Methylmercury and methane production potentials in North Carolina Piedmont stream sediments

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Abstract:

Methylated mercury (MeHg) can be produced by all microbes possessing the genes *hgcA* and *hgcB*, which can include sulfate-reducing bacteria (SRB), iron-reducing bacteria (FeRB), methane-producing archaea (MPA), and other anaerobic microbes. These microbial groups compete for substrates, including hydrogen and acetate. When sulfate is in excess, SRB can outcompete other anaerobic microbes. However, low concentrations of sulfate, which often occur in stream sediments, are thought to reduce the relative importance of SRB. Although SRB are regarded as the primary contributors of MeHg in many aquatic environments, their significance may not be universal, and stream sediments are poorly studied with respect to microbial Hg methylation. We evaluated suppression of methanogenesis by SRB and the potential contributions from SRB, MPA and other MeHg producing microbes (including FeRB) to the production of MeHg in stream sediments from the North Carolina Piedmont region. Lower methanogenesis rates were observed when SRB were not inhibited, however, application of a sulfate-reduction inhibitor stimulated methanogenesis. Greater MeHg production occurred when SRB were active. Other MeHg producing microbes (i.e., FeRB) contributed significantly less MeHg production than SRB. MPA produced MeHg in negligible amounts. Our results suggest that SRB are responsible for the majority of MeHg production and suppress methanogenesis in mid-order stream sediments, similar to other freshwater sediments. Further investigation is needed to evaluate the generality of these findings to streams in other regions, and to determine the mechanisms regulating sulfate and electron acceptor availability and other potential factors governing Hg methylation and methane production in stream sediments.

Keywords: Methanogenesis | Methanogens | Methylmercury | Microbial ecology | Streams | Sulfate-reducing bacteria

Article:

Abbreviations

AFDM: Ash free dry mass
ANOVA: Analysis of variance
BESA: 2-Bromoethanesulfonic acid
FeRB: Iron-reducing bacteria
GC-FID: Gas chromatography flame ionization detection
GC-ICP-MS: Gas chromatography inductively coupled plasma mass spectrometry
ICP-AES: Inductively coupled plasma atomic emission spectrometry
ICP-MS: Inductively coupled plasma mass spectrometry
MeHg: Methylmercury
MPA: Methane-producing archaea
SRB: Sulfate-reducing bacteria

Introduction

Methylmercury (MeHg) is a potent neurotoxin which can extensively bioaccumulate and biomagnify in food webs and making it a human health concern due to fish consumption (Scheuhammer et al. 2007). Sublethal exposure to MeHg can result in various toxic effects, including decreased reproduction rates and altered behavior in wildlife (Scheuhammer et al. 2015), and neurological damage in humans (Ha et al. 2017). Anaerobic microbes are primarily responsible for the conversion of inorganic mercury to MeHg in a variety of habitats, including freshwater, estuarine, and marine sediments (Gilmour et al. 2013). Thus, MeHg producing microbes in bodies of water, including streams, are of interest to public health.

Several lineages of anaerobic prokaryotes can methylate inorganic Hg into highly bioavailable and toxic MeHg (Gilmour et al. 2013). The ability to methylate Hg is thought to depend on the presence and expression of the *hgcAB* gene cluster or an ortholog, which collectively encode putative corrinoid protein (HgcA) and a 2[4Fe-4S] ferredoxin (HgcB) (Parks et al. 2013). In the environment, SRB have been implicated in production of MeHg in estuarine (Compeau and Bartha 1985) and freshwater ecosystems (Gilmour et al. 1992; Yu et al. 2012). Further, SRB have been demonstrated to produce MeHg in laboratory culture (Bridou et al. 2011; Gilmour et al. 2011, 2013; Parks et al. 2013) with high conversion rates (> 70%) of inorganic Hg to MeHg (Gilmour et al. 2013). However, other microbial groups can also produce MeHg. Some FeRB are known to methylate Hg in freshwater sediment (Fleming et al. 2006; Kerin et al. 2006; Yu et al. 2012) and have been documented to methylate > 30% of added Hg in culture (Gilmour et al. 2013). Recently, some MPA were confirmed to convert relatively low fractions of inorganic Hg (1–4%) to MeHg in culture (Yu et al. 2013; Gilmour et al. 2013) and have been implicated as the primary methylator of inorganic Hg in periphyton in a recent lake study (Hamelin et al. 2011). A variety of other microbes including firmicutes and acetogens also are also known to possess the *hgcA* and *hgcB* genes required for MeHg production but their environmental relevance is less known (Gilmour et al. 2013).

Observations of competition between SRB and MPA have been facilitated by use of specific microbial inhibitors (Lovley and Klug 1983). The terminal steps of methanogenesis are competitively inhibited by 2-bromoethane sulfonic acid (BESA), rendering that metabolic pathway unusable for MPA (Bouwer and McCarty 1983). Thus, BESA has been used to inhibit methanogenic activity experimentally (Lovley and Klug 1983). Molybdate has been similarly used to inhibit sulfate reduction. Sulfate and molybdate are structurally analogous, allowing SRB to reduce molybdate when concentrations of molybdate exceed those of sulfate. However, molybdate reduction inhibits sulfate metabolism and is toxic to SRB (Biswas et al. 2009). These inhibitors have suggested that MPA are more metabolically active when SRB are inhibited, and more MeHg is produced by SRB when MPA are inhibited in estuarine sediment (Compeau and Bartha 1985). There is no known specific microbial inhibitor for FeRB.

