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

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BIOGEOCHEMICAL RESPONSE TO AQUEOUS SULFATE ADDITIONS IN WCA-3A MESOCOSMS

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INTRODUCTION

The wetland ecosystems of the Everglades have undergone varying degrees of eutrophication as a result of increased loading of nutrients from anthropogenic sources, facilitated by the shift during the last century from a rainfall-driven, sheet flow wetland mosaic to a hydrologic regime dominated by canal discharges (McCormick et al., 2011). Phosphorus enrichment of surface waters, soils and vegetation in the vicinity of drainage canal outflows was documented in great detail during the 1990s (e.g., Davis, 1994; Koch and Reddy, 1992; DeBusk et al., 1994). Subsequent research efforts led to management programs that have succeeded in markedly decreasing P loading to the Everglades (Baker et al., 2014). More recently, attention has been focused on sulfate enrichment in the Everglades, which is similarly attributed to loading from external anthropogenic sources (Orem et al., 2011).

Historically, the surface water sulfate concentration in the Everglades was likely ≤ 1.0 mg/L (Scheidt and Kalla, 2007). Today, sulfate concentrations range from 70 mg/L to ≤1.0 mg/L, along a rough north-to-south gradient (Julian et al., 2014). Agricultural runoff represents the largest source of sulfate loading to the Everglades, by mass (Bates et al., 2002; Julian et al., 2014). However, other potentially important sources, of both natural and anthropogenic origin, have been identified. Based on regional estimates, atmospheric deposition of sulfate (e.g., from sea water aerosols, fossil fuel combustion and other sources) was equal to about 12% of the load delivered by surface waters to the EPA. The contribution of atmospheric sulfate may be relatively greater in the unenriched interior portions of the central and southern Everglades (Bates et al., 2002; Orem et al., 2011), since rainfall sulfate concentrations (1.0 – 4.3 mg/L; Bates et al., 2002) may be several times the water column concentration. Groundwater is a source of sulfate to Everglades surface waters, although the magnitude of its contribution to surface water sulfate is uncertain, and confounded by complex groundwater-surface water interactions (Bates et al., 2002). Bates et al. (2002) measured sulfate concentrations of 3.8 – 16 mg/L in shallow groundwater underlying Water Conservation Area (WCA) 2A in the northern Everglades, while a mean sulfate concentration of 23 mg/L was reported by Radell and Katz (1991) for the shallow Biscayne Aquifer underlying the southeastern Everglades.

Sulfate has been implicated in contributing to two major biogeochemical threats to the well-being of the Everglades: P enrichment and methylmercury contamination (Gilmour et al., 1998; Orem et al., 2011). Enhanced mobilization of P, referred to as "internal eutrophication" (Lamers et al., 1998), may occur by several mechanisms in sulfate-enriched wetlands (Smolders et al., 2006). As a terminal electron acceptor, excess sulfate can increase the rate of organic matter decomposition (D'Angelo and Reddy, 1999), with resultant release of stored P (Smolders et al., 2006), when C:P ratios are sufficiently low (Lamers et al., 2001). In areas where much of the total P is bound to iron minerals, sulfide produced from the reduction of sulfate can precipitate with that iron and reduce the number of binding sites available for P, thereby increasing P mobility (Lamers et al., 1998; Smolders et al., 2006). Less commonly, sulfate ions can compete directly with P for anion binding sites in the soils (Beltman et al., 2000).

Studies of methylmercury (MeHg) production in various wetland and aquatic ecosystems have demonstrated a strong relationship between sulfate and Hg methylation by sulfate-reducing bacteria (SRB) (Orem et al., 2011; Gilmour, 2011). This is thought to occur as a result of increased metabolic activity of SRB in the presence of sulfate (Gilmour, 2011). Although most regions of the Everglades exhibit excessive tissue Hg levels in fish and predator animals (Orem et al., 2011), the overall role and relative importance of sulfate (as well as sulfide) in the production and bioaccumulation of MeHg in the Everglades is not well understood (Liu et al., 2008; Julian et al., 2014). Several studies have reported links between sulfate concentration and MeHg levels in the Everglades based on broad-scale environmental monitoring data (e.g., Orem et al., 2011; Gabriel et al., 2014), but attempts to establish this relationship experimentally have not proved definitive (Gilmour et al., 1998; Gilmour et al., 2007b). Unfortunately, myriad interactions among other factors such as inorganic Hg bioavailability and water/porewater constituents such as dissolved organic matter (DOM) and dissolved iron influence or control Hg cycling in other ecosystems (Hsu-Kim et al., 2013). Evidence for the importance of inorganic Hg loading (Pollman, 2012), DOM (Liu et al., 2008; Aiken et al., 2011), and a suite of other surface water constituents (Zheng et al., 2013) on MeHg production and accumulation in the Everglades has emerged recently. In particular, the seasonal dry-down/reflood cycle may be especially important to the Everglades Hg cycle by liberating key "ingredients" for Hg methylation, for example, sulfate, inorganic Hg, DOM (Krabbenhoft and Fink, 2001; Zheng et al., 2013).

Most of the current knowledge of the role of sulfate in the Everglades is based on data from research in other ecosystems or regions, and on data collected from field monitoring of water, soils and biota in the Everglades. Thus, there is a paucity of hypothesis-driven experimental data on which to base ecosystem models and management decisions for the Everglades. In order to address this data gap we conducted a multi-year mesocosm field study in a relatively unimpacted region of the Everglades, using a sulfate dose-response approach to assess biogeochemical changes under sulfate-enriched conditions.

The primary objective of the mesocosm study was to determine changes in surface water, porewater and soil pools of sulfur (sulfate, sulfide and total S), total Hg and MeHg, nutrients, and dissolved organic carbon (DOC), and concurrent changes in relevant physicochemical parameters, at varying levels of sulfate enrichment.

METHODS

Description, operation and routine sampling of mesocosm enclosures

In late 2009 and early 2010, we established *in situ* mesocosm enclosures in a ridge and slough community in WCA-3A, a large (ca. 200,000 ha) hydraulic impoundment containing much of the remaining pristine Everglades marsh. The mesocosm site is located 9 km NNW of long-term water quality monitoring station CA315 (herein noted as site 3A-15) in south-central WCA-3A, in a relatively unimpacted (surface water TP concentration of about 4 µg/L; sulfate concentration typically <1 mg/L) region of the Everglades (Figure 1). The mesocosm enclosures consisted of translucent 1.5 m-diameter, open-top, open-bottom, fiberglass cylinders that were carefully pushed into the soils until contacting bedrock. Each enclosure effectively isolated a parcel of water column, soils and vegetation on the ecotone between a ridge and a slough. The wall of each mesocosm enclosure was fitted with two, 2-way valves to maintain a hydrostatic head pressure within the enclosures equal to the outside water. This important design feature ensured that differences in head pressures inside and outside of the enclosures, which may arise from pumping events or heavy rainfall, did not result in the bulk transport of porewaters into or out of the enclosures. Each mesocosm enclosure had two 5-cm holes with tightly-fitting rubber stoppers that were opened during water exchanges and closed otherwise, to maintain experimental water quality conditions within each enclosure.

