems. Four mesocosms (duplicates at each of 2 dosing levels) were used to examine the effects of DOC, again all with a 22 μg/m^2 Hg dose. DOC for the experiment was isolated previously from Everglades surface waters by Dr. George Aiken at USGS Boulder. Target addition levels for DOC were 30 and 50 mg/l. Finally, two mesocosms were used to examine the DOC by sulfate interaction; these were dosed with 30 mg/L DOC and 12 mg/L SO4. Two mesocosms were used as unamended controls, and two areas outside the mesocosms served as unenclosed control areas.

Table 1. Dosing levels for 2003–2004 3A-15 loading experiment.

Meso	Target Sulfate (ppm)	Added DOC (mg/L)	Added Hg (ug/m^2)
SM 1A	4	0	22
SM 2A	8	0	22
SM 3A	12	0	22
SM 4A	16	0	22
SM 5A	20	0	22
SDM A	12	10	22
DM 1 A	0	10	22
DM 2 A	0	20	22
Meso Ctr A	0	0	0
Out Ctr A	XX	XX	XX
SM 1B	4	0	22
SM 2B	8	0	22
SM 3B	12	0	22
SM 4B	16	0	22
SM 5B	20	0	22
SDM B	12	10	22
DM 1 B	0	10	22
DM 2 B	0	20	22
Meso Ctr B	0	0	0
Out Ctr B	XX	XX	XX

Mesocosm dosing began in June 2003. Mesocosms were sampled in June, August and November of 2003, and in fall 2004. This allowed ACME to examine a longer time frame for response to sulfate than had been examined in past mesocosm studies.

Sulfate concentrations were maintained as best possible throughout the 17-month study. Surface water samples were taken approximately monthly during the wet season for sulfate analysis, water volumes were measured, and sulfate was then added as needed to maintain the desired surface water sulfate concentration. USGS Reston arranged for sulfate dosing of the

mesocosms during the wet seasons. DOC additions were made only once at the beginning of the study because of the cost and time required to prepare the DOC additions.

All mesocosms except controls were dosed with 22 μg/m^2 of mercury as enriched $^{202}HgCl_2$ at the beginning of the study (June 2003), and again, with 22 μg/m^2 of enriched $^{200}HgCl_2$, about 2 months later (August 2003). This allowed us to examine the response to Hg load at the time when sulfate loads were first increased, and later, after the mesocosms had equilibrated to some extent with the sulfate dose.

The first spikes (Hg, DOC, and sulfate) were made in June 2003. All mesocosms plus two outside control sites were sampled as described below in June, August, and November 2003. The original study design called for an end to the experiment in November 2003, but the team agreed to at least one more sampling in spring 2004, because initial data suggest that sulfate levels in the mesocosms did not stabilize at their new levels until fall of 2003. Because of an extended dry season in 2004, sulfate dosing was suspended from March until late summer. A final sampling was conducted in Nov 2004.

At each main sampling time, soils, surface and pore waters and gambusia were sampled from each mesocosm and outside control sites. Hg and MeHg concentrations in each matrix were characterized using ICP-MS methods tailored for Hg analysis, either by SERC or USGS Middleton. Other biogeochemical characteristics examined included DO, DOC, dissolved sulfide, Fe, Mn, anions, cations, nutrient, and pH in surface and soil interstitial waters; and solid-phase Fe(II)/Fe(III), acid-volatile, chromium-reducible and total reduced sulfides, organic carbon content; and bulk density in surficial soils. In all cases, "soils" are defined as the top 4 cm of solid material, which was often unconsolidated. Most soils samples consisted of multiple cores, composited for analysis. Pore water samples were taken from 5 cm depth using an *in situ* probe. For the outside control sites, we also characterized mercury methylation rates in surficial soils using stable isotope techniques. Methods are defined in our previous reports and publications (Orihel et al. 2006; Gilmour et al. 1998, 2000, 2004; Branfireun et al. 2005; Krabbenhoft et al 1998; Bates et al. 1998, 2002; Hintelmann et al. 2004).

Results: Mesocosm surface water sulfate levels were checked and adjusted roughly monthly from June 2003 to March 2004, and again from August to November 2004. Surface water sulfate concentrations through time in the mesocosms dosed with sulfate are shown in **Figure 3.** The desired progression of increasing sulfate concentration across the treatments was generally achieved, although with considerable variability through time, and between duplicate mesocosms.

The concentration of MeHg produced from the stable Hg isotope spikes increased in response to sulfate additions to mesocosm surface waters. Ambient MeHg concentrations in surface waters also responded significantly to sulfate load, although surface soil ambient MeHg concentrations did not. Results are shown in **Figures 3–9**. In these graphs, "Out" is the outside control, "Control" is the mesocosm control, "D10" and "D20" are DOC only treatments at roughly 10 and 20 mg/L added DOC, the S treatments are sulfate-only treatments with the number representing the target surface water sulfate level, and the S12/D10 were the mesocosms treated to 12 mg/L sulfate and amended with 10 mg/L DOC.

In the graphics, "ambient" mercury or MeHg represents all of the mercury in the mesocosms that was not added or derived from spike. The majority of ambient mercury, especially in soils, has accumulated over long time periods, although a small fraction of "ambient" mercury has been newly deposited to the system in rainwater. Spike mercury is that mercury added as enriched stable isotope spikes, and spike MeHg is the MeHg produced from that spike. Mass spectrometry, combined with isotope dilution mathematics, allows separation of these pools (c.f. Hintelmann and Ogrinc 2003).

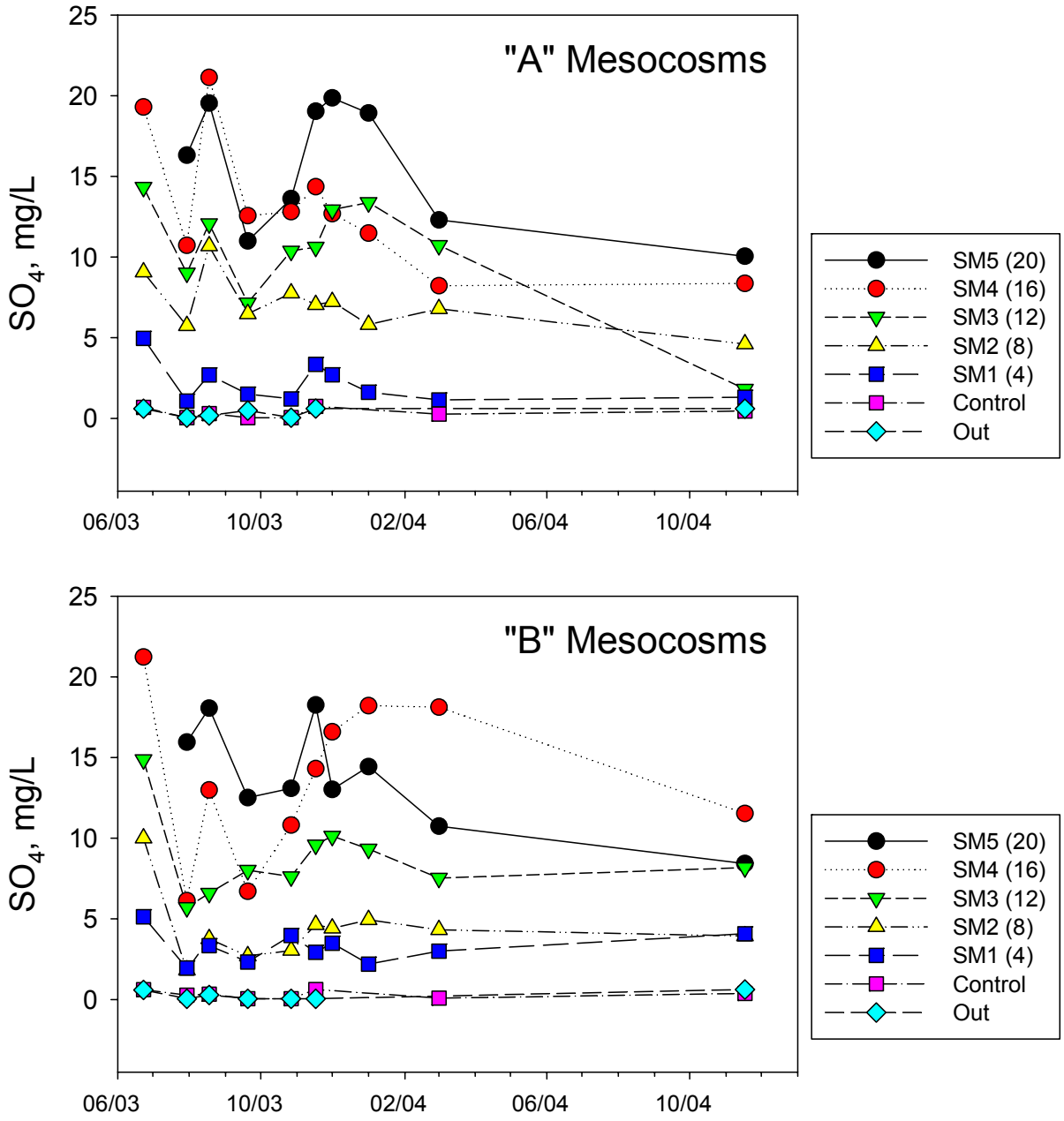


Figure 3. Surface water sulfate concentration through time in the 3A-15 2003–2004 mesocosm study. "Out" is the outside controls, "Control" is the mesocosm controls, the S treatments are sulfate-only treatments with the number representing the target sulfate level in mg/L.