Although streams are generally well-oxygenated systems, anaerobic metabolism is an important form of respiration in the hyporheic zone (Grimm and Fisher 1984; Mulholland et al. 1997). The heterogeneity of stream sediments provides areas of low flow with small pore spaces and high accumulation of organic matter (Nogaro et al. 2010). These characteristics create a mosaic of anoxic microsites in stream sediments (Baker et al. 1999). Oxic biofilms can support anoxic microsites for anaerobic metabolism (Fenchel and Finlay 1995). Additionally, some anaerobes such as sulfate-reducing bacteria (SRB) have adaptations to withstand oxic conditions (Cypionka 2000). Sulfate reduction and methanogenesis are important anaerobic biogeochemical processes in stream sediments (Baker et al. 2000). Streams can be significant sources of methane (CH_4) and act as 'sulfate sinks' from microbial sulfate reduction (Hlaváčová et al. 2005). Variations in temperature and availability of electron donors and acceptors drive the relative importance of anaerobic pathways in stream sediments (Takii and Fukui 1991).

Methane-producing archaea (MPA) are obligate CH_4 producers and use methanogenesis for most, if not all, of their energy production (Liu and Whitman 2008). In freshwater ecosystems, methanogenesis is generally viewed as the primary anaerobic metabolic process for the decomposition of organic matter (Maerki et al. 2009). The majority of CH_4 production in freshwater is from acetoclastic and hydrogenotrophic methanogenesis (Whalen 2005). Availability of H_2 relative to acetate influences the importance of the two pathways in MPA communities (Liu and Whitman 2008).

Competition for substrate and electron donor availability influences the ecology of anaerobic microbes. Interactions between SRB and MPA are driven by sulfate availability. SRB are known to outcompete MPA for H_2 and acetate when sulfate is in excess (Stams et al. 2003). Additionally, other anaerobic microbes compete with SRB and MPA for resources, including iron-reducing bacteria (FeRB) and acetogens (Muyzer and Stams 2008). FeRB may compete with SRB in environments rich in Fe(III) (Lovley and Phillips 1986) and compete with MPA for acetate and H_2 (Roden and Wetzel 2003).

The specific concentration of sulfate that limits SRB growth is unclear. Localized sediment thermodynamics controls the favorability of sulfate-reduction. Additionally, models have suggested that methanogenesis suppression can occur at sulfate concentrations above 2.9 mg L^{-1} (Lovley and Klug 1986). While favorability of sulfate reduction is variable, in natural freshwater systems sulfate is found at concentrations that are thought to restrict SRB growth

(Whitman et al. 2006), such that low sulfate concentrations in streams may promote methanogenic conditions as MPA are released from competition with SRB (Liu and Whitman 2008). Further, under redox conditions, sulfate-reduction is thermodynamically favored over methanogenesis (Baker et al. 1999). SRB are reliant on H_2 and propionate rather than sulfate (Muyzer and Stams 2008) at very low sulfate concentrations found in freshwater systems (Lovley and Klug 1983). Furthermore, SRB may compete for H_2, but not for acetate (Muyzer and Stams 2008) in low sulfate conditions.

Competition between SRB and MPA in freshwater has been examined in lake sediments (Lovley and Klug 1983) and contaminated river sediments (Avramescu et al. 2011), but to our knowledge has not been studied in mid-order background streams. Additionally, the relative contributions of different Hg methylating microbial groups have not been characterized in mid-order streams. Because MeHg contamination is an important concern in freshwater fish that spend part or all their life cycle in streams (Chasar et al. 2009), it is important to evaluate the significance of these groups of microbes in the production of MeHg.

This study investigated the production of methane from MPA and MeHg from anaerobic microbial guilds using microcosm experiments containing sediments from four North Carolina Piedmont streams. The ability of SRB to inhibit CH_4 production under low concentrations of sulfate in stream water was also assessed. Additionally, the potential production of MeHg from SRB, MPA and other MeHg producing microbes (including FeRB) was compared to determine the potential contributions of these microbial guilds to the production of MeHg in stream sediments. We recognize that laboratory microcosms remove the natural areal and vertical spatial heterogeneity in streams, but expect that such sediment homogenization can be used to effectively evaluate mechanisms of biogeochemical processes.

Materials and methods

Study sites and sample collection

Sediments were collected from streams in Guilford County, North Carolina, USA, for two experiments initiated in January 2015 and January 2016, respectively. For the late January 2015 experiment, stream sediments were collected over three consecutive days from four selected streams. Streams examined in the study were a part of the Cape Fear River Basin and included North Buffalo Creek, South Buffalo Creek, Little Alamance Creek, and Reedy Fork Creek. Discharge data were available via USGS monitoring stations for North Buffalo Creek (ID: 02095271), South Buffalo Creek (ID: 02094659), and Reedy Fork Creek (ID: 02093800). All streams were either 4th or 5th order and were sampled during base flow conditions (Table 1). North Buffalo Creek and South Buffalo Creek had similar urban drainages, with streams originating inside the city of Greensboro and containing large impervious surface areas, while Reedy Fork Creek and Little Alamance Creek drainages had primarily forested and cultivated land cover (2011 landsat data USGS Stream Stats Beta Version 4; Table 1). At each site, 35 sediment cores of 38 mm internal diameter and 5 cm depth were taken by driving acid-cleaned polycarbonate core tubes (total length of 25 cm) into sediment along a reach of 75 m. Water above the sediment–water interface was discarded on site, taking care to not disturb the sediment–water interface. Sediment cores were pooled from each stream, transported to the

laboratory on ice, and then stored at 4 °C in the dark until subsequent processing on the day of collection. On January 2016, sediment was collected from North Buffalo Creek only at the same location of the first experiment, with 35 sediment cores taken from a 75 m reach. Procedures for the collection and processing of sediment were identical to the first experiment.