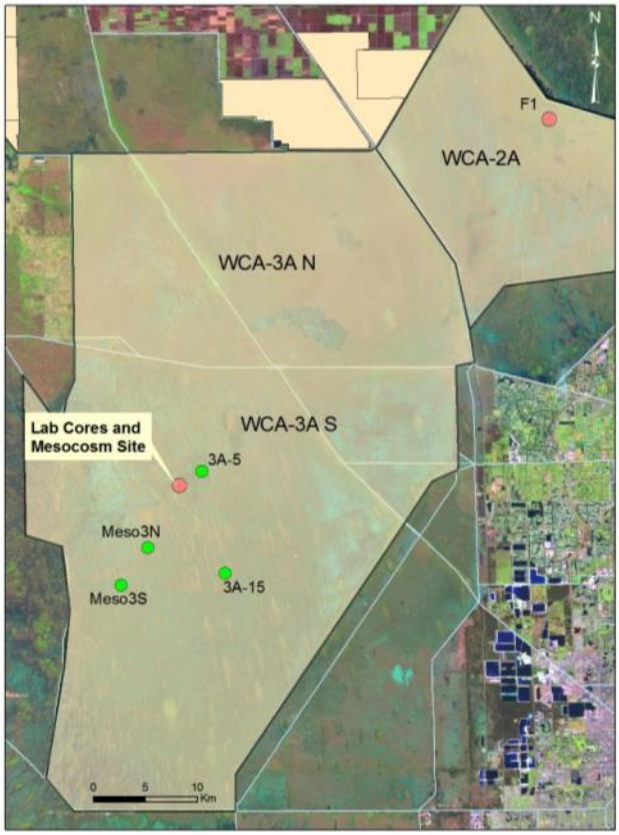

Figure 1. Location of mesocosm facility in Everglades WCA-3A

After an eight-month post-installation recovery period, initial amendment spiking and water sample collection was initiated (July 2010). We followed a detailed process of replacing previously amended site water with fresh, unamended water prior to re-amending (Figure 2). The nearby outside surface water in the slough served as the raw water source. The interval between consecutive flushing and amending cycles was two weeks. The slough surface water was first collected in a large sump, then drawn to smaller "mini-sumps" (Figure 3), and then pumped to 2-3 enclosures per "mini-sump" via a manifold (Figure 4). Prior to pumping, stoppers were removed from the sidewalls of the enclosures to allow exchange between the inside and outside surface waters. Care was exercised during the exchanges so as not to agitate the nearby vegetation or soil surface in order to prevent a sampling artifact by dislodging or resuspending particles. Prior to the exchange of water in the mesocosms, the feed lines supplying the slough water were flushed for approximately 0.5 hour, or until the water was particle-free. The two-week-old unamended (control) and amended water inside the enclosures were exchanged a total of five times their standing water volumes with the fresh outside slough water. After the five-fold water exchange, the enclosure sidewalls were stoppered and surface waters collected and analyzed for the same chemical constituents that were being added as amendments later that day (Figure 2). This was performed to determine whether the water exchange had been complete enough to flush the residual amendments added two weeks prior. The amendments were then added to the water column of selected enclosures by flow-controlled gravity from a 5-gal bucket containing stock solutions and a wand fitted with a porous tip (Figure 5) so as to deliver the stock solutions in even amounts throughout the enclosures without resuspending particles associated with the submerged aquatic vegetation (SAV). Then, approximately two weeks after amending, all enclosure surface

waters were sampled (including the unamended control enclosures and unenclosed, unamended "control" areas in the ridge and slough outside the enclosures) for sulfate, sulfide, soluble reactive P (SRP), total P (TP), ammonia-nitrogen (N), pH, temperature, dissolved oxygen (DO), dissolved calcium (Ca) and iron (Fe), and alkalinity prior to starting the cycle over again by exchanging the standing water volume 5 times with outside slough water (Figure 2).

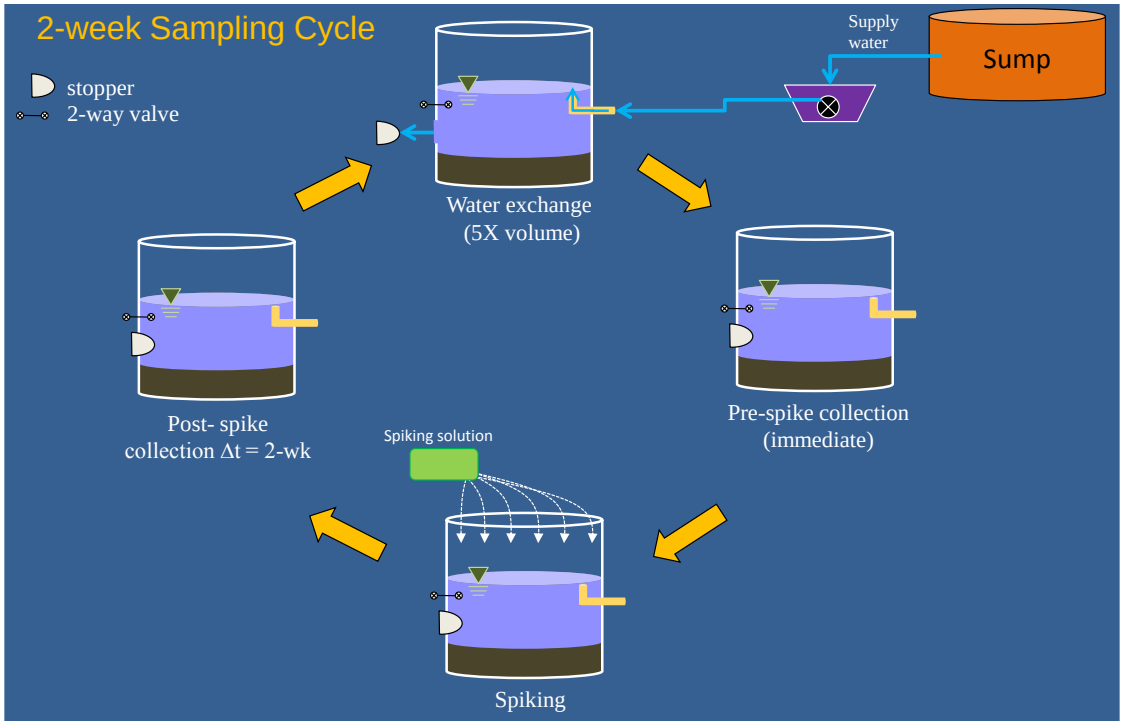

Figure 2. Operational cycle followed for flushing, spiking, and sampling the enclosures over a two-week period at the mesocosm platform in WCA-3A.

Figure 3. A "mini-sump" receiving outside slough water prior to being delivered to three of the 18 enclosures during the flushing and exchange performed once every two weeks at the mesocosm platform in WCA-3A.

Figure 4. Delivery of low-sulfate outside slough water via a manifold into one of the slough enclosures at the WCA-3A site during the flushing and exchange performed once every two weeks. Note the gentle turbulence without resuspending any of the particulates associated with the nearby *Utricularia purpurea* or the soil interface.

Figure 5. Subsurface injection of stock solutions of amendments into one of the enclosures. The stock solutions were gravity-fed at a controlled flow rate from a 5-gal bucket to a PVC wand (see inset) fitted with a porous tip.

Mesocosm enclosures were batch-fed slough water and experimental chemical amendments on a bi-weekly basis for a total of three wet seasons (2010-2012; Figure 6). The experimental design for the first wet season was as follows (Figure 7): three control mesocosms (no amendments); three groups of three mesocosms that received bi-weekly doses of sulfate amendments (12, 24, or 48 mg/L); three enclosures that received sulfate (48 mg/L) + Ca (40 mg/L) + alkalinity (100 mg $CaCO_3$/L); and three enclosures that received Ca (40 mg/L) + alkalinity (100 mg $CaCO_3$/L). During the 2nd and 3rd wet seasons, we discontinued dosing of Ca and alkalinity, since no responses to these amendments were observed; the sulfate + Ca + alkalinity mesocosms continued to receive sulfate at 48 mg/L and the former Ca + alkalinity group was converted to sulfate-only amendments at 3 mg/L. In addition to the mesocosms, three locations in the adjacent (within about 25 m) ridge and slough communities were monitored as "unenclosed controls."

During Season 1, surface water sulfate was measured 1 – 3 days (typically, 2 days) after each sulfate dose. Amendments of sulfate effectively and reliably achieved target surface water sulfate concentrations (Figure 8a). These immediate post-dose measurements were discontinued for monitoring Seasons 2 and 3.