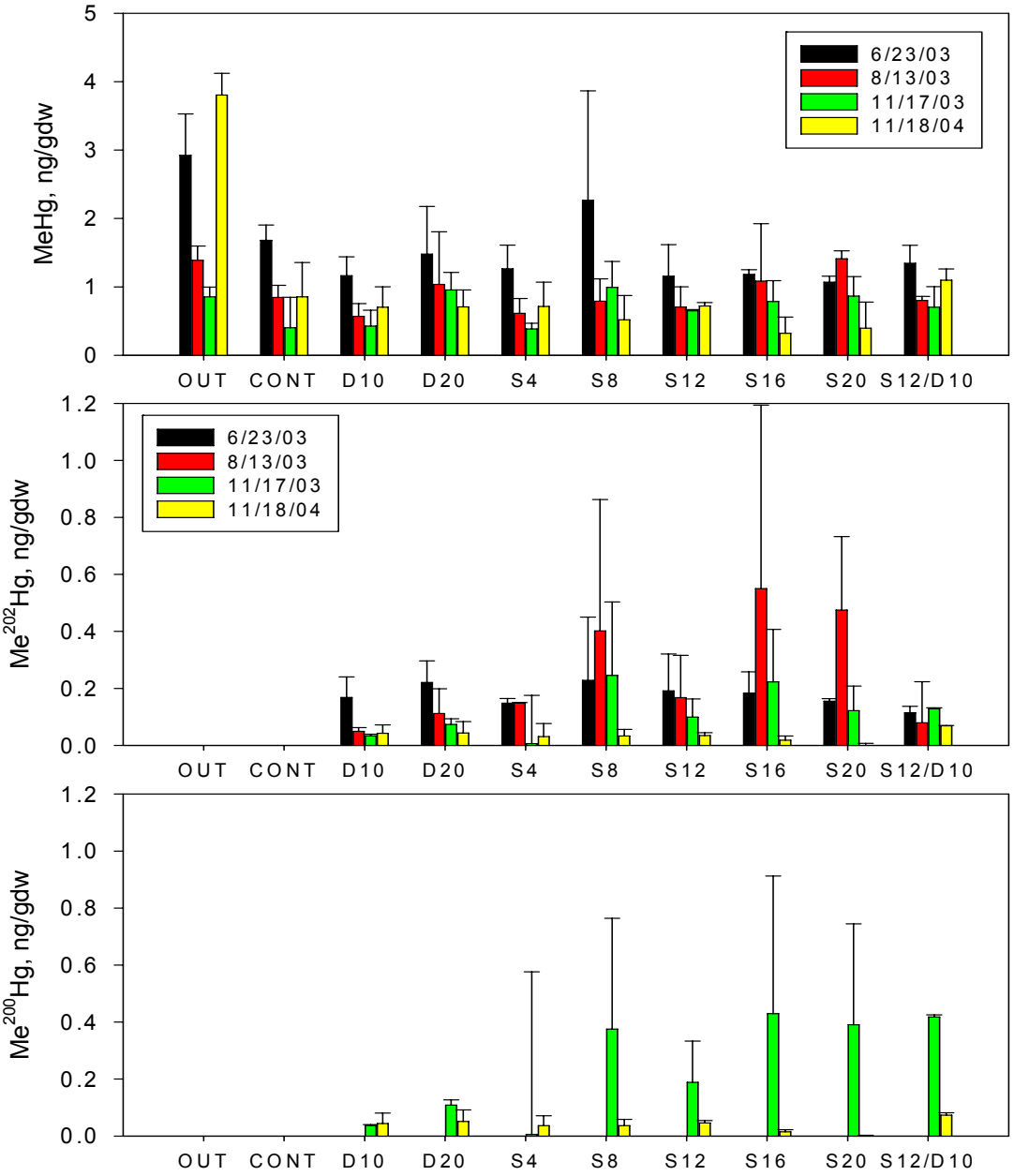


Figure 4. Average concentrations of MeHg in surface (0–2 cm) soils in the 3A-15 mesocosms. Top – ambient MeHg, middle – excess $Me^{202}Hg$, bottom – excess $Me^{200}Hg$. Each bar represents the average and standard deviation for duplicate mesocosms, on each sampling date. Triplicate cores from each mesocosm were pooled on each date. "Out" is the outside control, "Cont" is the mesocosm control, "D10" and "D30" are DOC only treatments at roughly 10 and 20 mg/L added DOC, S treatments are sulfate-only treatments with the number representing the target sulfate level, and S12/D10 are mesocosms that were treated to 12 mg/L sulfate and had 10 mg/L added DOC.

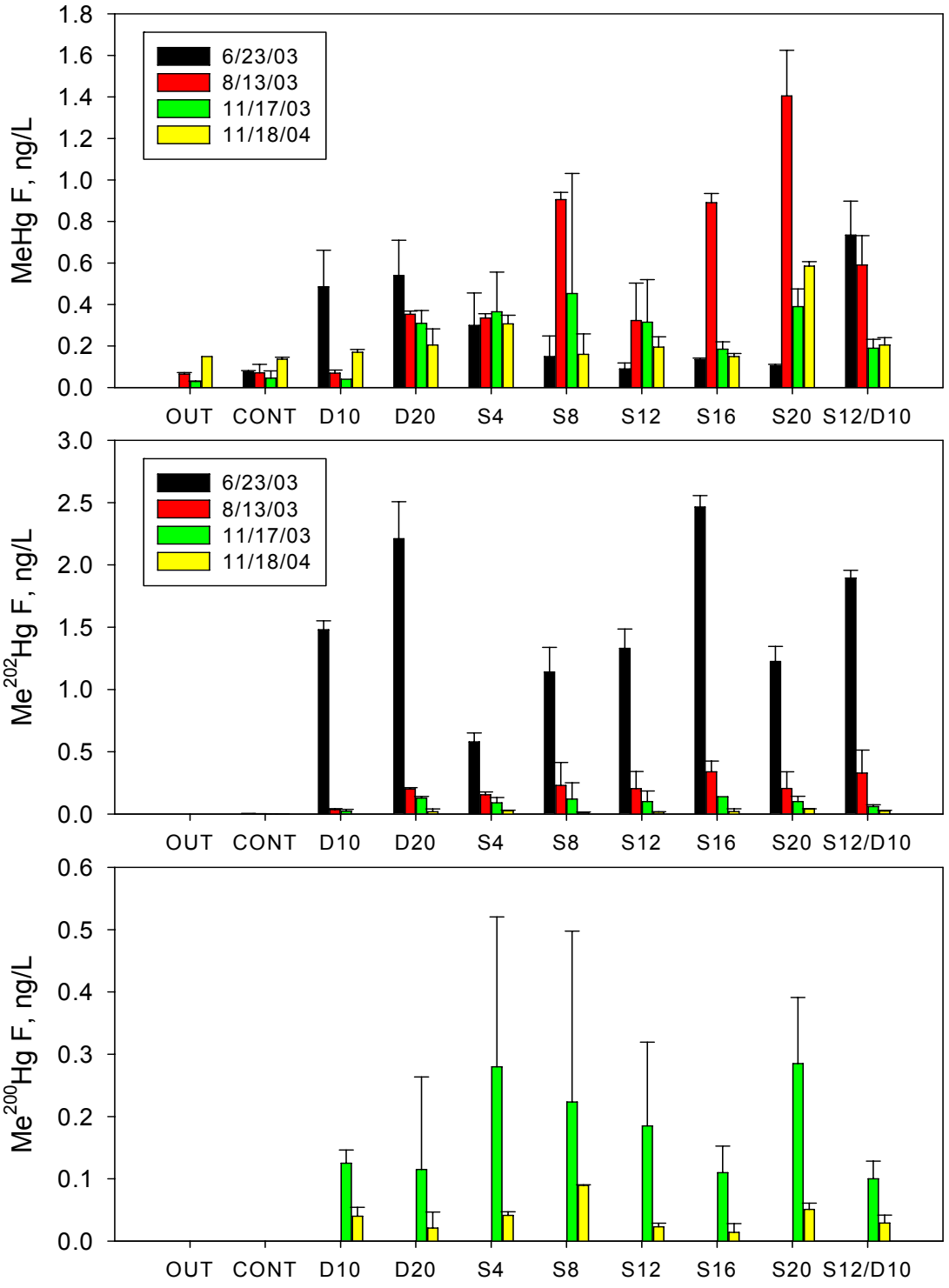


Figure 5. Average concentrations of filterable MeHg in surface water in the 3A-15 mesocosms. Top – ambient MeHg, middle – excess $Me^{202}Hg$, bottom – excess $Me^{200}Hg$. Each bar represents the average and standard deviation for duplicate mesocosms on each sampling date. See treatment descriptions in Figure 3.

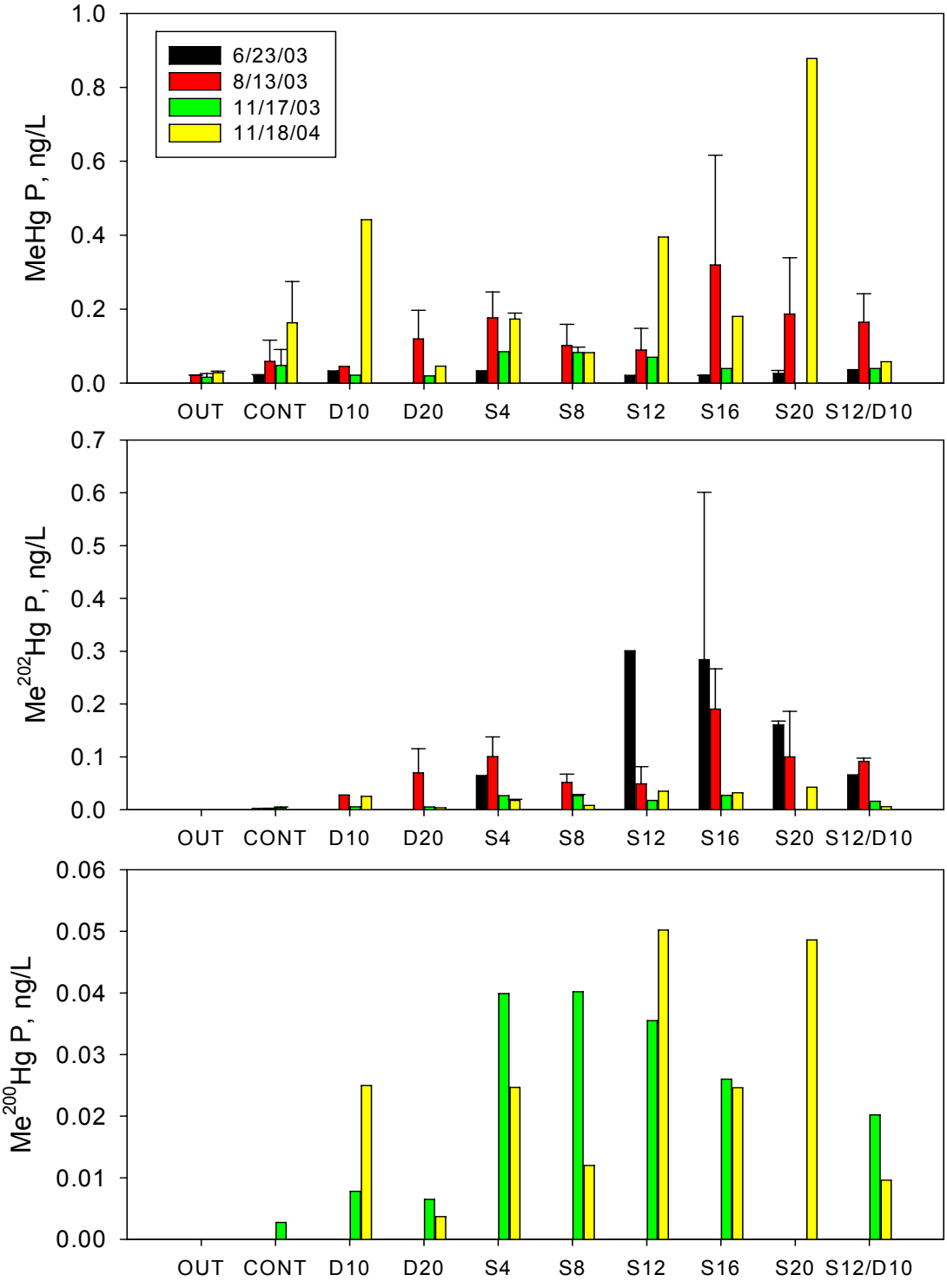


Figure 6. Average concentrations of particulate MeHg in surface water in the 3A-15 mesocosms, Top – ambient MeHg, middle – excess $Me^{202}Hg$, bottom – excess $Me^{200}Hg$. Each bar represents the average and standard deviation for duplicate mesocosms on each sampling date, except data without error bars, which were single samples. See treatment descriptions in Figure 3.