Table 1. Location of sediment collection, discharge of streams, and drainage cover of study streams

	North Buffalo Creek	South Buffalo Creek	Little Alamance Creek	Reedy Fork Creek
Geographic coordinates	36°4′45.1446″, − 79°48′46.0182″	36°2′57.3246″, 79°51′17.7192″	36°2′37.5612″, −79°39′54.7878″	36°10′22.008″, − 79°57′12.1572″
Discharge ($m^3\ s^{-1}$)[a]	0.07	0.04	N/A	0.31
Stream order	5	4	4	5
Drainage cover				
% Bare	0.0	0.0	0.2	0.0
% Cultivated	0.1	0.1	23.4	27.0
% Developed	98.4	97.4	27.3	24.9
% Forest	1.3	2.3	40.9	40.6
% Grass	0.0	0.1	4.1	3.3
% Impervious surface	28.5	42.8	6.7	5.6
% Shrub	0.0	0.0	2.5	1.5
% Water	0.2	0.1	1.3	0.8
% Wetland	0.0	0.0	0.4	2.0

Drainage cover was calculated using 2011 landsat data with USGS Stream Stats version 4 beta

[a] Discharge on date of collection of the first experiment

Sediment characterization

A subset of sediment from the 2015 experiment was lyophilized and used to measure bulk sediment pH, AFDM, total S, total Na, and total Fe. Cations from subsamples of the pooled sediment from each stream were analyzed with a PerkinElmer NeXion 300S inductively coupled plasma mass spectrometer (ICP-MS) after digestion with concentrated trace-metal grade nitric acid in Teflon digestion vessels at 60 °C overnight. Extractible sulfur from sediment subsamples was measured by inductively coupled plasma atomic emission spectrometry (ICP-AES). The pH of sediment was measured using an ion-selective glass electrode in extracted oxic porewater (Sikora and Kissel 2014). Ash free dry mass (AFDM) was measured by weighing lyophilized sediment subsamples before and after combustion in a muffle furnace for 2 h at 500 °C. Total sulfur ($\mu g\ g^{-1}$) in sediment was determined using a LECO S144-DR Sulfur Analyzer. Samples of stream water taken from the same locations in January 2016 were analyzed for dissolved sulfate concentration ($\mu g\ L^{-1}$) using a Dionex 120 ion chromatograph.

Methane and methylmercury production experiment

Experimental design

In January 2015, an 18-day microcosm incubation experiment was conducted to compare the activities of SRB, MPA, and all other microbes in production of CH_4 and methylation of Hg from sediments in the four study streams. Five treatments were used to compare microbial communities across stream sediments, with four replicates for each treatment. The experimental

treatments included: (1) control without inhibitors, (2) MPA inhibition treatment using 50 mM of BESA, (3) SRB inhibition treatment using 2 mM molybdate, (4) MPA and SRB inhibition treatment (50 mM BESA + 2 mM molybdate), and (5) a general bacterial inhibition treatment (2 mM chloramphenicol). Inhibitor concentrations were chosen based on our preliminary experiments, which established effectiveness of those concentrations in the study sediments. Concentrations given were of added inhibitor. This approach was expected to allow non-targeted microbial groups to remain metabolically active in the microcosms.

Microcosm construction

Microcosms consisted of acid cleaned 200-mL glass serum bottles (Zhang et al. 2012; Randall et al. 2013) containing 100.1 ± 0.1 g (± SE) of wet surficial sediment from pooled January 2015 samples and 101.1 ± 0.2 mL (± SE) of argon-purged reconstituted soft water (ultrapure water amended with 30 mg^{-1} L^2 $CaSO_4$·H2O, 30 mg^{-1} L^2 $MgSO_4$, 48 mg^{-1} L^2 NaHCO3, and 2 mg^{-1} L^2 KCl; USEPA 2002a). Pooled sediments from each stream were manually homogenized in sealed plastic bags for 5 min and large particles (e.g., stones and leaves) were removed before 100 g of wet sediment subsamples were distributed into microcosms. Artificial soft water purged with argon was used to reduce chemical variation in microcosm water and to promote anoxia in microcosms. Inhibitors were prepared separately in reconstituted soft water used in their respective experimental treatments before being introduced to the microcosms.

Microcosms were sealed with gas impermeable butyl rubber stoppers (Geo-Microbial Technologies) and crimped with aluminum seals. After sealing, microcosms were shaken vigorously for 30 s to equilibrate CH_4 between the water and headspace before evacuating headspace gas and filling with high-purity N_2 for six 30 s cycles to create an anoxic headspace free of CH_4. Microcosms were incubated at 22.5 ± 0.6 °C (± SD) for 18 days in the dark to prevent photoreduction of Hg(II), photodegradation of MeHg, and algal growth.

Methane sampling

Microcosms were sampled for CH_4 over 18 days (sample days: 0, 0.5, 1, 1.5, 2, 3, 5, 7, 10, 13, 18). Acid cleaned 20 mL glass serum vials with crimped aluminum seals served as CH_4 sample vials. Before use, sample vials were evacuated and filled with high-purity N_2 for six cycles, equalized to atmospheric pressure, treated with 0.1 mL of 10% HCl to prevent microbial activity, and then injected with 3 mL of N_2 purged deionized water (Lofton et al. 2014). Sample vials were stored in an inverted position to form a water barrier between the headspace gas and septum, acting as seal. Before sampling, each microcosm was shaken for 30 s to release trapped CH_4 in the sediment slurry. Prior to withdrawing samples, the headspace gas was further mixed by the withdrawal and reintroduction of 5 mL headspace gas for ten times via a syringe. Five millilitre samples of gas were taken from the microcosm headspace using a 22-gauge gas-tight syringe needle, and replaced with 5 mL of high purity N_2. Samples were injected into sample vials sealed with butyl rubber stoppers, from which 5 mL of gas had been previously evacuated. Sample vials were inverted during storage prior to processing, with acidified water.

Methane analysis

CH_4 concentrations were measured with a Shimadzu GC-8A gas chromatograph equipped with a flame ionization detection unit (GC-FID) operating with a 1/8″ diameter × 1 m length molecular sieve 5A (60/80) column at 140 °C, using ultra-high purity N_2 at 33 L min^{-1} flow rate for the carrier gas (Lofton et al. 2014). The GC-FID was calibrated using two points of NIST certified CH_4 standards before use. Output from the GC-FID was used to calculate concentrations of CH_4 from sample serum vials, which was then converted to nmol concentrations in each microcosm, accounting for headspace volume, total water volume, temperature, atmospheric pressure, ratio of microcosm gas sample to non-sample gas volume in sample serum vials, and the equilibration between aqueous and gaseous phases of CH_4 determined by multiplying Henry's constant for methane in water by the partial pressure of gas $CH_4(aq) = KPg$ (Lofton et al. 2014). Concentrations of CH_4 were normalized to the dry weight of sediment (g). Dry-weight concentrations were plotted over time for each microcosm to estimate the rate of CH_4 production (nmol g^{-1} DW day^{-1}).