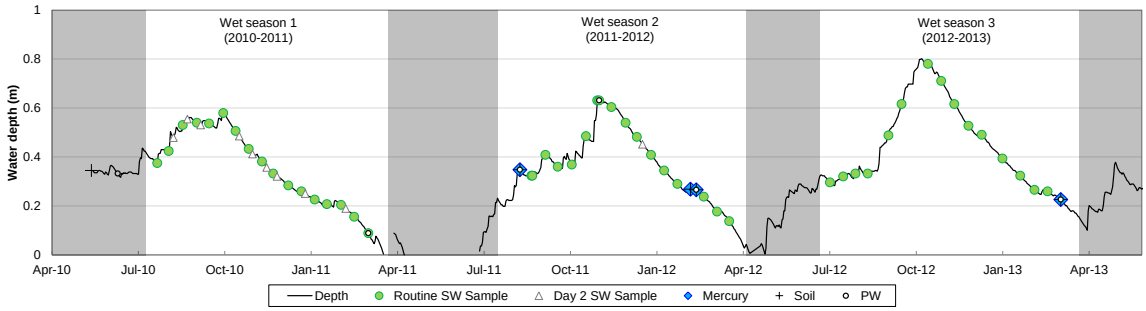

Figure 6. Water depth at monitoring station 3AS, approximately 5.5 km NE of our experimental site. Sampling events are superimposed. Round green markers indicate routine surface water sampling, at the end of each two-week batch incubation cycle. Open triangles show surface water (SW) collection immediately (1-3 days) following sulfate amendment at the start of each batch incubation cycle during Season 1. Blue diamonds mark sampling events that included analysis for mercury species. Soil and porewater (PW) sampling events are given by crosses and open circles, respectively. Shaded periods approximate spans between monitoring seasons, when no samples were collected and no maintenance (including amendment application) was conducted.

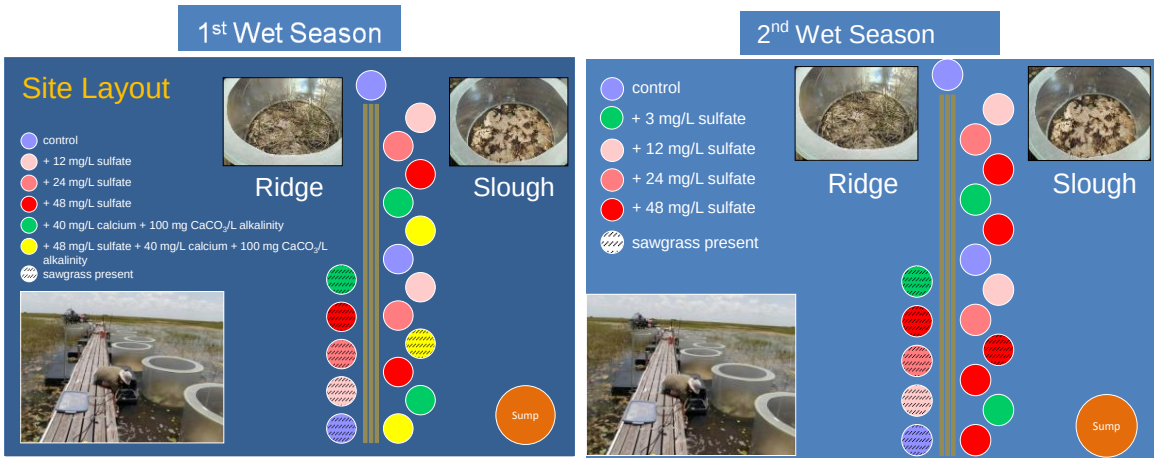

Figure 7. Site layout of the enclosures during the first (July 22, 2010 to March 3, 2011) and second (July 14, 2011 to March 20, 2012) wet seasons in WCA-3A. The two insets at the top are overhead views of an enclosure within a ridge community (left) and another enclosure within a slough (right) community. The photo at the bottom left depicts the mesocosm infrastructure.

Porewater and soil sampling

Porewaters in each enclosure and the adjacent, unenclosed control sites were sampled (6-10 cm below soil surface) at the end of each monitoring season (Figure 6) using a "sipper", a minimally-intrusive *in-situ* sampler for collecting a composite porewater sample over a specified range of depth. Porewaters were analyzed for pH, redox potential, temperature, sulfate, sulfide, alkalinity, ammonia-N, total soluble P (TSP), SRP, DOC, dissolved Ca and dissolved Fe.

Additionally, during Season 2, we sampled porewaters at the beginning (August 2011) and middle (Nov 2011; pH, redox, temperature and sulfide only) of the monitoring period to detect changes over time within the dosing season.

Surface soils (0 – 5 cm) were sampled from each enclosure and the unenclosed controls in March 2013, as part of the experiment closeout. Intact soil cores were retrieved with a Teflon coring device, extruded in the field into cleaned polyethylene bags, and stored on ice. Rhizomes and large roots, if present, were removed from the soil sample. Duplicate cores were collected in each mesocosm and composited in the field.

Mercury dosing and sampling protocol

THg and MeHg were analyzed in the surface waters and soils of the mesocosms during four sampling events (Figure 6). For the first Hg sampling event, water and surficial soils were collected from a subset of the enclosures and the surrounding slough on August 11, 2011, approximately one month after the end of a four-month seasonal dryout in WCA-3A (Figure 6). The second Hg sampling event was performed in February 2012, after approximately seven months of continuous biweekly sulfate dosing. Total Hg and MeHg were measured in surface water and soils immediately before dosing 15.8 μg Hg(II)/m^2 into each enclosure. Six days later, surface waters and soils were re-sampled for THg and MeHg. This Hg spiking study was performed to determine whether the addition of "new" Hg would result in an increase of MeHg levels at elevated sulfate doses (Gilmour et al., 2004). The fourth Hg sampling event was conducted during the March 2013, during the experimental closeout. This event was not associated with either Hg dosing or seasonal dryout events.

For Hg sampling, clean techniques were practiced, which entailed "clean" and "dirty" hands partnering, and wearing ultra-trace clean gloves when collecting samples (USEPA, 1996). Core barrels re-used in the field were rinsed with trace metal grade HCl (4%) in the field, before and after rinsing with ultra-pure reagent water, and pre-cleaned and field-cleaned equipment blanks were collected to insure that all equipment and sample containers were uncontaminated with MeHg or THg.

Surface water was sampled by inserting a pre-tested (checked for Hg contamination at a frequency of 1 in 10) 250-mL fluoropolymer (FLPE) bottle under the surface to a depth of 2 cm. An aliquot of the sampled water was immediately poured into a second 250-mL FLPE sample bottle, which contained trace metal grade HCl to yield a final concentration of 0.4%, and then capped with FLPE-lined lid for later MeHg analysis at Brooks Rand. The sample was stored in the dark at 4°C until analyzed. For THg, surface water remaining in the first FLPE bottle was kept refrigerated until preserved with BrCl within 28 days of collection.

For Hg analysis of soils, Teflon core barrels (4.7 cm i.d.) with a beveled end were pre-cleaned in the lab with trace metal grade combined 10% (2.3 N) HNO_3 and 10% (3.5 N) HCl acid followed by an ultra-pure distilled water rinse. A pre-cleaned core barrel was inserted into the soil to obtain a core representing the surface-to-5 cm soil depth. Each experimental unit (that is, the selected mesocosm enclosures and an unenclosed, unamended location in the adjacent slough) was cored twice; the two samples were composited in a 4-oz plastic wide-mouth jar, homogenized with an acid-rinsed Teflon policeman, immediately placed on ice in the field and frozen within 24 hr. All soil samples were analyzed for THg and MeHg.

All Hg analyses were completed within one month, well within statutory holding times of six months (MeHg in water,) one year (MeHg in soil), three months (THg in water), and one year (THg in soil) (USEPA, 2001a and 2001b). EPA Methods 1630 and 1631E Appendix (USEPA, 2001a and 2001b) were followed for MeHg and THg analyses, respectively, in both water and

soil. The method detection limits (MDLs) were 0.020 ng/L and 0.008 ng/g wet wt for water and soil, respectively, for MeHg; corresponding MDLs for THg were 0.15 ng/L and 0.05 ng/g wet wt.

Data handling

No significant differences in any analyte were detected between unenclosed ridge and slough sites, therefore ridge and slough were pooled (averaged) as "unenclosed controls." The SO4(3) treatment was established in monitoring Season 2, in triplicate mesocosms that had received Ca amendment (with no sulfate added) during Season 1. There was no detectable effect of Ca amendment on any measured analyte, thus SO4(3) data are included without caveat. Three of the SO4(48) mesocosms were established in monitoring Season 2 in mesocosms that had received SO4(48)+Ca during Season 1. Ca amendment seemed to reduce the surface water sulfate concentration in these mesocosms during Season 1, but had no detectable effect on any other measured analyte (except dissolved Ca). Therefore, the SO4(48)+Ca mesocosms were excluded from Season 1 analysis, but included in the SO4(48) treatment group for analysis of Seasons 2 and 3 data, without distinction.