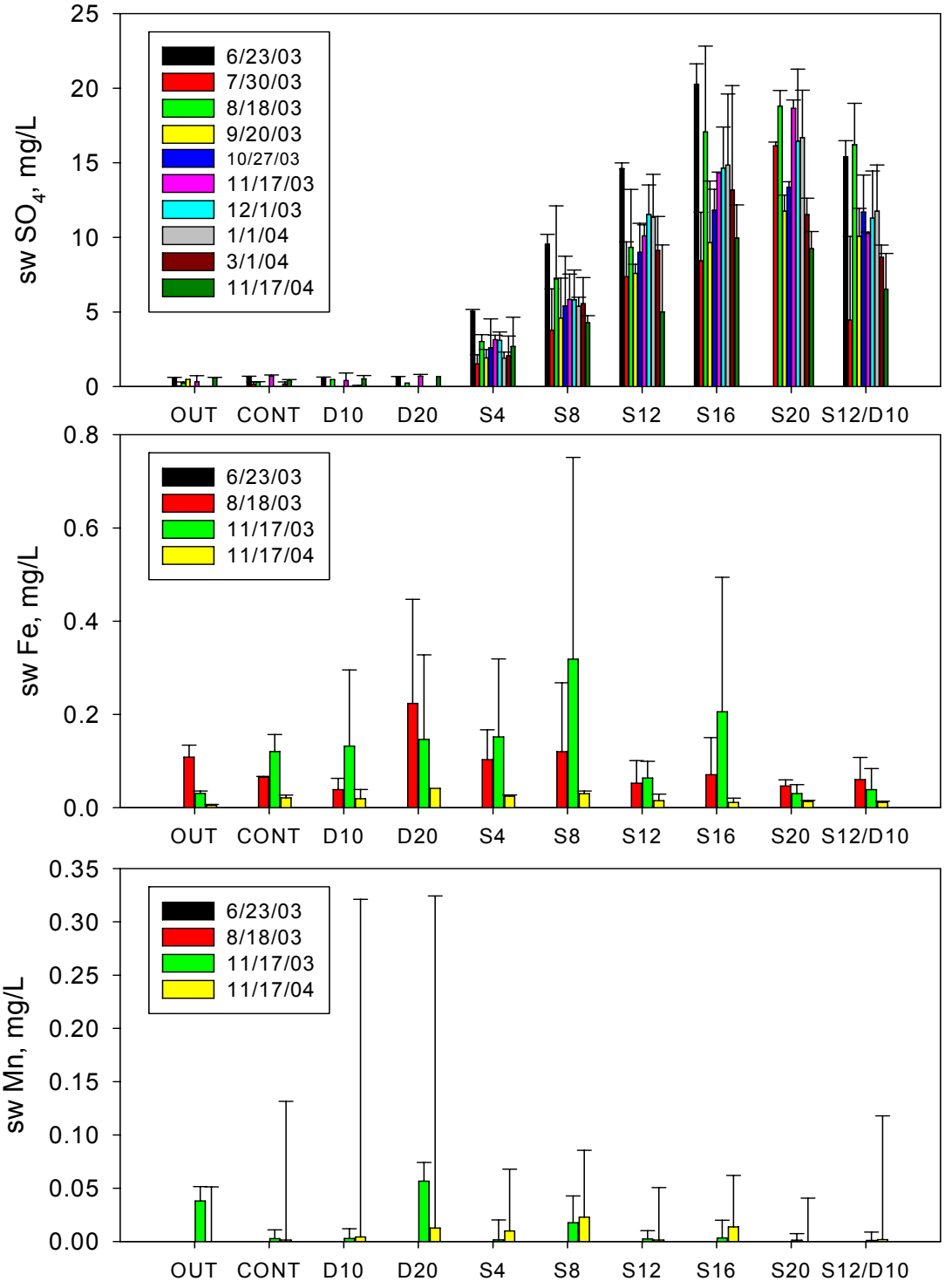


Figure 7. Average concentrations of filterable surface water sulfate (top), iron (middle) and manganese (bottom). ach bar represents the average and standard deviation for duplicate mesocosms on each sampling date. See treatment descriptions in Figure 3.

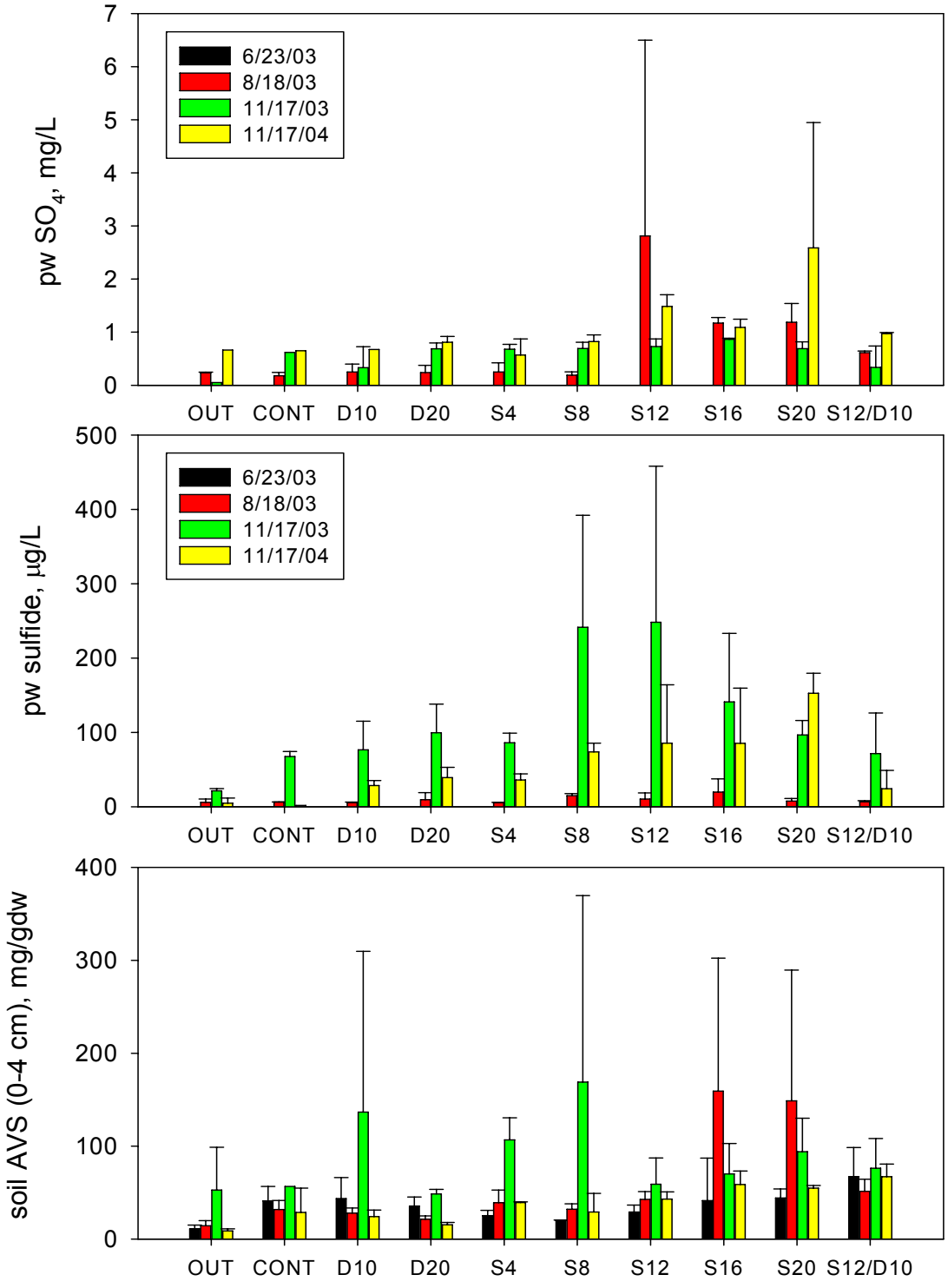


Figure 8. Average concentrations of soil pore water sulfate (top), pore water sulfide (middle), and solid phase acid volatile sulfides. Each bar represents the average and standard deviation for duplicate mesocosms on each sampling date. See treatment descriptions in Figure 3.