Mercury analysis in sediment and enriched stable mercury isotope spiking setup

The concentration of total Hg in sediment from each stream was determined after acid digestion. Wet sediment samples were digested with concentrated trace-metal grade HNO_3 and H_2O_2 (4:1, v:v) in a tightly closed Teflon digestion vessel at 80 °C overnight. Sample Hg(II) was reduced to Hg(0) with stannous chloride, purged with Hg-free nitrogen gas, concentrated onto gold-coated glass beads, and quantified with a cold vapor atomic fluorescence spectrometer (Brooks Rand Model III) (USEPA 2002b).

After (pre-)incubation of sediment for 10 days, the total amount of Hg in the sediment was doubled by injecting via syringe a solution of enriched stable isotope of inorganic Hg(II), $^{200}Hg(NO_3)_2$ (96.4% purity, Oak Ridge National Laboratory), into the microcosm bottles created for also examining methane production. These microcosms had the same treatments as outlined previously. Microcosm bottles were incubated for 8 days after $^{200}Hg(NO_3)_2$ additions and were shaken daily to ensure homogenization of the contents. Following incubation, overlying water was removed and centrifuged (2000 rpm) for 10 min to remove suspended particulate matter. Overlying water from the control treatments was analyzed for multiple cations by ICP-MS (i.e., Na, Al, Cr, Mn, Fe, Ni, Zn, As, Se, and Pb). Suspended particulate matter collected from the overlying water was lyophilized with remaining sediment in microcosms. Freeze-dried particulate matter was combined with sediment from the same microcosm and then pulverized and homogenized by a mixer mill for subsequent analyses of methylated ^{200}Hg ($Me^{200}Hg$) and sediment chemistry.

Methylmercury isotope analysis

The amount of added $^{200}Hg(II)$ methylated to $Me^{200}Hg$ was measured by isotope dilution gas chromatography ICP-MS (GC-ICP-MS; Hintelmann and Evans 1997). Subsamples of lyophilized sediment were weighed into 50-mL centrifuge tubes, amended with $Me^{199}Hg$ internal standard, and extracted for MeHg with methylene chloride and back extracted to aqueous phase (Bloom et al. 1997; Hammerschmidt and Fitzgerald 2004). Samples (aqueous phase) received additions of sodium tetraethylborate to derivatize MeHg to ethyl-MeHg, were purged with high-purity N_2, and collected on Tenax resin traps (USEPA 1998). $Me^{200}Hg$ was quantified by GC-

ICP-MS (Hintelmann and Evans 1997; Hintelmann et al. 2000). The amount of measured $Me^{200}Hg$ was normalized to the concentration of $^{200}Hg(II)$ in sediment, both that which was naturally present and added experimentally. Simultaneous demethylation of MeHg may have occurred, and therefore a rate was not calculated (Schaefer et al. 2004). Percent of added total Hg as MeHg (i.e., % $Me^{200}Hg$) in sediment samples was interpreted as net MeHg production potential in each microcosm.

Soft water concentration experiment

A second microcosm experiment was conducted in January 2016 to compare CH_4 production in waters of differing soft water concentrations because soft water contains sulfate, which was expected to impact activity of SRB. Microcosm construction (including mass of sediment and water) were identical to the first experiment to allow for comparison to the first experiment. Treatments included unaltered stream water collected with sediment (2.9 mg L^{-1} sulfate), reconstituted soft water (40.8 mg L^{-1} sulfate), and reconstituted very soft water (10.2 mg L^{-1} sulfate) (USEPA 1991). Each water treatment was subdivided into BESA (50 mM), sodium molybdate (2 mM) and control treatments with four replications for each water and microbial inhibitor combination. This experiment investigated the influence of reconstituted water and stream water on methanogenesis and effectiveness of microbial inhibitors. On January 2016, sediment was collected from North Buffalo Creek only at the same location of the first experiment, with 35 sediment cores taken from a 75 m reach. Procedures for the collection and processing of sediment, microcosm construction (including mass of sediment and water) were identical to the first experiment to allow for comparison to the first experiment. Microcosms were incubated for 20 days, and CH_4 production was measured in samples on days 0, 1, 2, 3, 5, 7, 10, 15, and 20. Sampling of microcosms for methane, and subsequent methane analysis used the same procedures outlined for the methane and methylmercury production experiment.

Statistical analysis

Before statistical analysis, CH_4 data from the methane and methylmercury production and the soft water concentration experiments were examined for outliers and conformation to heterogeneity and normality assumptions of ANOVA analysis by residuals. Significant outliers were detected with Cook's distance and then removed. The data had a non-normal distribution with very small negative values as indicated with a Shapiro–Wilk test (Shapiro and Wilk 1965), so the data were rank transformed (Conover and Iman 1981). A two-tailed one-way ANOVA was used to assess treatment effects in ranked data, with streams viewed as units of replication since sediments were pooled within streams. Comparison between rank transformed treatments were made using Tukey–Kramer post hoc analysis. A two-tailed two-way ANOVA assessed microbial inhibitor and water type effects from the soft water concentration experiment. Tukey–Kramer post hoc analysis was used for treatment comparisons from the soft water concentration experiment.