Several individual mesocosms developed high densities of crayfish (*Procambarus fallax* and a few *P. alleni*) since they may have been protected from predators in our enclosures (Kellog and Dorn, 2012). Crayfish damaged the macrophytes and periphyton, and shifted the community of primary producers away from these toward phytoplankton. The following individual enclosures were dually identified as severely impacted by crayfish through independent reviews of field vegetation observations and surface water TP concentrations, and thus excluded from all analyses presented herein: Enclosed control – Rep 2; SO4(3) – Rep 3; SO4(12) – Rep 2; SO4(48) – Rep 2. Finally, the rim of mesocosm SO4(48) – Rep 6 was a frequent roost for local piscivorous birds, as evidenced by guano stains and regurgitated fish bones. The TP concentration in this unit averaged about 20 µg/L, more than twice the average concentration measured in any other SO4(48) replicates. Therefore, it was excluded from all analyses as well.

RESULTS

Sulfur chemistry

During Season 1, measurement of sulfate concentrations within 2-3 days of application of each biweekly sulfate dose indicated that actual sulfate levels closely reflected target concentrations (Figure 8a). The rate of loss of sulfate from the water column was strongly related to the sulfate addition level (Figure 8b), but our amendments were frequent enough that the added sulfate was not fully depleted, and the batch-end sulfate concentrations were still well stratified by treatment group (Figure 8a). Also, the average sulfate depletion rate within each of the high-sulfate treatment groups (12-48 mg/L) was correlated to surface water temperature (r = 0.72 to 0.81; not shown).

Porewater sulfate and sulfide concentrations were also strongly affected by sulfate dosing, although the 3-mg/L treatment had no effect on these porewater constituents, relative to the unamended control mesocosms (Figure 8c & 8d). Surface water sulfide was elevated in the 12-, 24- and 48-mg/L sulfate-amended mesocosms (average 0.009 – 0.011 mg/L, compared to 0.006 in the enclosed controls), but not correlated to the sulfate dose across those groups (not shown). However, surface water sulfide was strongly correlated with soil TS across individual mesocosms (r = 0.89).

On average, the higher (12 – 48 mg/L) experimental sulfate amendments increased soil sulfur concentrations by 20 to 70% over background levels (Table 1). The soil TS concentration in individual mesocosms varied, in part, with the average surface water sulfate concentration measured in each mesocosm (r = 0.70).

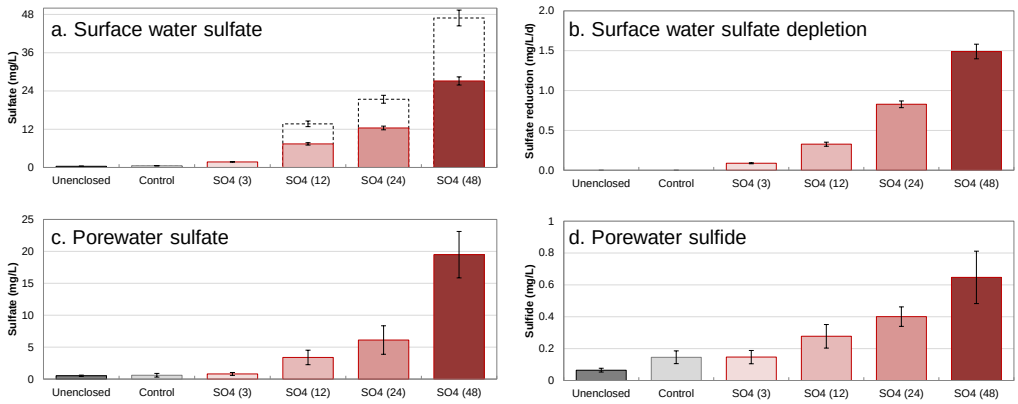

Figure 8. Average surface water a) sulfate concentration and b) sulfate depletion and porewater c) sulfate and d) sulfide concentrations for each mesocosm treatment group and the adjacent unenclosed controls. Surface water constituents were measured two weeks after each sulfate dose, resulting in 51 measurements of each individual mesocosm over three dosing seasons (Figure 6). Porewater was measured once at the end of each of three dosing seasons. Values shown here are averaged across two to six replicates within each group, as detailed in the Data Handling section above. Error bars give ± 1 S.E. In panel (a), the dashed bars give the average surface water sulfate concentration measured 1 – 3 days (typically 2 days) after each sulfate dose during Season 1.

Table 1. Average concentrations of selected soil (0-5 cm) constituents in each treatment group at the end of three dosing seasons. N = 2 for each treatment, as a result of the data screening procedures described above. Standard error is given in italic. "Initial" samples were collected from the ridge and slough communities (n = 4 each) at the experimental site prior to installation of mesocosms or initiation of sulfate additions.

	Initial	Unenclosed Control	Enclosed Control	SO4(3)	SO4(12)	SO4(24)	SO4(48)
TC (%)	38.3	39.5	42.5	33.2	39.7	35.3	37.7
	1.1	--*	*1.8*	*2.5*	*1.7*	*3.3*	*4.7*
TOC (%)	35.9	37.2	41.7	28.9	37.2	31.6	37.1
	1.6	--*	*2.3*	*3.3*	*2.4*	*4.2*	*4.5*
TN (%)	3.1	3.6	3.8	2.7	3.4	3.0	3.3
	0.1	--*	*0.2*	*0.2*	*0.1*	*0.4*	*0.4*
TP (mg/kg)	398	363	587	336	413	363	428
	30	--*	*84*	*41*	*25*	*79*	*14*
TS (%)	0.78[+]	0.84	0.89	0.73	1.11	0.96	1.38
	0.7	--*	*0.02*	*0.07*	*0.07*	*0.16*	*0.33*
TFe (mg/kg)	--[‡]	7500	7600	7300	9066	8900	9100
	--	--*	*400*	*602*	*656*	*800*	*1100*

* Only a single sample was collected from the unenclosed control station
[+] Average of two samples, each the field-composite of four individual samples
[‡] Soil total iron was not measured in the initial sampling event

Dissolved oxygen, alkalinity, pH and redox potential

Average surface water dissolved oxygen (DO) concentration varied seasonally from about 2.5 mg/L in early fall to about 5.5 mg/L in late winter. On average, DO in the enclosures was about 1 mg/L less than the unenclosed site water, except in the SO4(24) treatment group. Surface water alkalinity averaged about 150 mg/L $CaCO_3$ (3.0 meq/L), with no significant differences detected between treatments (Figure 9). On average, alkalinity in all treatments, including unenclosed controls, did tend to increase from about 140 mg/L $CaCO_3$ at the beginning of the monitoring season to about 180 mg/L $CaCO_3$ at the end. Throughout the experiment, surface water pH averaged 7.79 and did not change over time, or with respect to sulfate treatment (not shown). Average porewater pH was about 0.25 units higher in the enclosures (including unamended controls; 7.37) than in the unenclosed controls (7.12). Soil redox potentials displayed an inverse relationship with the sulfate amendment rate (Figure 10), and were markedly depressed (ca. -200 mV) in mesocosms that received high sulfate doses relative to enclosed and unenclosed controls (ca. -50 mV). Season-end mean redox potentials did not change appreciably among monitoring seasons, but a decreasing trend in redox potentials was observed during Season 2, when redox was measured on three occasions (Figure 11).