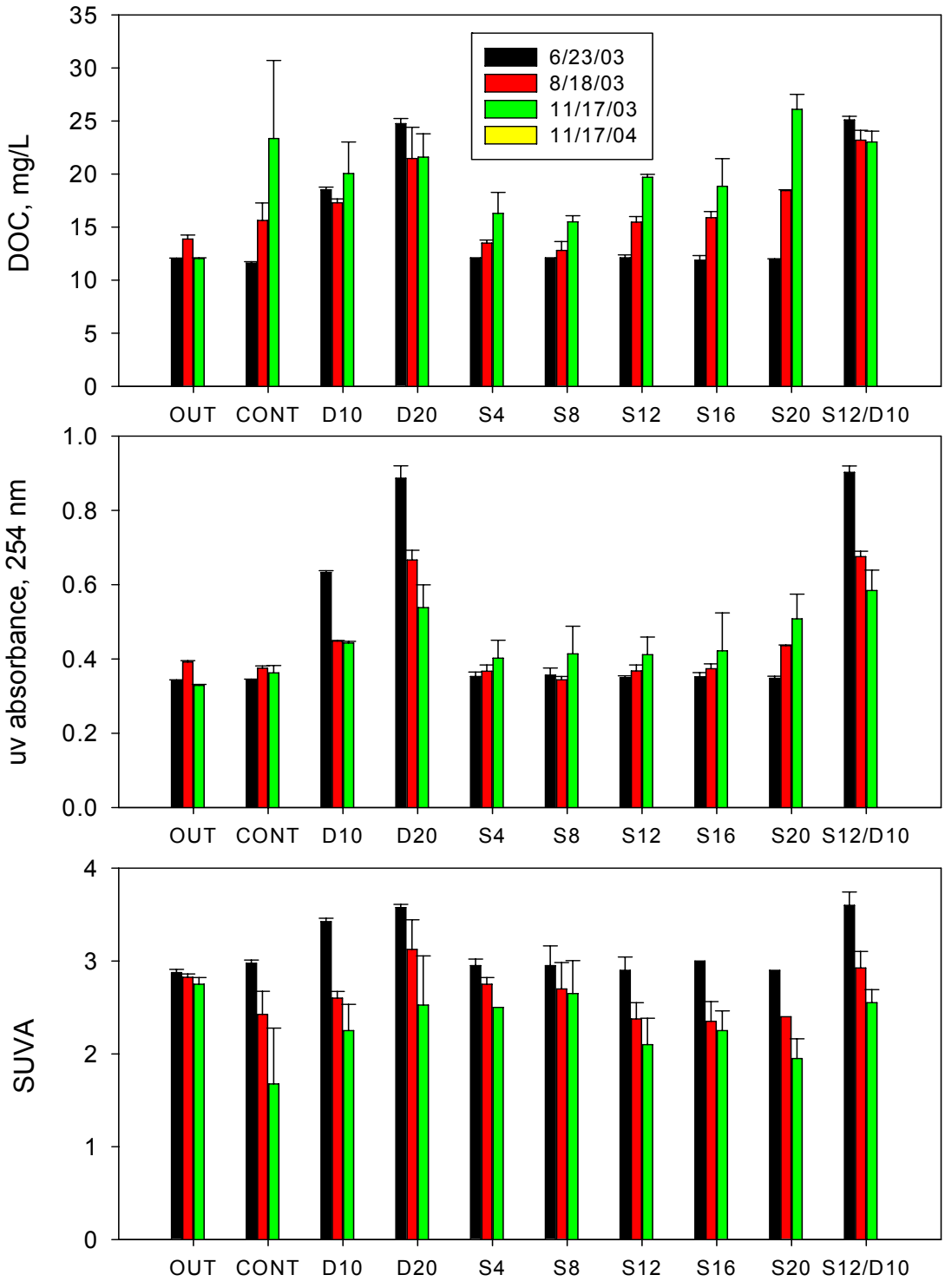


Figure 9. Average concentrations of total dissolved organic carbon (top), uv absorbance 254 nm (middle), and specific uv absorbance (bottom). Each bar represents the average and standard deviation for duplicate mesocosms on each sampling date.

The average MeHg concentrations in mesocosm surface (0–4 cm) flocs/soils over the duration of the study are shown in **Figure 4.** Spike MeHg concentrations were highest and most strongly related to sulfate additions 2–3 months after the spike was added. Excess $Me^{202}Hg$ concentrations were highest in August 2003 following the June 2003 ^{202}Hg spike; and excess $Me^{200}Hg$ concentrations were highest in November 2003 following the August 2003 ^{202}Hg spike. Spike MeHg concentrations declined significantly thereafter. Regression analysis showed significant linear relationships between sulfate dose and MeHg concentration at each of these time points. Two-way ANOVAs on date and treatment showed significant differences among dates and treatments, and significant interactions between date and treatment.

There were large differences in net MeHg production in soils from spikes between the replicate mesocosms for each treatment, as shown by the error bars. Although the mesocosms were placed in adjacent rows (<5m apart) in open slough habitat, variability among field enclosures is expected, and the B set was somewhat closer to a stand of sawgrass (still ~10 m away). Concentrations of other components (for example surface water Fe and manganese concentrations, and pore water sulfide, **Figure 4**) showed that redox conditions were often different between the A and B mesocosms sets.

Methylmercury (MeHg) concentrations in mesocosm surface waters over time are shown in **Figures 5** and **6**. Surface water ambient filterable MeHg concentrations were maximal at the time point two months after the first sulfate additions (**Figure 5**). Spike $Me^{202}Hg$ concentrations were maximal 3 days after the ^{202}Hg spike, and were strongly related to sulfate dose. The relationship between $Me^{202}Hg$ produced from spike and sulfate dose remained significant through Aug 2003. Two-way ANOVAs on date and treatment showed significant differences among dates and treatments, and significant interactions between date and treatment. After the August 2003 ^{200}Hg spike, $Me^{200}Hg$ was maximal at the first time point sampled, November 2003, and declined thereafter. However, the magnitude of the response was much less in the fall than in the summer. These experiments again confirm that MeHg concentrations in the Everglades are affected sulfate concentrations, and by the "age" of the substrate Hg pool in soils, where MeHg is formed.

Sulfate is reduced to sulfide by sulfate-reducing bacteria (SRB) in soils. In this experiment, dissolved sulfide concentrations in soil pore waters (**Figure 8**), and the most labile pool of solid phase sulfides (acid volatile sulfides) increased in response to sulfate additions. Both pools represent sulfate load that has been converted to sulfide and stored in soils. Sulfide build-up in soils reduces soil redox potential. Higher concentrations of sulfide may also inhibit MeHg production through the formation of dissolved Hg-S complexes that are less available to cells (Benoit et al. 1999a,b, 2001a,b). Dissolved sulfide remained below detection limits in surface waters (**Figure 7**).

Figure 10 shows the relationships between sulfate dosing levels and resultant MeHg concentrations in water and surface soils, at the time of maximum response. For ambient MeHg, the maximal response was about 2 months after the first sulfate dose. For both Hg spikes, the relationship between sulfate and MeHg had the steepest slope at first time point examined. Later time points showed weaker of no relationship between MeHg and sulfate. All of the relationships shown in **Figure 8** could be fit with a significant linear model.

The addition of DOM to mesocosm surface waters led to increased concentrations of both total (data not shown) and MeHg in surface waters (**Figures 4** and **5**). Resultant levels of DOC in surface waters are shown in **Figure 9**. Data from this study and prior mesocosm studies suggest that DOC influences MeHg concentrations in surface waters by enhancing the solubility of MeHg itself, and by enhancing the solubility of inorganic Hg, which is the substrate for methylation. This experiment shows that the effects of DOC on MeHg are concentration-dependent. Statistical analysis of potential interactions between DOC and sulfate are pending.

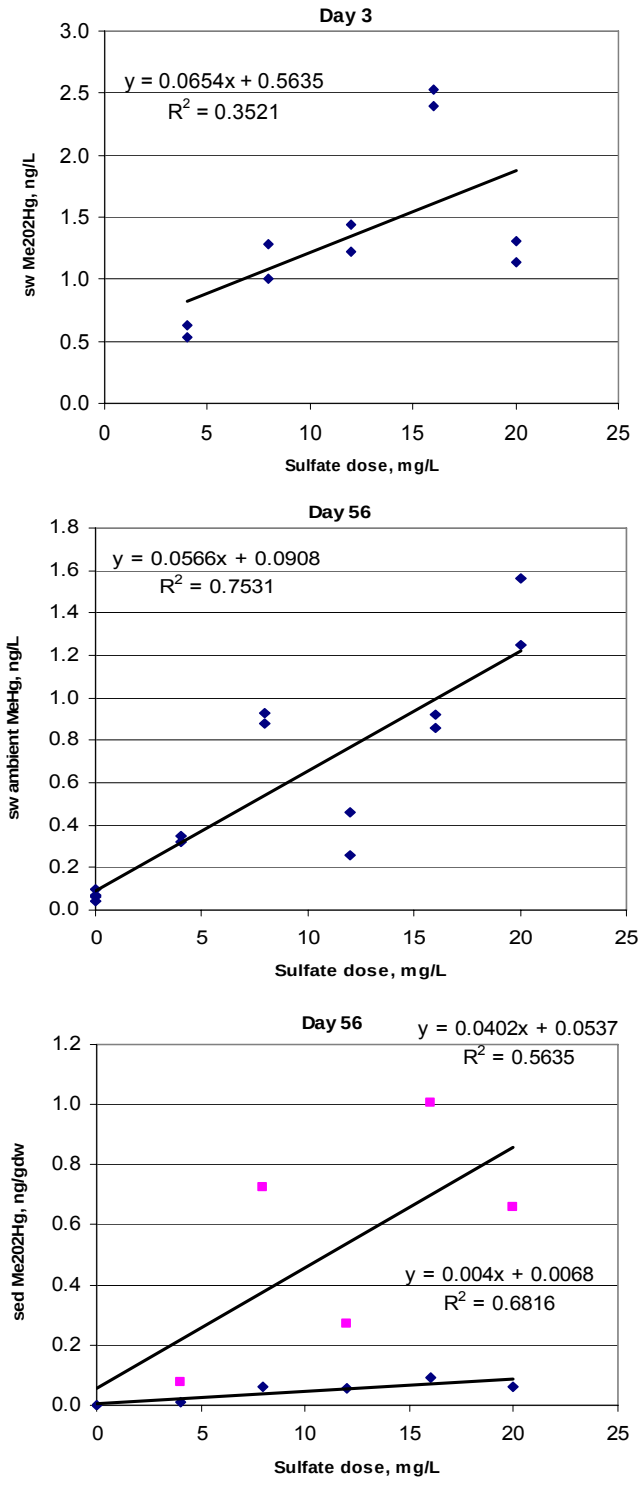


Figure 10. Relationships between sulfate dose and MeHg for surface water $Me^{202}Hg$ (top); surface water ambient MeHg (middle); and $Me^{202}Hg$ in surface soils (bottom); each at the time of their maximum response. dose. Linear regressions are shown for each relationship. For water, all of the data were regressed together. For soils, each series of mesocosms was regressed separately because of the very different responses of MeHg production in soils between the A and B series of mesocosms.

This experiment was designed to examine the quantitative response of MeHg to sulfate in surface waters, and interactions between sulfate, DOC, and Hg. Based on previous mesocosm experiments in the Everglades and on many studies in other ecosystems, we anticipated that sulfate would stimulate net MeHg production and increase MeHg concentrations in soils and surface waters. This was the case. However, we also anticipated that sulfide accumulation in soils at the higher sulfate doses would counterbalance that effect, and reduce net MeHg production. In general, however, MeHg concentrations increased linearly with the sulfate dose, up to the highest dose used in this experiment. Although the sulfate concentrations used in the experiment approach those found in more sulfate-impacted areas of the northern Everglades, the dissolved sulfide concentrations achieved in this mesocosm experiment were well below those found in WCA-2A and other sulfate impacted areas. The site of this experiment in central 3A-15 is currently a site with very low ambient surface water sulfate and pore water sulfide concentrations. We hypothesize that sediment solid phase sulfide pools (both organic sulfide and iron sulfides) were not saturated during this experiment, therefore limiting the buildup of dissolved sulfide.