Net MeHg production potentials from the methane and methylmercury production experiment, expressed as % $Me^{200}Hg$, were examined for outliers and conformation to heterogeneity and normality assumptions of ANOVA by residuals. Data were log transformed to correct for non-normality. One-way ANOVA was used to assess treatment effects. Stream was excluded as an

explanatory variable, because the differences between individual stream MeHg production rates was not the focus of our study and sediments were pooled for each stream. Comparisons between treatments were made with Tukey–Kramer post hoc analysis. To establish the relationship between % $Me^{200}Hg$ and final CH_4 concentration, a linear regression model was created with data from control treatments. No outliers were found in the regression model and data transformation was not necessary. MPA inhibited treatments were excluded because there was very low production of CH_4 in those treatments. As net MeHg production potential represented the production of MeHg at the conclusion of the experiment, it was compared with the final concentrations of CH_4 in the control treatment. % $Me^{200}Hg$ from the control treatment was also regressed against total S (µg/g) to explore if total S (µg/g), a portion of which is sulfate, influences MeHg production.

Results

Sediment characterization

North Buffalo Creek, South Buffalo Creek, and Little Alamance Creek had similar ion concentrations in water and sediment (Table 2). Reedy Fork Creek had the lowest concentrations of Na, Al, Fe, S, Mg, Zn, Pb, Ni and had the lowest total S and AFDM among study streams (Table 2).

Table 2. Physiochemical measurements from stream sediments, and cations from control treatment supernatant from the conclusion of the study

	North Buffalo Creek	South Buffalo Creek	Little Alamance Creek	Reedy Fork Creek
Sediment				
pH	7.35	7.45	7.05	7.15
AFDM (mg g^{-1})	3.03	2.92	4.76	2.67
Total S (µg g^{-1})	190	195	175	150
Total Hg (ng g^{-1})	3.79	3.87	4.56	1.13
Na (µg g^{-1})	553.26	661.46	277.77	119.57
S (µg g^{-1})	7.50	7.00	8.00	4.00
Fe (mg g^{-1})	17.46	25.29	20.34	11.4
Water				
Na (mg L^{-1})	26.47	27.4	28.03	23.88
Al (µg L^{-1})	201.31	329.04	291.81	174.35
Fe (mg L^{-1})	4.39	4.61	4.65	0.90
Ni (µg L^{-1})	2.96	3.99	3.52	2.45
Zn (µg L^{-1})	71.7	54.87	162.56	49.38
Se (µg L^{-1})	0.26	0.41	1.64	0.62
SO^{-2}_4 (mg L^{-1})[a]	2.90	2.30	2.85	1.20

Elemental concentrations of sediment were measured directly (Fe and NA) and as extractible forms (S). Total Hg units were derived per unit of wet sediment. All other sediment chemistry was calculated per gram of dry sediment
[a]Measures of SO^{-2}_4 from surface water in spring 2016

Methane and methylmercury production experiment

Methane production

The time course of CH_4 production was distinctive between control and molybdate treatments (Fig. 1). CH_4 production in control treatments began to decline after 10 days, except in Reedy Fork Creek, where the CH_4 production was very low overall and declined after day 3. In contrast, the CH_4 production rate in molybdate treatments did not decline during the 18-day incubation.

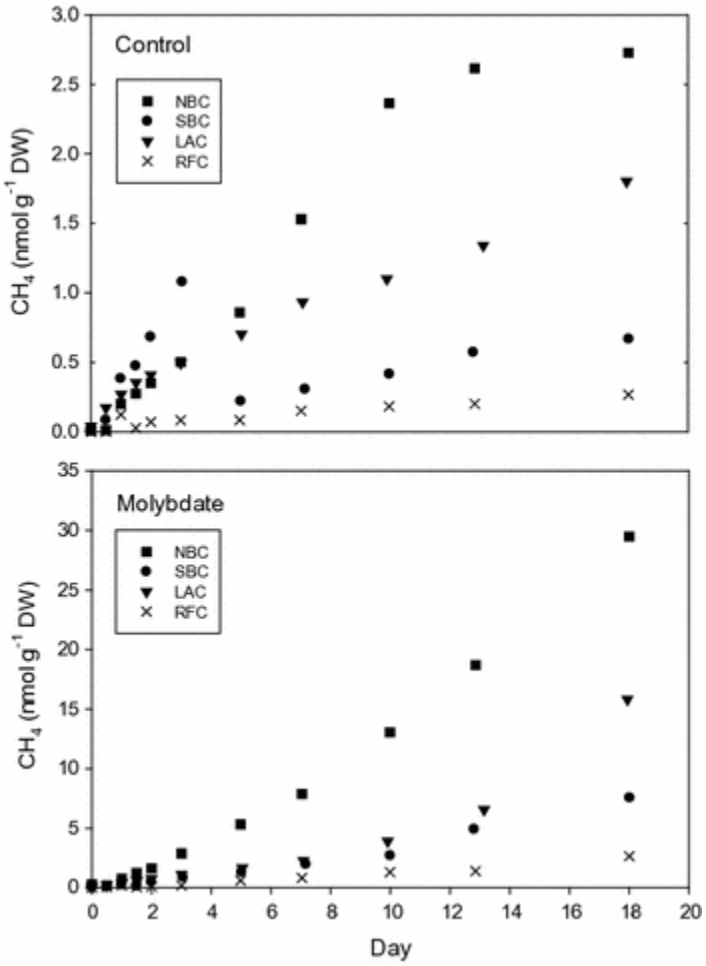

Figure 1. Average dissolved CH_4 concentrations in microcosms of stream water plotted across day of sample collection for control and molybdate treatments. *NBC* North Buffalo Creek, *SBC* South Buffalo Creek, *LAC* Little Alamance Creek, *RFC* Reedy Fork Creek

Methane production in sediment varied among treatments (Fig. 2). The molybdate treatment, which was intended to inhibit SRB but leave MPA and other anaerobes active, had the greatest average CH_4 production rate, 0.879 ± 0.16 nmol CH_4 g^{-1} DW day^{-1} ($\bar{x} \pm$ SE), followed by the control treatment (0.143 ± 0.036 nmol CH_4 g^{-1} DW day^{-1}). BESA, BESA + molybdate, and chloramphenicol treatments had uniformly low CH_4 production (< 0.03 nmol CH_4 g^{-1} DW day^{-1}). Differences in CH_4 production among treatments were significant (ANOVA, f-value = 23.18, df = 4, two-tailed p value < 0.001). Both molybdate and control treatments were statistically different from each other and distinct from the treatments that inhibited MPA (BESA, BESA + molybdate, and chloramphenicol). While treatment effects were consistent among streams, the magnitude of CH_4 production varied among streams (Fig. 2). Little

Alamance Creek and North Buffalo Creek had similarly higher CH_4 production than sediments from the other two sites. Reedy Fork Creek had the lowest CH_4 production of the four streams.