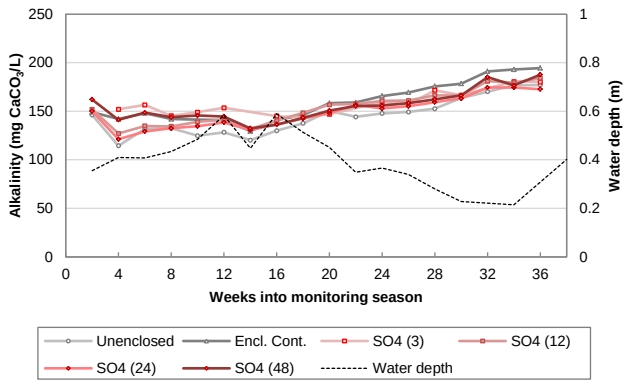

Figure 9. Surface water alkalinity concentration measured approximately two weeks after sulfate amendment of mesocosms averaged over three monitoring seasons. Each point gives the average of 6 – 18 individual measurements (number of replicates within each treatment group: 2 – 6 [see Data Handling section]). Error bars omitted for clarity. Water depth at SFWMD monitoring site 3AS, about 5.5 km NE of the mesocosm site, is also shown.

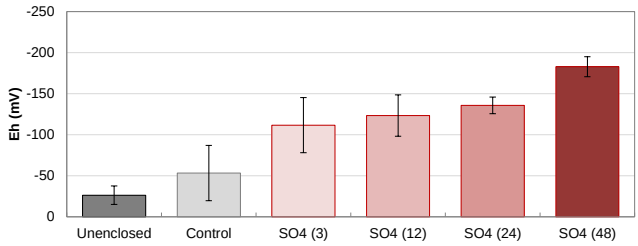

Figure 10. Average porewater redox potential (Eh; mV) within each treatment group. Note the inverted vertical axis. Porewater redox was measured once at the end of each of three dosing seasons (Figure 6). Values shown here are averaged across two to six replicates within each group, as detailed in the Data Handling section. Error bars show ± 1 S.E.

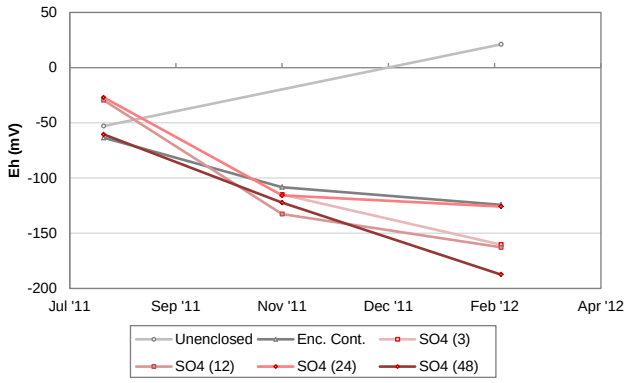

Figure 11. Average porewater redox potential (Eh) within each treatment group during monitoring Season 2. Values shown here are averages of two to six replicates within each group, as detailed in the Data Handling section. Error bars omitted for clarity.

Nutrients

TP in enclosures was about 50% higher than in the unenclosed ridge and slough. This included the enclosed controls (T-test of enclosed versus unenclosed controls: t-value = -10.75, DF = 395, $p < 0.01$), indicating an effect of the mesocosm platform itself. The TP pool was dominated by PP (≥ 50%), with lesser contributions from DOP and SRP (Figure 12). Relative to the unenclosed controls, the increased P in the enclosures was principally as PP and DOP (Figure 12). Total soluble P in porewaters responded similarly (not shown), with ~50% higher concentrations in the enclosures (including unamended controls) than in the unenclosed controls.

Within the mesocosms, we observed no response of P to sulfate amendments. Mesocosms receiving sulfate amendments produced TP concentrations equal to or less than the TP concentration in the unamended control mesocosms, significant only for the 12 ($p < 0.01$) and 24 mg/L ($p < 0.01$) treatments. The season-average surface water TP concentration was not correlated to the surface water sulfate concentration measured in individual mesocosms ($r = 0.22$, $p = 0.16$). Further, among the enclosures, no significant correlations were detected between season average TP, PP, DOP, SRP in surface water, or DOP, SRP in porewater, and any of surface water sulfate, porewater sulfate, porewater sulfide or redox potential.

After three seasons of sulfate dosing, the soil phosphorus concentration had not changed systematically from the initial, pre-study condition (Table 1), nor was the soil TP concentration in individual mesocosms related to the period-of-record average surface water sulfate concentration ($r = -0.12$).

Surface water ammonia-N concentrations averaged 0.05 mg/L in the unenclosed controls, but were 2-3 times higher in the mesocosm enclosures, including the un-amended controls (not shown). No response to sulfate amendment was detected. In porewaters, ammonia-N demonstrated no enclosure effect or response to sulfate amendment, except to be about 35% lower in the SO4(48) treatment group (0.67 mg/L) than in the enclosed controls (1.04 mg/L).

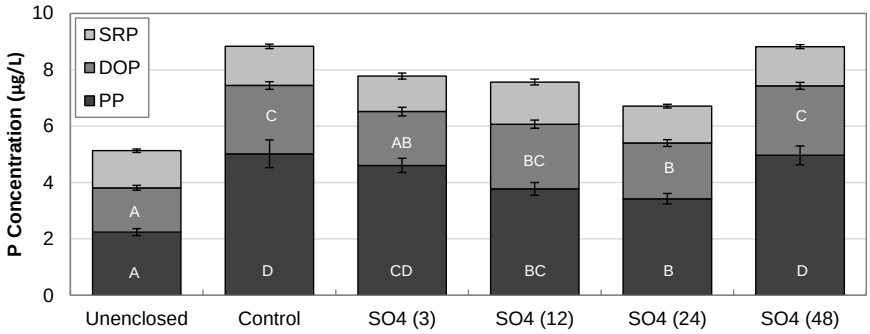

Figure 12. Period-of-record average concentrations of phosphorus (P) species (PP = particulate P; DOP = dissolved organic P; SRP = soluble reactive P) within each treatment group. Statistical comparison of means was conducted within each species; values sharing a common letter are not statistically different ($\alpha = 0.05$). No statistical differences were detected between mean SRP concentrations. Error bars give ± 1 S.E. Total height of each bar approximates the average TP concentration.

Carbon

Sulfate additions had very little effect on DOC concentrations in surface waters. Statistical tests included one-way ANOVA of mesocosm treatment groups ($F[4,424] = 0.43$, $p = 0.78$) and a t-test of all sulfate-amended mesocosms versus enclosed controls (t-value = 0.82, DF = 427, $p = 0.41$). Season-average DOC was positively correlated ($r = 0.51$, $p < 0.01$) to the measured surface water sulfate concentration among individual mesocosms, but the response was relatively small (about 7% over the full range of sulfate concentrations). The possible influence of sulfate on DOC was dwarfed by the seasonal fluctuation in the site water DOC (Figure 13). Sulfate amendments had no measureable effect on porewater DOC concentrations.

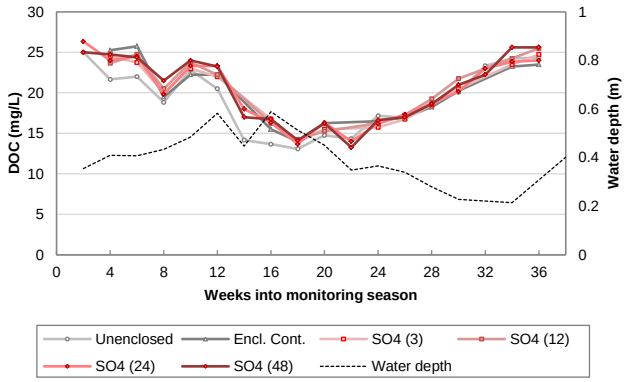

Figure 13. Average surface water dissolved organic carbon (DOC) concentration measured approximately two weeks after sulfate amendment of mesocosms, with respect to time elapsed since initiation of sulfate dosing each monitoring season. Each point gives the average of 6 – 18 individual measurements (number of replicates within each group: 2 – 6 [see section 0] averaged over 3 dosing seasons). The water depth at SFWMD monitoring site 3AS, about 5.5. km NE of the mesocosm site is also shown.

After three seasons of sulfate dosing, the soil total organic C concentration had not changed systematically from the initial, pre-study condition (Table 1), nor was the soil TOC concentration in individual mesocosms related to the period-of-record average surface water sulfate concentration (r = 0.08).