Based on this experiment and on previous sulfate addition experiments at multiple sites, we hypothesize that the relationship between surface water sulfate concentration and net MeHg production will vary somewhat across the Everglades, depending in large part on sulfide accumulation from sulfate inputs. The history of sulfate loading, the mineral content of soils, and the rate of organic matter accumulation would all affect this relationship.

In this experiment at 3A-15, MeHg production was generally a linear function of sulfate loading, through 20 mg/L surface water sulfate concentrations. Sulfide concentrations in pore waters were insufficient to significantly inhibit net MeHg production. However, the zones of the Everglades where surface water sulfate is currently in the 20 mg/L range (WCA-2A and areas around canals in WCA-3A) have much higher average pore water sulfide concentrations. A model for MeHg concentrations based on both sulfate and sulfide concentrations will more accurately predict MeHg concentrations than surface water sulfate concentration alone, and biogeochemical, process-based model would provide the most comprehensive information. However, we believe that a simple tool for predicting sulfate impacts on MeHg production in the Everglades can be achieved if we can gather good information on the relationships between sulfate, sulfide, and organic matter for each of the major sulfate regimes in the Everglades Protection Area (EPA).

To summarize the findings to date from this study, sulfate additions that resulted in surface water sulfate concentrations up to 20 mg/L resulted in increased MeHg concentrations in both surface soils and surface waters. The relative magnitude of the sulfate additions was higher on the newly added Hg spikes than on ambient Hg in the mesocosms. The addition of DOM also enhanced MeHg accumulation in the mesocosms and was concentration dependent.

This study confirms and extends previous smaller-scale mesocosm studies, suggesting that net MeHg production is proportional to sulfate at sulfate concentration found across most of the EPA. Only the most impacted areas of WCA-2A and 3A have average surface water sulfate concentrations above 10–20 mg/L, concentrations at which sulfide production may begin to limit methylation in Everglades soils. This hypothesis should be tested through sulfate addition studies at other sites in the Everglades, particularly those with a history of higher sulfur loading. This study confirms prior mesocosm studies in the Everglades and in other ecosystems showing that Hg in sediments and soils becomes less available for methylation as it ages in place. Therefore, MeHg concentrations in fish reflect primarily the recent history of Hg deposition.

B. MESOCOSM STUDIES TO ASSESS THE IMPACT OF FE(III) ADDITIONS ON MEHG PRODUCTION IN EVERGLADES SOILS, SITE 3A-15, JUNE–AUGUST 2005

Iron biogeochemistry is poorly understood in relation to Hg methylation, but recent research suggests that Fe impacts Hg methylation through both chemical controls on Hg complexation and through the activity of Fe-reducing bacteria. In a study of wetland sediment slurries from San Francisco Bay, California, Mehrotra et al. (2005) observed decreases in Hg methylation rates with the addition of Fe(III), suggesting that interactions between Fe and sulfur cycles inhibit methylation in areas where sulfate reduction dominates. However, Warner et al. (2003, 2005) found measurable methylation in sediments where Fe reduction was the dominant terminal electron acceptor, although rates of methylation were lower than those observed in sulfate-reducing or methanogenic sediments. Furthermore, Hg methylation was reported in a *Geobacter* strain isolated from Clear Lake, CA (Fleming et al. 2006). Chemical inhibition of sulfate reduction did not result in complete inhibition of methylation in Clear Lake sediment cores; this decoupling of methylation from sulfate reduction suggests that another process (i.e., Fe reduction) may be responsible for some amount of *in situ* Hg methylation. In another section of this appendix, we report Hg methylation by a number of Fe-reducing bacteria, all *Geobacter* strains, in pure culture (Kerin et al. 2006).

In this study, we investigated the impact of Fe oxyhydroxide additions on net MeHg production in Everglades soils. Mesocosms installed at WCA-3A site 3A-15 were amended with three concentrations of Fe(III) oxyhydroxide (or a no-addition control) plus a stable isotope Hg spike. The effect of each Fe concentration on MeHg production was observed over a period of two months during summer 2005.

Methods. A total of 8 mesocosms (each ~1m diameter) was installed at 3A-15 in March 2005, allowing for duplicates of three Fe addition concentrations plus controls. Two control plots on adjacent Everglades slough were marked with PVC piping. In June 2005, three concentrations of Fe(III) oxyhydroxide (11.5, 23.1, and 46.2 mmoles Fe) were added to the surface water of the mesocosms. These concentrations were designated low, medium, and high Fe treatments and represent roughly one-half, one, and two times the amount of Fe accumulated in the soils at this site in one year. All mesocosms received a 7.69 μg $^{200}HgCl_2$ spike that represents roughly the annual deposition of Hg at this site. Mesocosms and control plots were sampled before dosing, 3 days following dosing, and 59 days following dosing. At each time point, 3 separate 4.8 cm diameter soils cores were removed manually from the mesocosms and combined prior to analysis. Soil interstitial waters were also sampled using a sipper (USGS). Soil analysis included solid and aqueous phase Fe, acid volatile sulfides/chromium reducible sulfides, and total and methyl Hg. Pore water and surface water analysis included for pH, temperature, sulfide, nutrients, DOC, Hg, and MeHg.

Figure 11. Fe-addition mesocosms in place at site 3A15 in central WCA 3A (photo by Cynthia Gilmour, Smithsonian Environmental Research Center).

Figure 12. Soil (left) and interstitial water (right) sampling of mesocosms from an airboat. Airboats and operators were generously provided by the South Florida Water Management District (photo by Cynthia Gilmour, Smithsonian Environmental Research Center).

Results/Discussion. The responses of MeHg to Fe(III) were mixed. MeHg was produced rapidly in surface soils from added ^{200}Hg in all mesocosms.

Figure 13 shows the concentration of $Me^{200}Hg$ in 0–2 cm depth soils 3 days and 59 days after the Hg and Fe spikes. $Me^{200}Hg$ concentrations continued to increase through 59 days in all treatments except the high-Fe treatment. Based upon a single factor ANOVA and using $\alpha = 0.05$, there was a significant difference among treatments in the production of $Me^{200}Hg$ between day 3 and day 59. The MeHg data were log-transformed to achieve normality. Significantly less $Me^{200}Hg$ was produced from ^{200}Hg in soils in the high Fe-addition mesocosms than in controls. The apparent stimulation of net methylation in the low Fe mesocosms was not significant for soils. There was no significant effect of the Fe additions on ambient net MeHg production.

In the water column, both spike and ambient MeHg concentrations increased in the low Fe treatment mesocosms relative to controls (**Figure 14**). However, Fe additions stripped total Hg out of the water column (**Figure 15**). There were no significant effects of the Fe additions on pore water sulfide concentrations (**Figure 16**). Solid phase sulfide concentrations (acid volatile and chromium reducible sulfides) were also unaffected by the Fe additions (data not shown). There was no difference between total bulk phase Fe concentrations among treatments or between dates (**Figure 17**) based upon a two factor ANOVA with $\alpha = 0.05$ ($n = 2$); error bars represent standard deviations. Similarly, no differences in dissolved Fe concentrations in soil pore waters or 0.5M HCl extractable Fe were observed (data not shown). Although Fe additions represented roughly one-half, one, and two times the amount accumulated in soils over one year, it appears that these additions were too small to create a measurable difference in the soil Fe pool in these mesocosms.

Conclusions:

- Fe oxyhydroxide additions to Everglades mesocosms had mixed effects on MeHg production. Fe(III) inhibited MeHg production in soils at the highest dose, but enhanced MeHg concentrations in surface waters at low doses.
- The Hg spike added to the mesocosms was more responsive to Fe dosing than was ambient Hg.
- Fe additions enhanced the partitioning of Hg to the solid phase.
- However, the changes in MeHg production or concentration could not be linked to significant differences in soil Fe or sulfur concentrations or speciation among treatments.
- We hypothesize that Fe may have affected Hg availability for methylation through changes in filterable Hg concentrations. Microbial Hg methylation appears to require soluble Hg in a bioavailable form.
- Additionally, Fe(III) additions may have affected the balance between microbial sulfate reduction and microbial Fe(III) reduction. Microbial activities were not examined, but not changes in pore water sulfate concentrations were observed.

In summary, we found the effects of Fe oxyhydroxide additions on MeHg production to be complex. This study illustrates that Fe oxyhydroxide additions to wetlands may either enhance or inhibit MeHg production, probably depending on initial biogeochemical conditions.