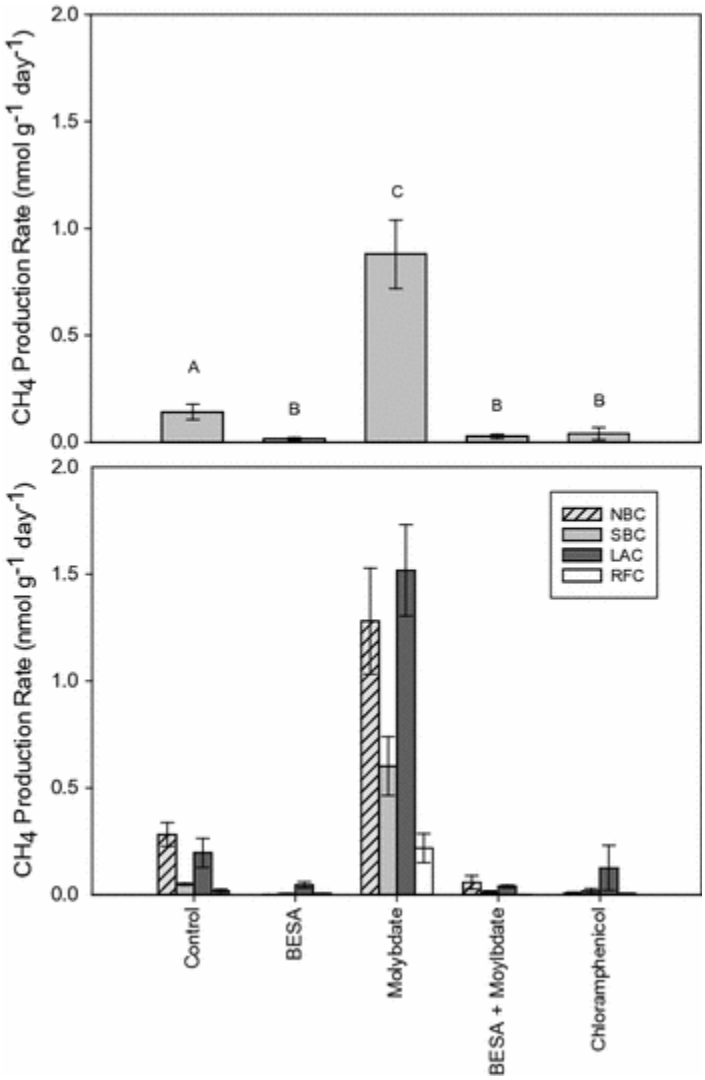

Figure 2. Average rates of CH_4 production for sediment samples from four streams exposed to specific microbial inhibitors, and comparison of CH_4 production from each individual stream. Error bars represent ± 1 SE. Similar letters above bars indicate rates that are not significantly different as determined by Tukey–Kramer HSD post hoc comparisons, while different letters indicate rates that are significantly different. *NBC* North Buffalo Creek, *SBC* South Buffalo Creek, *LAC* Little Alamance Creek, *RFC* Reedy Fork Creek

Methylmercury production potential

Both the control and BESA treatments had similar % $Me^{200}Hg$ and both had significantly greater net MeHg production potential than other treatments (f-value = 83.25, df = 4, two-tailed p-value < 0.001), with an average of 7.43 and 7.27%, respectively (Fig. 3). Molybdate and BESA + molybdate treatments had similarly low %$Me^{200}Hg$, 1.15 and 1.74%, respectively. The

chloramphenicol treatment had the lowest %Me^{200}Hg and was statistically different from all other treatments (0.36%).