Iron

Dissolved iron concentrations at our experimental site were relatively high; porewater and surface water iron averaged about 1.25 and 0.2 mg/L, respectively, in the enclosed and unenclosed controls. Sulfate dosing decreased iron concentrations in both matrices, down to about 0.4 and 0.1 mg/L, respectively, in the 48 mg/L sulfate treatment (Figure 14). The depleted iron was likely precipitated with sulfide and deposited in the soils as FeS_x; average soil iron concentration was elevated (compared to controls) in the sulfate-dosed enclosures (Table 1) and was moderately well correlated to the average surface water sulfate concentration (r = 0.68) and to the soil sulfur content (r = 0.72) in individual mesocosms.

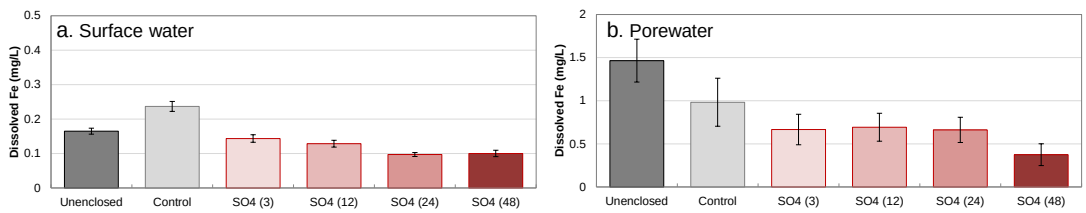

Figure 14. Average a) surface water and b) porewater dissolved iron (Fe) concentrations within each treatment group. Porewater iron was measured once at the end of each of three dosing seasons (Figure 6). Values shown here are averaged across two to six replicates within each group, as detailed in the Data Handling section. Error bars show ± 1 S.E.

Mercury

Total mercury

Surface water total mercury concentrations were typically around 1-2 ng/L, and were not related to treatment group (one-way ANOVA overall, and within sampling events: $p > 0.05$). The experimental addition of inorganic Hg in February 2012 increased surface water THg concentrations temporarily to 6-10 ng/L; in all treatment groups, surface water THg had returned to < 1.0 ng/L by the next sampling event in March 2013. In surface soils, total mercury ranged from about 200 – 350 ng/g dry, and was not related to the sulfate treatment group (one-way ANOVA overall, and within sampling events: $p > 0.05$). The inorganic Hg addition in February 2012 did not affect soil THg concentrations.

Methylmercury

Following the one-time addition of inorganic Hg in February 2012, the surface water MeHg concentration actually declined (two-tailed matched-pairs t-test: $t = -2.51$, $p = 0.04$) six days later, though the difference was very small (average 0.026 ng/L, or about 0-20%, depending on the pre-spike value). Further, the pre- and post-spike MeHg concentrations in individual mesocosms were highly correlated ($r = 0.98$, $p < 0.01$). To demonstrate these similarities, the data from the two February 2012 events are presented separately in Figure 15, but otherwise all MeHg data from the two events were averaged and consolidated for subsequent analyses.

In both surface waters and soils, overall average MeHg was higher in August 2011 (following site drydown/reflood) than for either of the subsequent Hg sampling events, but overall, there was no treatment effect on surface water MeHg (one-way ANOVA: $F[5,37] = 1.66$, $p = 0.17$). The conditions during Hg sampling events were diverse, so each event was considered separately. Only in the February 2012 sampling event (pre- and post-Hg-spike combined) was a significant treatment effect detected (one-way ANOVA: $F[4,11] = 5.35$, $p = 0.03$; Figure 15a). Surface water MeHg was weakly but significantly correlated to sulfate concentration among individual mesocosms [$r = 0.37$, $p = 0.03$]; Figure 16a). There was never a detectable treatment effect on soil MeHg (one-way ANOVA overall, and within sampling events: $p > 0.05$; Figure 15b).

Significant correlations were detected between surface water MeHg and several surface water and porewater constituents. Some examples are given in Figure 16. Other significant correlates for surface water MeHg included surface water SRP ($r = 0.35$, $p = 0.04$) and porewater TSP ($r = -0.38$, $p = 0.04$).

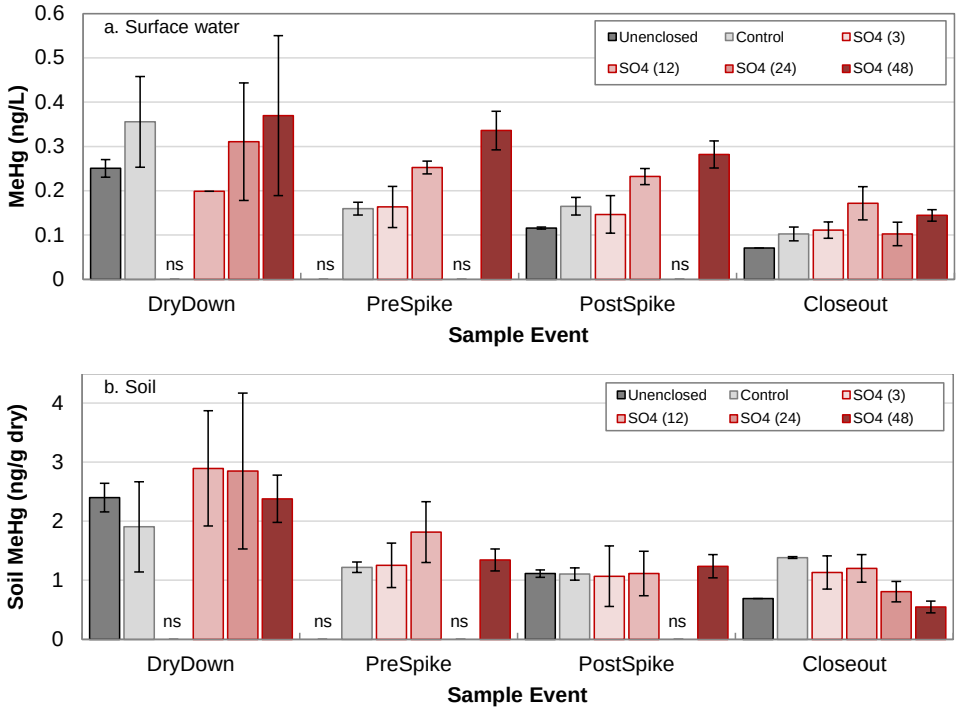

Figure 15. Average a) surface water and b) soil (0–5 cm) methylmercury (MeHg) concentrations by experimental treatment group and each sampling event. N = 2 for each value. Error bars give ± 1 S.E.