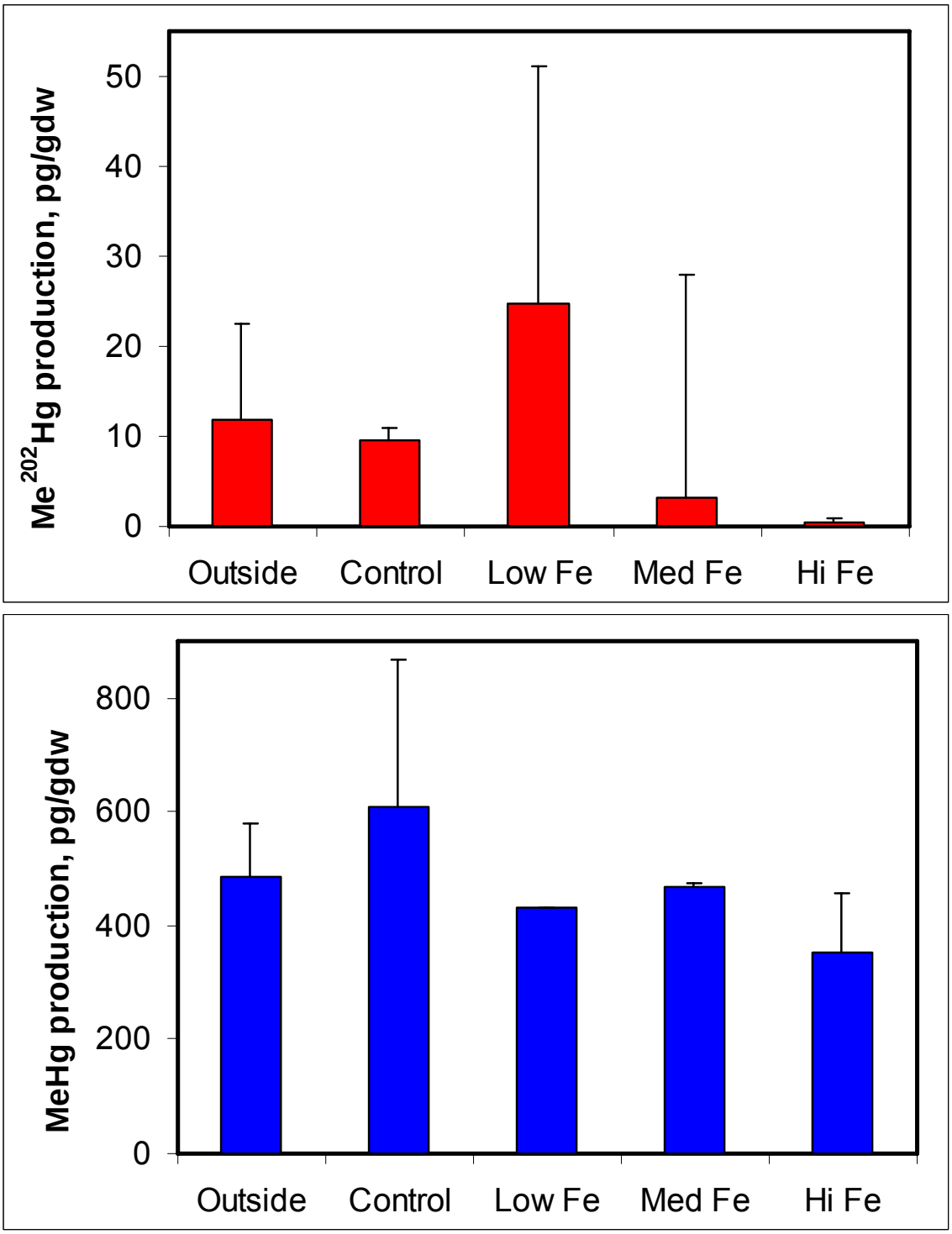
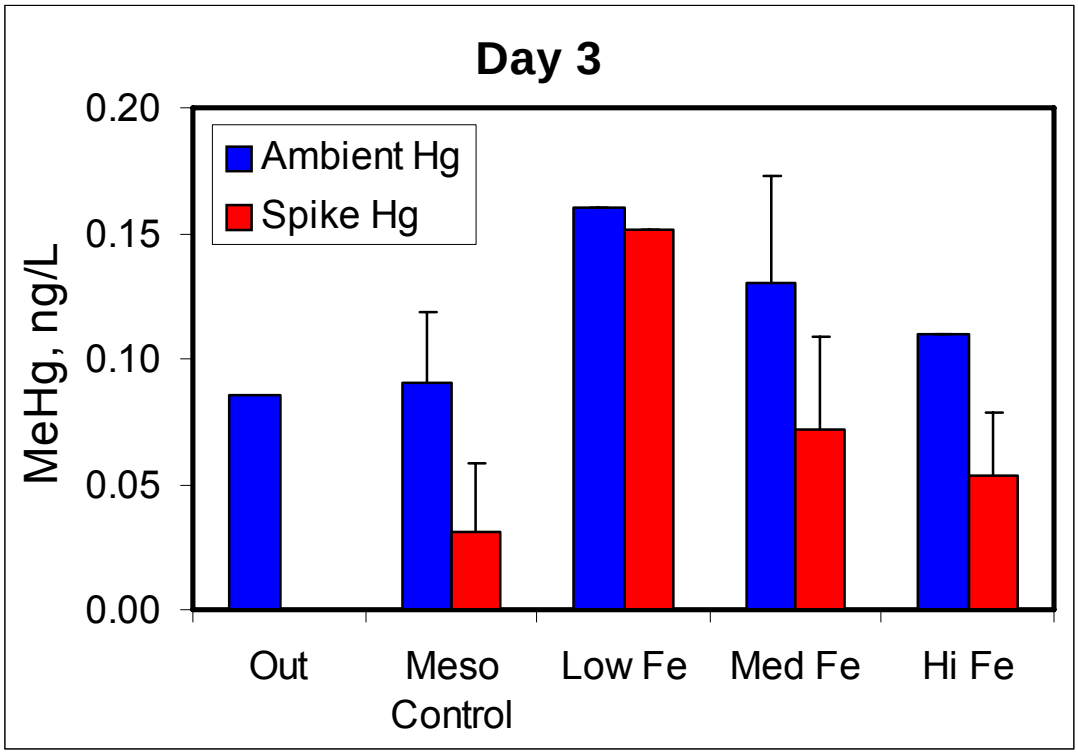


Figure 13. Net increase in surface soil spike (top) and ambient (bottom) MeHg concentration in the two months after Fe and Hg spikes.



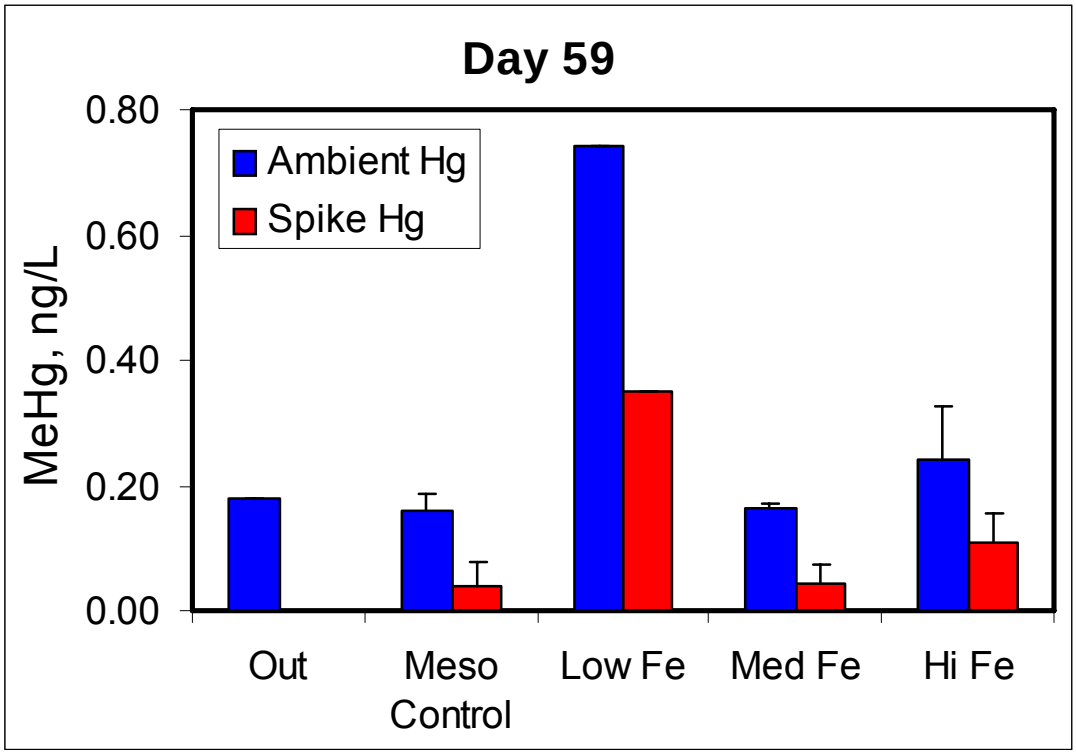


Figure 14. Filterable MeHg in mesocosm surface waters, in ng/L, at day 3 (top) and day 59 (bottom) after the Fe and Hg spikes.

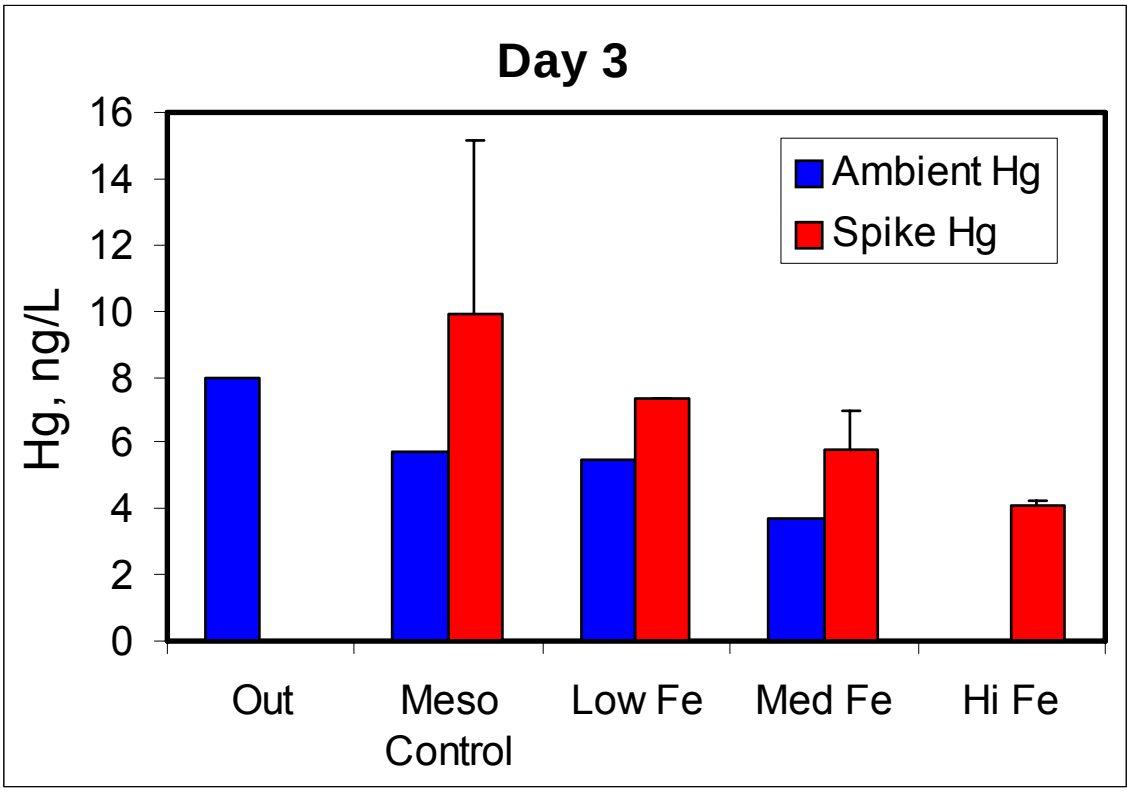


Figure 15. Filterable Hg in mesocosm surface waters.