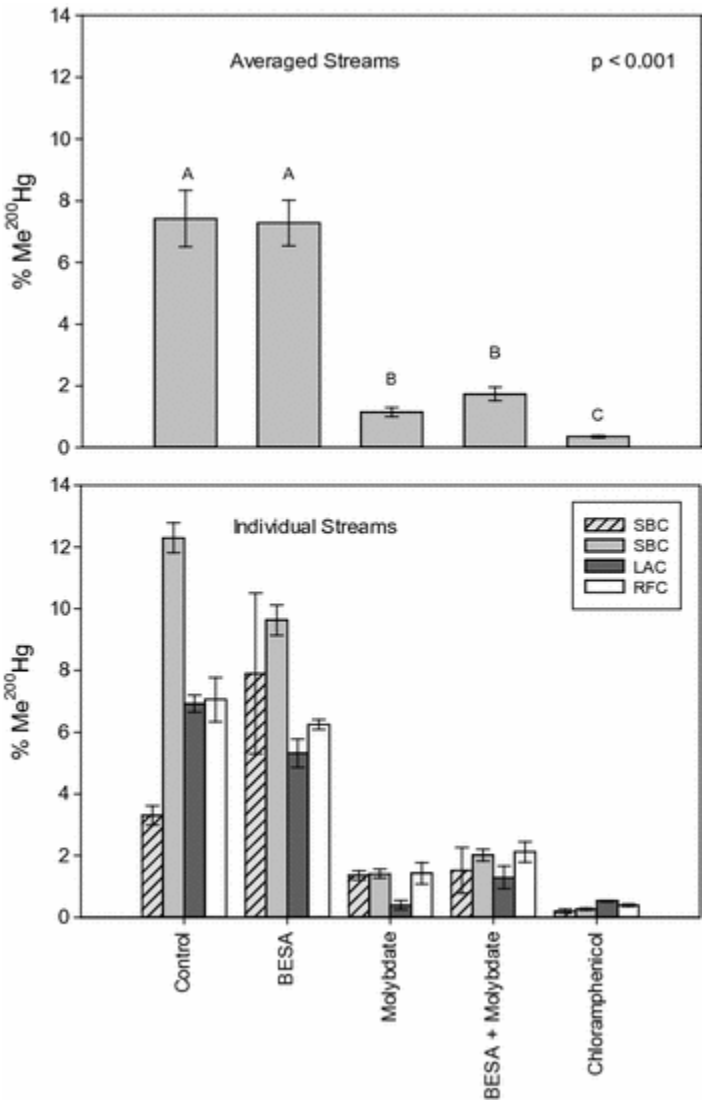

Figure 3. Average methylmercury production from sediment exposed to specific microbial inhibitors, and comparisons of MeHg production from each individual stream. Error bars represent ± 1 SE. Similar letters above bars indicate rates that are not significantly different as determined by Tukey–Kramer HSD post hoc comparisons, while different letters indicate rates that are significantly different. *NBC* North Buffalo Creek, *SBC* South Buffalo Creek, *LAC* Little Alamance Creek, *RFC* Reedy Fork Creek

Sediments from all four streams had a similar pattern of MeHg production among treatments, with higher % Me^{200}Hg in control and BESA treatments (Fig. 3). Similarly, there was consistently low methylation in chloramphenicol, molybdate and BESA + Molybdate treatments. However, and as observed for CH_4, net MeHg production potential varied among streams, with Reedy Fork Creek control sediments having the lowest MeHg production compared to others.

Net production of MeHg in control samples was strongly correlated with CH_4 production among all study sites (f-value, 39.11, df = 13, two-tailed p-value < 0.001; Fig. 4). Interestingly, % $Me^{200}Hg$ also was positively related to total S in stream sediment (f-value = 17.19, df = 13, two-tailed p-value = 0.001; Fig. 5).

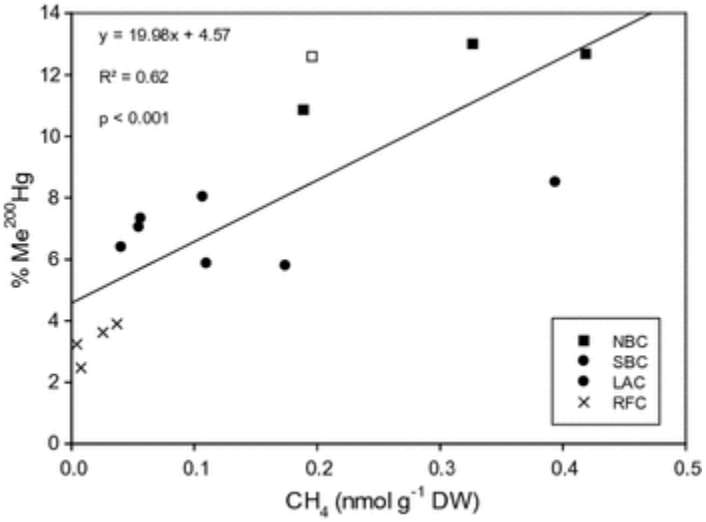

Figure 4. Control $Me^{200}Hg$ production and methane concentration regression. *NBC* North Buffalo Creek, *SBC* South Buffalo Creek, *LAC* Little Alamance Creek, *RFC* Reedy Fork Creek

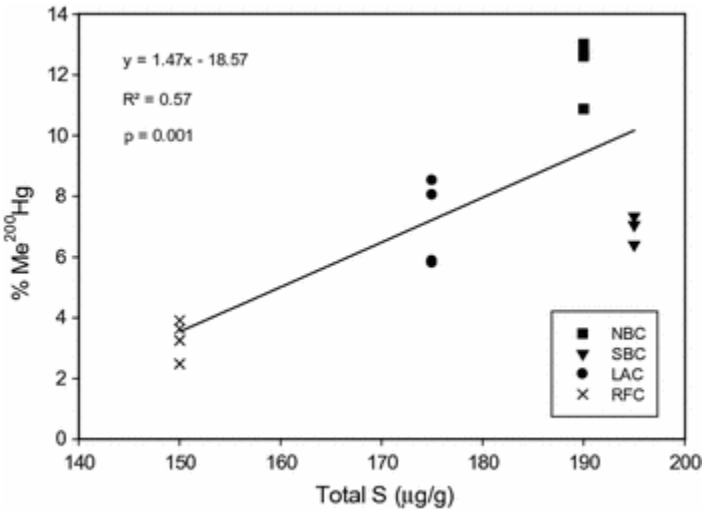

Figure 5. Control total sulfur and $Me^{200}Hg$ regression. *NBC* North Buffalo Creek, *SBC* South Buffalo Creek, *LAC* Little Alamance Creek, *RFC* Reedy Fork Creek

Sulfate concentration experiment

The molybdate treatment had the highest CH_4 production, and the BESA treatment had the lowest in the second experiment (Fig. 6). The two-way ANOVA for CH_4 production explained by sulfate-reduction inhibitory treatment (molybdate) and water type was statistically significant (f-value = 9.43, df = 30, $p < 0.0001$). Only the sulfate-reduction inhibitor treatment showed a significant effect ($p < 0.001$).