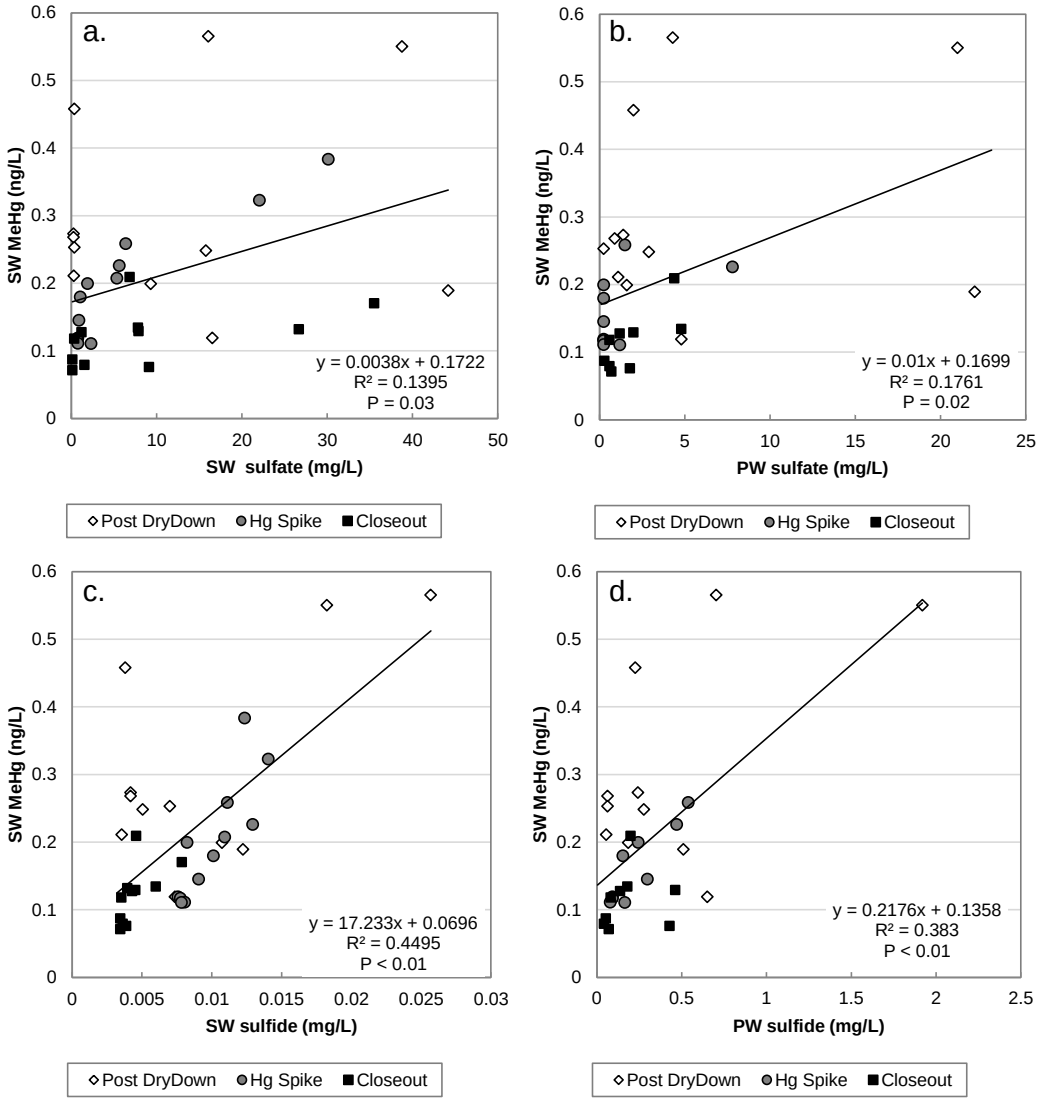

Figure 16. Scatterplots of the relationship between surface water (SW) methylmercury (MeHg) and a) surface water sulfate, b) porewater (PW) sulfate, c) surface water sulfide and d) porewater sulfide concentrations. Each point represents the MeHg concentration in a given sampling event and the sulfate or sulfide concentration measured during the preceding monitoring season. The individual mercury sampling events are distinguished, and the data from the two events in February 2012 are averaged and presented as "Hg Spike".

DISCUSSION

Sulfur chemistry

Sulfate additions resulted in proportional increases in sulfate levels in mesocosm surface water and porewater, mimicking the gradient of sulfate enrichment observed across the marshes of the Everglades (Scheidt and Kalla, 2007; Julian et al., 2014). Despite native soil redox potentials (> -50 mV) too high to support reduction of sulfate to sulfide (Reddy and DeLaune, 2008), sulfide accumulated in the porewaters of sulfate-amended mesocosms (Figure 8d), probably originating in highly-reducing soil microsites conducive to sulfate reduction. However, porewater sulfide levels in high-sulfate treatments (average 0.3 – 0.6 mg/L; Figure 8d) were lower than those typically found in sulfate-enriched areas of the Everglades (1 – 5 mg/L; Scheidt and Kalla, 2007). This was likely the result of precipitation of sulfide with porewater iron (Smolders et al., 1995), evidenced by lower iron concentrations in sulfate-amended mesocosms (Figure 14) and co-accumulation of iron and sulfur in soils (Table 1). Nonetheless, sulfide accumulated in porewaters proportionally to the sulfate amendment rate (Figure 8d), and concomitantly depressed the bulk redox potentials of soils in sulfate-amended enclosures (Figure 10) over the course of each dosing season (Figure 11). Thus, this experiment effectively altered the sulfur chemistry (surface water and porewater sulfate and sulfide, and soil sulfur) and redox potentials such that it offered a very rigorous evaluation of the effects of sulfate on the critical processes of internal P loading and Hg methylation in the Everglades.

Increased sulfate availability stimulated microbial sulfate reduction rates, as evidenced by the general depletion of added sulfate and the accumulation of sulfide (Lamers et al., 1998). Although alkalinity is generated during organic matter decomposition by sulfate reduction, we did not detect any treatment-related change in alkalinity (Figure 9). We suggest that we did not observe any sulfidogenic alkalization because of buffering by the diurnal cycle of mineral $CaCO_3$ precipitation mediated by photosynthetic algae (Scinto and Reddy, 2003). That the rate of sulfate loss was correlated to the water temperature suggests that the reaction was biotically-mediated, and not due to some physical process like dilution (by rainfall) or diffusive or advective flux out of the mesocosms. Finally, the rate of sulfate depletion was proportional to the concentration of the sulfate additions, which indicates that SRB were increasingly metabolically active with increasing sulfate levels. Therefore, we conclude that SRB 1) were indeed present in the native microbial consortium (Drake et al., 1996; Bae et al., in press), and 2) were sulfate-limited, at least in part, at native sulfate concentrations (Lamers et al., 1998, 2002).

Phosphorus release (internal eutrophication)

The clear lack of P mobilization at all levels of sulfate addition contrasts with many studies in other wetland environments (Roden and Edmonds, 1997; Lamers et al., 1998, 2002; Zak et al., 2006; Geurts et al., 2008) and portions of an earlier experiment in WCA-3A (Gilmour et al., 2007a). However, sulfate enrichment without concomitant soil P release has been observed occasionally at other locations (Lamers et al., 2002; Geurts et al., 2008), and particularly in Everglades environments including central WCA-3A (Dierberg et al., 2011, 2012). These studies illustrated that certain biogeochemical conditions can dampen the sensitivity of soil P to sulfate enrichment.

Smolders et al. (2006) outlined four inter-dependent mechanisms by which excess sulfate can mobilize soil P: i) direct competition of sulfate (SO_4^{2-}) with phosphate (PO_4^{3-}) for anion-sorption sites; ii) increases in pH due to alkalinity generated during the microbial reduction of sulfate to sulfide, creating conditions more favorable to organic matter decomposition (specifically, P mineralization); iii) increased soil organic matter decomposition by SRB stimulated by sulfate availability, and; iv) redox-mediated reduction and dissolution of ferric iron minerals and the

associated P. Mechanism (iv) is coupled with and exacerbated by the precipitation of reduced (ferrous) iron with sulfide as FeS_x minerals, which have relatively low affinities for P. The present study corroborates the laboratory findings of Dierberg et al. (2011) that these four processes, while activated to varying degrees by experimental sulfate additions, do not result in internal P eutrophication in central WCA-3A.

First, displacement of exchangeable P by sulfate is not thought to be prominent in most ecosystems, since the number of anion-binding sites is typically small (Lamers et al., 2002; Smolders et al., 2006). Indeed, sequential P extraction conducted on surface soils (0-5 cm) collected from the site at the initiation of this mesocosm study confirmed that the exchangeable (i.e. ion-extractable) P was a very small pool (0.2 mg/kg; 0.05% of the soil TP).

Second, as discussed above we detected no response of alkalinity to sulfate addition, so there was no evidence of the mechanism of alkalization-enhanced P release. In addition to the aforementioned alkalinity buffering, the surface waters at our experimental site have high native alkalinity (150-200 mg/L $CaCO_3$), which likely reduced the relative importance of any potential alkalization from sulfidogenesis (calculated to be about 1.6 mg $CaCO_3$/L/d at the sulfate reduction rate in the highest sulfate treatment).