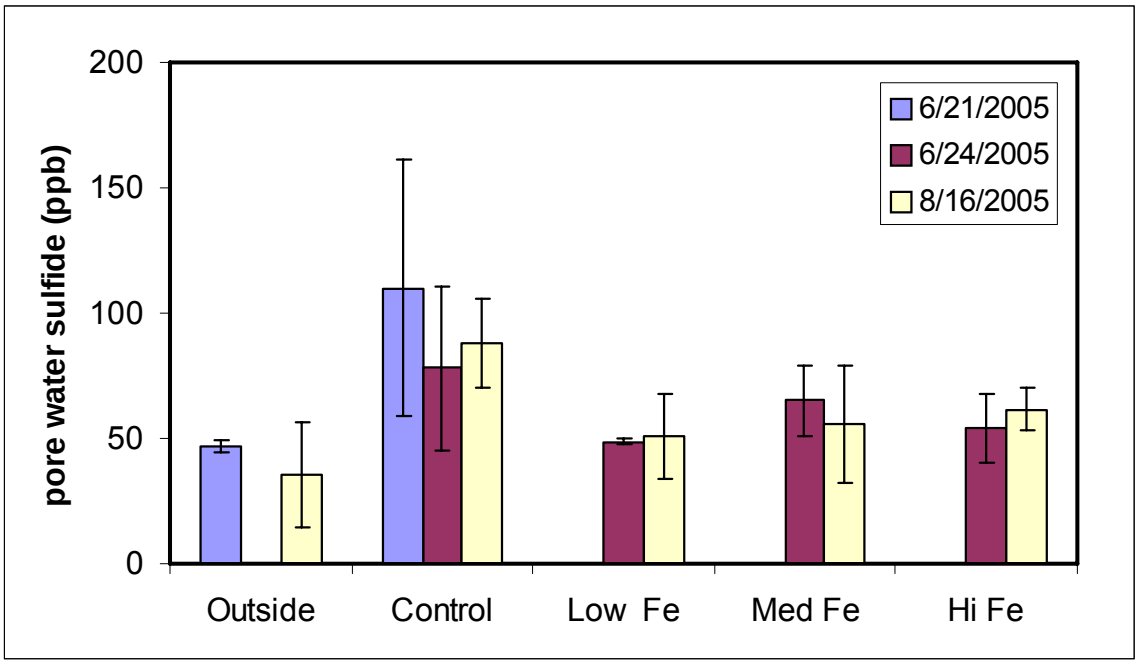


Figure 16. Mean pore water sulfide concentrations.

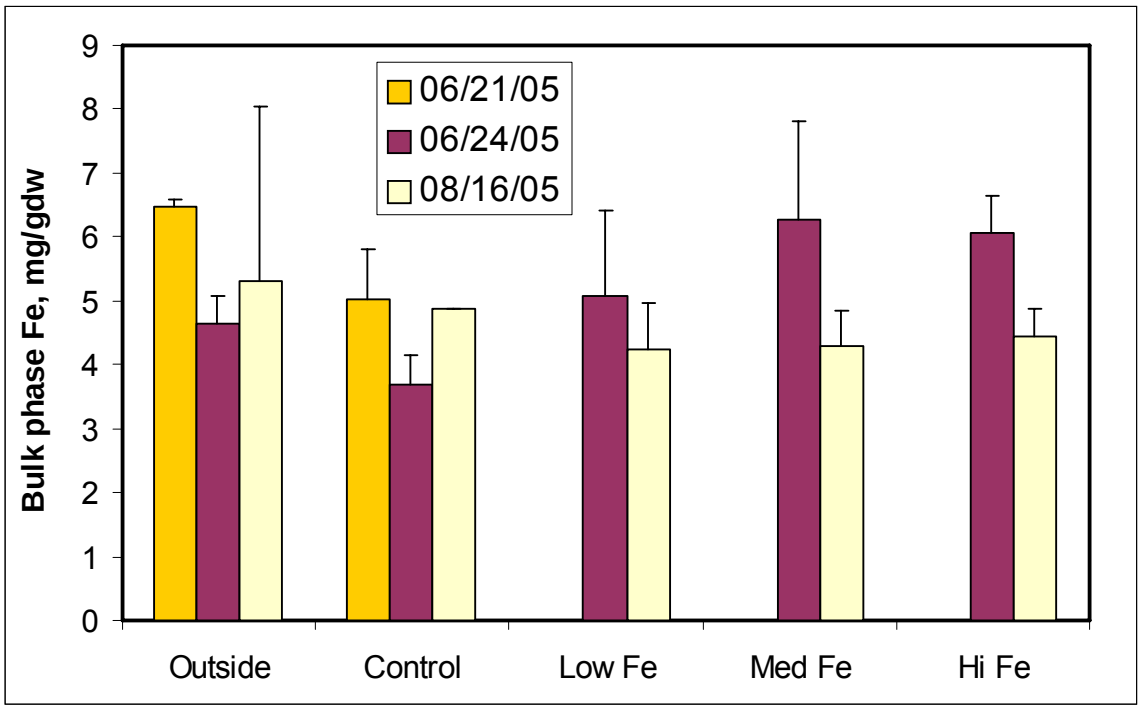


Figure 17. Mean soil concentrations of total bulk phase Fe. Bulk phase Fe concentrations in surface (0–2 cm) soils.

C. HG METHYLATION BY IRON-REDUCING BACTERIA AND SULFATE-REDUCING BACTERIA

Iron-reducing bacteria. To help understand how Fe affects Hg methylation, we sought to confirm and expand our earlier work, and the recent Fleming et al. (2006) paper, that show that some Fe-reducing bacteria have the ability to methylate Hg. This work was done in conjunction with Eric Roden of the U. Wisconsin, and M. Suzuki at U. Maryland/Chesapeake Biological Lab. Molecular work and sequencing were done by S. Werner and the Smithsonian Laboratory of Analytical Biology. This study has been published on line at Applied and Environmental Microbiology (Kerin et al. 2006), and further details can be found there.

The mercury-methylating ability of dissimilatory Fe-reducing bacteria in the genera *Geobacter, Desulfuromonas*, and *Shewanella* was examined. All of the *Geobacter* and *Desulfuromonas* strains tested methylated Hg while reducing Fe(III), nitrate, or fumarate. In contrast, none of the *Shewanella* strains produced MeHg above abiotic controls under similar culture conditions. *Geobacter* and *Desulfuromonas* are closely related to known Hg-methylating sulfate-reducing bacteria (SRB) within the *Deltaproteobacteria.*

Results are shown in **Figure 18**. Methylation was measured using Hg-stable isotopes and distillation/methylation/ICP-MS. Methylation of Hg significantly above that in uninoculated controls (T-tests, $p < 0.05$, two-tailed, unequal variances) was observed on Fe-reducing medium in *G. metallireducens, G. sulfurreducens*, *G. hydrogenophilus*, and *D. palmitatis*, but not *S. alga,* or *S. putrifaciens.* While growing on electron acceptors other than Fe(III), both *G. metallireducens* and *G. sulfurreducens* produced MeHg above abiotic controls while *S. oniedenis* and *S. putrifaciens* did not. The small percentages of methylation observed in abiotic controls are attributed to abiotic formation of MeHg in the experiment or during analysis. It is important to note that the *Geobacter* strains tested produced MeHg during growth on either Fe(III) or other electron acceptors (nitrate or fumarate). This indicates that active Fe(III)-reducing electron-transport chains are not neccessary for Hg methylation in these strains.

These results, in combination with the observation by Fleming et al. (2006) of methylation by a *Geobacter* isolate, suggest that ability to methylate Hg may be common among the *Geobacteraceae*. However, the observed lack of methylating capability among the *Shewanella* strains tested (all *Gammaproteobacteria*) shows that the ability to methylate Hg is not ubiquitous among Fe(III)-reducing bacteria.

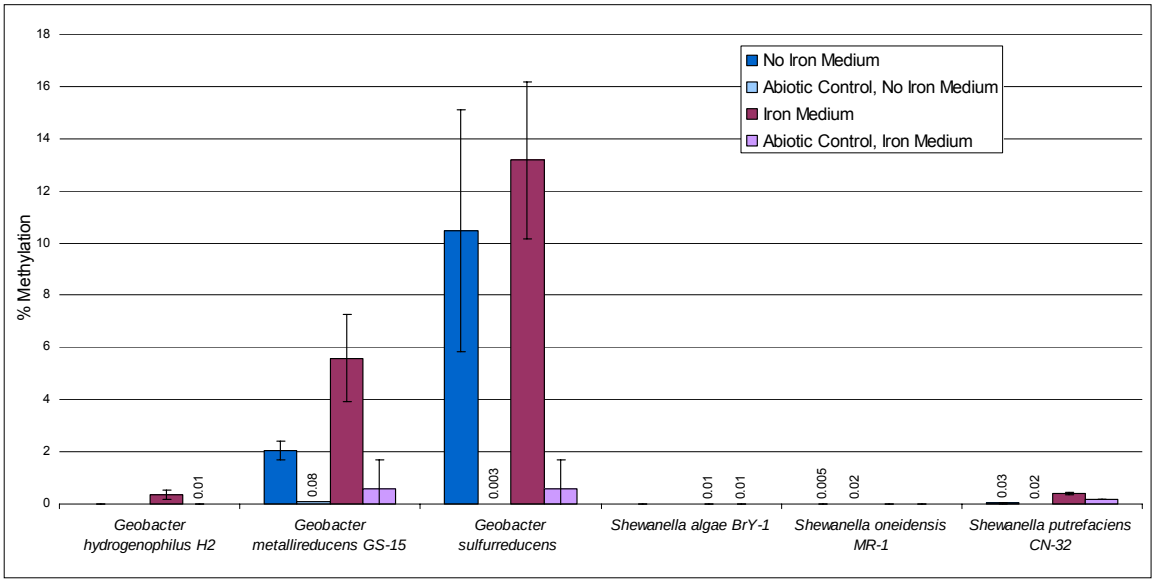


Figure 18. Observed MeHg production for six pure cultures of FeRB, expressed as a percentage of 10 ng/mL inorganic $HgCl_2$ methylated.

To date, essentially all strains for which Hg methylation has been demonstrated fall in the *Deltaproteobacteria* (Benoit et al. 2001; Compeau and Bartha 1985; King et al. 2000; Pak and Bartha 1998). These include SRB from the orders *Desulfovibrionales* and *Desulfobacterales*. However, it is important to note that the ability to produce MeHg is not ubiquitous among SRB in these families. Recently, we have applied molecular phylogenetic techniques to better understand the distribution of Hg-methylating ability among the SRB, Previously cultured strains of SRB from many environments were tested for methylation ability, and the organisms were identified by sequencing the 16S rRNA gene. This information was used to construct the first phylogenetic tree for Hg methylation (**Figure 19**). It suggests that mercury methylation is widespread but relatively rare among the SRB. Further studies are needed to ascertain whether Hg methylating capability is randomly distributed among *Proteobacteria* or related to phylogeny. Improved understanding of the phylogenetic distribution of Hg methylation capability may provide insight into the biochemical process of MeHg production within cells, and the relationships between microbial community structure and methylation rates among environments.