Third, although the sulfate reduction rate by SRB increased in response to sulfate amendments, our experimental infrastructure was not equipped to directly measure associated organic matter oxidation (e.g. as carbon dioxide or methane evolution). However, Dierberg et al. (2011) found no increase in carbon mineralization in slurries of soil from this site amended with 32 and 96 mg/L sulfate, despite observing sulfate reduction; this was attributed to metabolic limitation by other than sulfate, perhaps P or labile C. Further, Lamers et al. (2001) qualified this mechanism as dependent on the C:P ratio in soils. In soils with high C:P ratios, like those at our central WCA-3A site (TOC:TP = 700 - 1000 wt/wt), P is efficiently scavenged by microorganisms during decomposition, such that P is conserved despite mass loss of C (Cheesman et al., 2010). Indeed, data from the present experiment revealed that about 30% (130 mg/kg) of the soil TP was stored in microbial biomass (compared to 7% in the P-enriched STA-2 Cell 1 [Dierberg et al., 2011]). Therefore, accelerated organic matter mineralization by sulfate reduction may not be expected to mobilize mineralized organic P to the water column.

Finally, the sulfate additions in this experiment triggered the redox-sulfide-iron processes of mechanism (iv), but still did not mobilize P to the water column. Accumulated sulfide lowered redox potentials sufficiently such that ferric minerals would not have been stable, and sulfide apparently precipitated with ferrous iron (porewater dissolved iron concentrations were lower in sulfate-amended mesocosms than controls and the magnitudes of increases in soil total sulfur and total iron were correlated). However, the pool of P bound to soil iron was small at this site (10 – 20 mg/kg; 2.5 – 5% of TP), which can reduce soil susceptibility to sulfide-induced P release (Lamers et al., 2002; Smolders et al., 2006). Further, the porewater (as dissolved Fe:SRP wt/wt) and soil (as total Fe:total P wt/wt) Fe:P ratios were high (ca. 150 – 500 and 20 – 25, respectively), above the thresholds values for P mobilization identified by Geurts et al. (2008). That is, the pools of P were very small compared to the iron stores, so even large changes to iron chemistry resulted in negligible changes to P mobility.

Although the findings of the present study appear contradictory to those of Gilmour et al. (2007a; in Axelrad et al., 2007), any comparison of the results must be attempted with great caution. First, the data presented in Axelrad et al. (2007) apparently represent only a single sampling event, with no estimates of variability with treatments or over time given. Second, operational differences may have distinguished this study from that of Gilmour et al. (2007a). In particular, that study observed substantial P release to surface waters only at a sulfate concentration of 100 mg/L, more than double the highest concentration tested in the present experiment, and well above the highest concentrations observed in any Everglades marshes

(Scheidt and Kalla, 2007). In addition, based on a limited description of methodology, it appears that their mesocosm study did not include routine surface water exchanges, which likely introduced undocumented experimental artifacts.

The biogeochemical similarities between our experimental site in central WCA-3A and much of the greater Everglades (e.g. high alkalinity [Scheidt and Kalla, 2007]; high soil C:P ratios (DeBusk et al., 1994; Amador and Jones, 1995; Wright and Reddy, 2001; Dierberg et al., 2011); low ion-exchangable P and Fe-bound P [Reddy et al., 1998]), support the extension of these findings to the wider ecosystem. Generally, we conclude that sulfate enrichment of P-unimpacted Everglades surface waters does not mobilize soil P in any environmentally relevant fashion. Specifically, in the majority of the Everglades, where P is limiting, organic P is conserved during decomposition and not released to the water column, regardless of the rate of organic matter mineralization (Cheesman et al., 2010). Likewise, given the low percentage of soil TP occurring as Fe-bound P (Reddy et al., 1998), sulfide- and redox-induced reductive dissolution of ferric minerals should not mobilize notable amounts of P. Furthermore, even in P-enriched Everglades regions, to which the present study is not immediately applicable, sulfate was shown to not increase organic matter mineralization rates (Dierberg et al., 2011) or soil P release (Dierberg et al., 2011, 2012), which was attributed to labile C limitation and low levels of Fe-associated P.

Mercury methylation

Although sulfate amendment markedly increased the rate of sulfate reduction (as evidenced by sulfate depletion rates and PW sulfide accumulation), we observed a modest, at best, MeHg response. We did not observe the unimodal ("bell-shaped") MeHg response with respect to sulfate concentrations that has been predicted for ecosystems with gradients of sulfate and sulfide (Gilmour and Henry, 1991; Orem et al., 2011). Thus, net MeHg accumulation was not closely paired with sulfidogenic activity. This implies that the methylation rate was constrained only secondarily by sulfate concentration. Rigorous experimental data on the effects of sulfate on Hg methylation in the Everglades are scarce, but this finding appears to agree with the observation by Gilmour et al. (2004) that sulfate additions to *in situ* mesocosms at 3A-15 did not result in methylation of native inorganic Hg. The primary controls or limitations on Hg methylation at this site are not yet clear.

Primary limitations on methylation may have included availability of labile carbon or bioavailability of inorganic Hg. For example, labile C is necessary for microbial sulfate reduction to sulfide (Lamers et al,. 1998, 2001; Reddy and DeLaune, 2008), but low organic matter mineralization rates measured at WCA-3A (Dierberg et al., 2011) may indicate a limited supply of labile C. The bioavailability of inorganic Hg to methylating microorganisms can also be an important rate-limiting factor in the production of MeHg (e.g. Hsu-Kim et al., 2013), and is probably relevant to MeHg production in the Everglades (Aiken et al., 2011; Pollman, 2012).

An alternative hypothesis to explain the very muted MeHg response to sulfate comes from a recent novel investigation that identified syntrophs as the dominant class of SRB in the Everglades, including central WCA-3A (Bae et al., in press). These organisms can metabolize by sulfate reduction (high sulfate environment) or syntrophy (low sulfate environment). In a high sulfate environment, the syntrophic relationship between SRB and methanogens uncouples as they compete instead for fermented carbon compounds. The difference in net Hg methylation rate by these organisms during sulfidogenesis versus syntrophy is not known at this time. Further, Bae et al. (in press) suggested that the metabolism of these syntrophic SRB may be linked to that of methanogens, which was shown to have a negative net effect on MeHg accumulation. If the activity of SRB and methanogens both depend on sulfate concentrations, then the net effect of such on MeHg production may be more difficult to predict.

The apparent stimulation of Hg methylation by drydown/reflood is consistent with earlier work by Fink (2003), Krabbenhoft and Fink (2001), and Rumbold and Fink (2006). It is apparent that some rate-limiting constituent(s) is/are released during the drydown cycle. Notably, the sulfate amendments to our mesocosms from the preceding dry season did not have a significant effect on MeHg concentrations after reflood. Possibly one season that dosing was insufficient to affect soil TS (the presumed source of sulfate during drydown/reflood). Focused laboratory studies are currently underway to resolve the mechanics of this process.

The lack of MeHg response to added inorganic Hg was surprising, and contrasts with previous findings by other investigators (Gilmour et al., 2004, 2007b). It is uncertain why MeHg production was not stimulated by inorganic Hg added to the mesocosms. Possible reasons for this include (1) limited transport of added Hg to soil or periphyton methylation sites following placement of the Hg spike in the water column; (2) timing of follow-up sampling was either too late or too soon; (3) Hg spike event was conducted in February, when sulfate reduction depletion rate was ca. 20-30% of maximum rates, due to low water temperatures (ca. 20°C).

Despite these uncertainties, the results of this mesocosm study provide evidence that sulfate availability and reduction rates are not overriding master factors controlling MeHg levels in the Everglades. Future research should be focused on factors regulating the availability of Hg to methylators to explain spatial differences in net MeHg production. Our results suggest that seasonal drydown may be very important with regard to Hg methylation. It is possible that all wetland areas of the Everglades affected by soil desiccation and reflood cycles could be subject to enhanced Hg methylation and accumulation of MeHg during these periods.

CONCLUSIONS

Changes in surface and pore water chemistry caused by regular sulfate dosing of *in situ* mesocosms in unimpacted central WCA-3A were marked, but did not include soil P release or well-defined MeHg accumulation. The biogeochemical characteristics shared by this site and the greater Everglades suggest that these findings are representative of the larger ecosystem. Although P-enriched conditions persist in the northern Everglades, sulfate-induced internal eutrophication is not a contributor. The elevated MeHg concentrations in Everglades biota remain very concerning, but the role of sulfate in promoting the production of MeHg is less clear than once believed. Effective control of MeHg pollution will require greater understanding of the roles of sulfate, Hg bioavailability, DOM, seasonal climatic processes and the microbial consortia in this complex ecosystem.

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