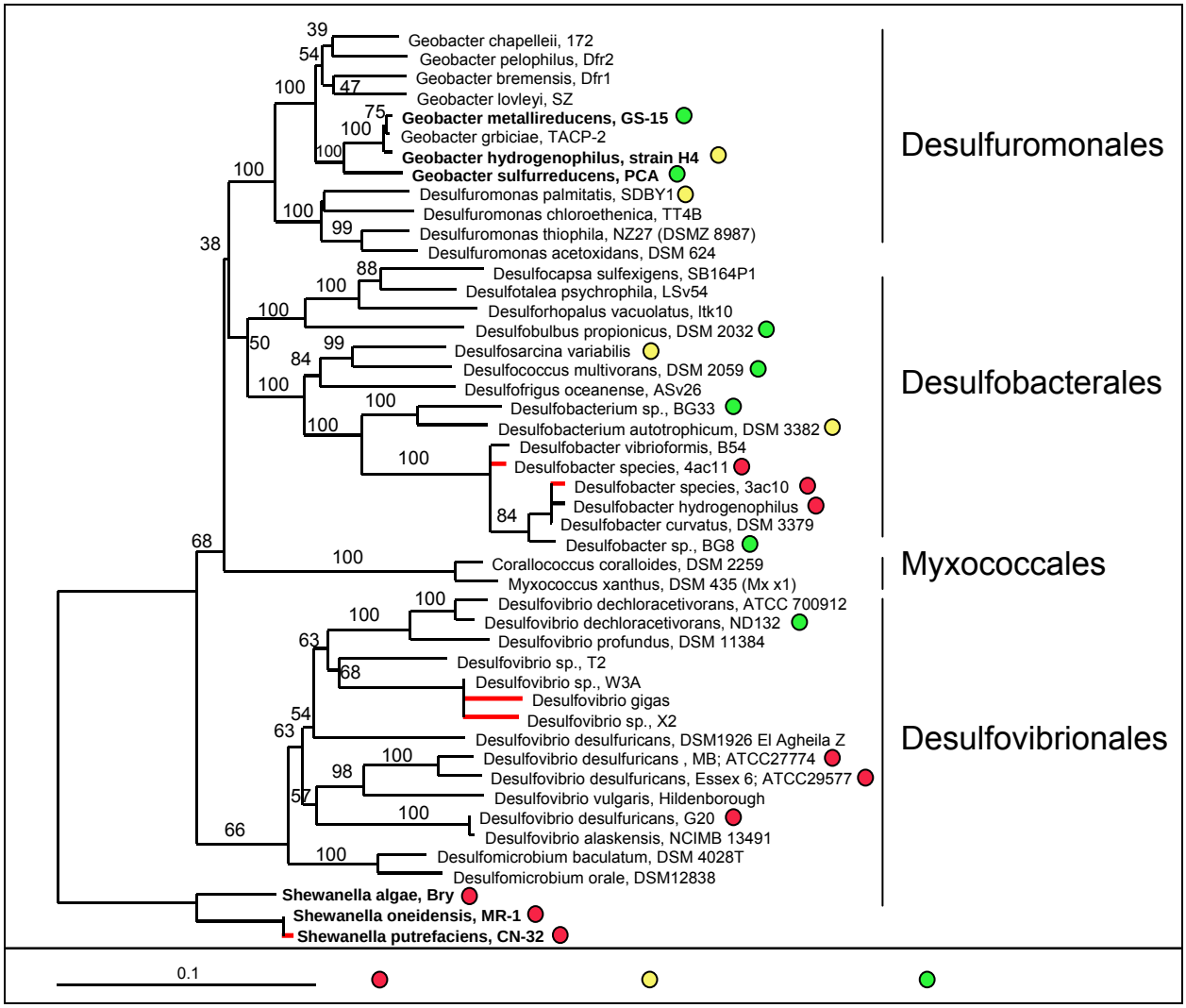


Figure 19. Phylogenetic reconstruction (Kimura Distance, Neighbor Joining, 1326 positions) of FeRB and SRB tested for Hg methylation, and related organisms. Red lines indicate sequences added by ARB parsimony with fixed original topology. Bootstrap values are for black branches only, and were based on 100 randomly resampled sets. Strong Hg methylators (green) methylate more than 0.5 percent of added (10 to 100 ng/ml) Hg. Weak methylators (yellow) methylate more than 0.05 percent of added Hg. Non-methylators (red) produced no significant MeHg above abiotic blanks (from Kerin et al. In review).

D. EXAMINATION OF THE EFFECT OF DOC ON HG SPECIATION IN ANOXIC WATERS

The dissolved phase complexation of Hg is a controlling factor in the bacterial production of MeHg (MeHg), the form of Hg that bioaccumulates in aquatic organism (Barkay et al. 1997; Benoit et al. 1999b; Benoit et al. 2001b; Benoit et al. 2001c). While both laboratory and field studies have shown that Hg complexed with DOM dominates the speciation of Hg under oxygenated conditions (Hintelmann et al. 1997a; Babiarz et al. 2001; Benoit et al. 2001a; Choe et al. 2004; Hsu et al. 2003; Lamborg et al. 2003; Ravichandran 2004), the complexation of Hg under natural anaerobic conditions has not been thoroughly investigated. Since mercury methylation occurs predominantly under anaerobic conditions and is partially controlled by the complexation of Hg in these environments (Benoit et al. 2003), the complexation of Hg under these conditions is extremely important. Under sulfidic conditions, thermodynamic models predict and laboratory studies have demonstrated that Hg-sulfide complexes dominate the Hg speciation as a result of the stronger affinity of Hg to reduced sulfur relative to DOM (Dyrssen et al. 1991; Benoit et al. 2001a), but the complexation of Hg under natural sulfidic conditions has not be measured. Since the speciation of Hg has never been measured under natural sulfidic conditions, thermodynamic speciation models used to predict the complexation of Hg in the presence of sulfide have not been validated.

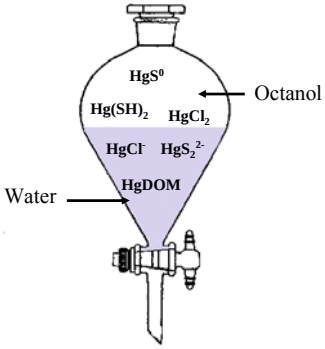
A series of studies was conducted to examine the complexation of Hg in natural sulfidic conditions and laboratory solutions (Miller 2006; Miller et al. in review). Octanol-water partitioning extractions (**Figure 20**) and centrifuge ultrafiltration were the techniques chosen to separate complexes by size and charge. While thermodynamic models indicate that inorganic Hg-sulfide complexes should dominate the speciation of Hg under these conditions, these species were not the most abundant species measured in natural samples. Using laboratory studies, we found that our current thermodynamic models did not accurately predict the complexation as a result of the interaction of Hg with DOM in the presence of sulfide.

Octanol-water extractions of anoxic pore waters showed much lower concentrations of neutrally charged Hg complexes than were predicted by our previous model (**Figure 21**). Experimental studies with model organic matter compounds and sulfide in the lab confirmed that DOC limits the formation of HgS^0 (**Figure 22**).

This work demonstrated the existence of a previously unknown complex between Hg, sulfide, and DOM. Miller et al. (2006) proposed a ternary structure for this complex, proposed as DOM-S-Hg-S-DOM, although the structure is not known. The discovery of this complex means that thermodynamic models for Hg complexation — required to construct biogeochemical models for Hg methylation — will need to be updated to consider this finding. Our findings suggest that thermodynamic models overestimate the abundance of inorganic Hg-sulfide, because they do not take into consideration the interaction of Hg, sulfide, and DOM. Estimation of a formation constant for this complex, required for modeling, is underway.Importantly, the interactions between Hg, DOC, and sulfide are likely to be highly dependent on the chemical characteristics of the DOC, and therefore additional research on those interactions will be needed to construct useful models.

The Use of D_{ow} to Measure Mercury Speciation in Natural Systems

- D_{ow} allows direct measurement of dissolved neutral Hg species
- Method developed for Hg in natural waters by Carrie Miller, to estimate the concentration of postulated dissolved neutral Hg-S species *in situ*



$$K_{ow} = \frac{[HgL]_{oct}}{[HgL]_{water}} \qquad D_{ow} = \sum \alpha_i (K_{ow})_i$$

Figure 20. Method diagram for the octanol-water extraction method for neutral Hg complexes in natural waters (from Miller 2006).

D_{ow} of Hg in natural samples

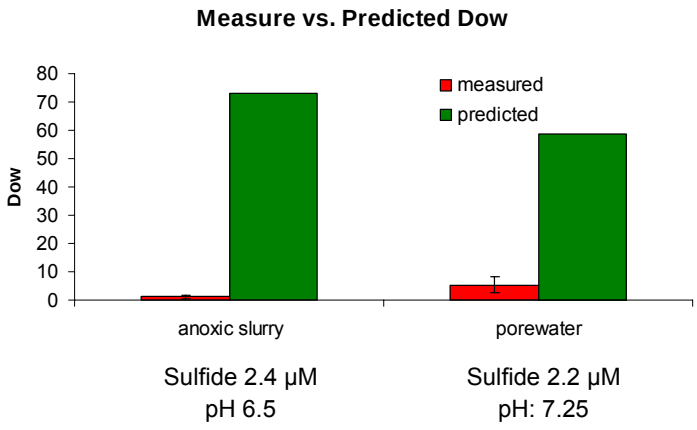


Figure 21. Measured (red) and predicted (green) concentrations of HgS in two sulfidic natural waters (from Miller 2006).

D_{ow} of Hg in DOM: sulfide experiments

- Solutions: Suwannee River or Everglades DOM, + sulfide and Hg-199
- Experiments
  A: HS 27.3 uM; pH 7.3; DOM 30 mg/L
  B: HS 20.8 uM; pH 6.3; DOM 30 mg/L
  C: HS 26.0 uM; pH 7.4; DOM 30 mg/L
  Everglades: HS 29.7 uM; pH 6.25; DOM 20 mg/L
- D_{ow} for DOM: 0.1-3.3

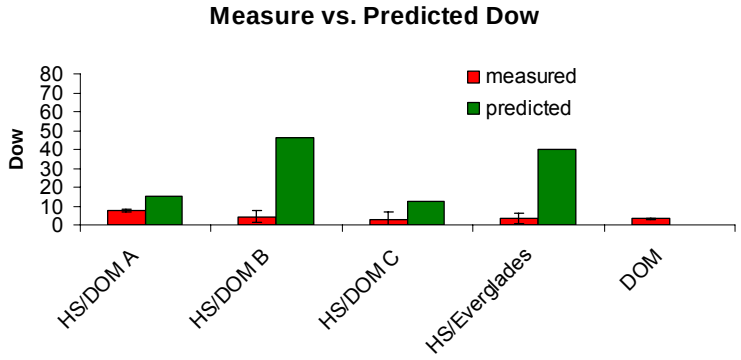


Figure 22. Measured (red) and predicted (green) concentrations of HgS in experimental mixtures of DOM and sulfide in the laboratory (from Miller 2006).

ACKNOWLEDGEMENTS

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