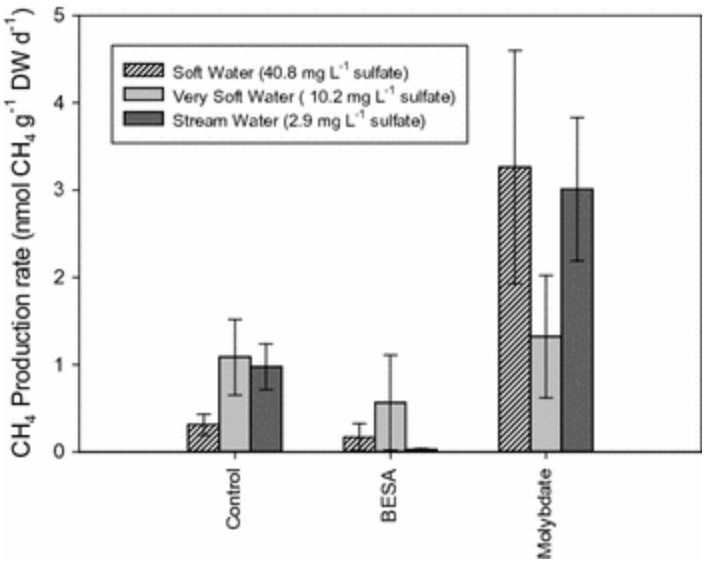

Figure 6. Methane production in soft, very soft, and stream water with dissolved microbial inhibitors. Error bars represent ± 1 SE

Discussion

Suppression of methanogenesis by sulfate-reducers

CH_4 emissions from lotic systems are significant to the global CH_4 budget (Bastviken et al. 2011) and contribute to global warming (Boucher et al. 2009). Our results show that CH_4 efflux from sediments is suppressed by SRB activity in North Carolina Piedmont stream sediments. Methane efflux in the control treatment was lower than in the molybdate treatment where only SRB were inhibited but emission from controls was still higher than the BESA treatment (Fig. 2). Bottles were anaerobic so there was no opportunity for aerobic oxidation, which is generally viewed as the major pathway for CH_4 oxidation in freshwater (Whalen 2005). Anaerobic oxidation of CH_4 (AOM) has recently been reported in freshwater wetlands, potentially coupled to sulfate reduction (Segarra et al. 2015), although its role in streams is unknown. Segarra et al. (2015) found that rates of AOM in wetlands were very low at low sulfate concentrations, but increased rapidly with increasing sulfate. In our soft water experiment, with variable sulfate concentration, we found no effect of water treatment on CH_4 emissions. This latter result provides no support for AOM as a mechanism regulating CH_4 emission, although does not conclusively rule it out. Thus, since our experiments were not designed to control for AOM, we cannot rule out that AOM occurred and could have accounted for some of the difference in CH_4 emissions between control and molybdate treatments. However, CH_4 emission from the control treatment was also significantly higher than from the BESA treatment. Thus, even if AOM occurred, it cannot account for all of the CH_4 emission. Thus, these results indicate that MPA were released from competition with SRB when sulfate reduction was inhibited, as previously observed in Lawrence Lake sediments in southwestern Michigan (Lovley and Klug 1983). Further investigation would be needed to conclude with certainty whether the mechanism of anaerobic oxidation of some of the CH_4 produced by methanogens via AOM also occurred, further reducing CH_4 emission. However, it is clear that SRB regulated the potential

for CH_4 emissions in our study. Thus, SRB may provide an ecosystem service by partially mitigating CH_4 emission from streams.

The dynamics of SRB-MPA interactions across streams may depend on sulfate concentrations and substrate availability (Takii and Fukui 1991; Lovley and Klug 1986), but did not appear to do so at the sulfate concentrations used in this study. We did not evaluate sulfate availability during the experiment, but sulfate concentrations measured post experiment (2.9–1.2 mg L^{-1}) were at or below the reported 2.9 mg L^{-1} threshold of sulfate needed for the suppression of methanogenesis by SRB suggested by Lovley and Klug (1986). In the first experiment, we used EPA reconstituted freshwater with a sulfate concentration of 40.8 mg L^{-1} to reduce within microcosm variability, which increased sulfate availability for SRB. This meant that ~ 8–10% of total sulfur in the microcosms was from sulfate in the added reconstituted soft water. Ambient stream conditions could have also been important in determining stream-specific microbial communities used to establish the microcosms. Initial (i.e., stream sediment) sulfate concentration from the four sites ranked SBC = NBC > LAC > RFC, whereas CH_4 production ranked LAC = NBC > SBC > RFC. Thus, the observed pattern is not consistent with CH_4 production being controlled primarily by initial sulfate availability.

Results from the second experiment supported the inference that sulfate availability was not the major factor controlling CH_4 production rate. The second experiment, which compared CH_4 production between stream water and artificial waters, showed that the same concentration of sulfate in reconstituted water used in the first experiment resulted in a statistically similar pattern of SRB control of CH_4 production, with no significant interaction between water and inhibition treatments. Furthermore, mean rates of CH_4 production in the control treatments were similar among natural stream water and the reconstituted soft waters (Fig. 6), whereas greater sulfate should have suppressed MPA activity if sulfate concentration was the primary controlling factor (i.e., a trend would be expected for: stream water ≥ very soft water > soft water). Thus, it appears that sulfate was not limiting SRB activity and not controlling the interaction of SRB and MPA microbial groups in the second experiment, and likely not limiting in the first experiment.

Microbial contributions to methylmercury production

SRB produced more MeHg in this study than other methylating microbes. BESA-amended treatments produced MeHg to levels like those in untreated controls (Fig. 3), suggesting a limited role for MPA in either mercury methylation or MeHg demethylation. SRB accounted for about three fourths of MeHg produced in the control (Fig. 3). To the extent that experimental conditions simulate natural processes in local streams, these results suggest that SRB are the primary producers of MeHg in sediment of the study streams, and potentially other North Carolina Piedmont streams. Furthermore, the positive relationship between total S and % MeHg (Fig. 5) also supports the conclusion that SRB are the primary methylators of Hg. Because a portion of total S represents sulfate, the positive relationship could be indicative of a correlation of favorable conditions for SRB activity and “net” Hg methylation. A positive relationship was also observed for potential net MeHg and final CH_4 concentration in the experimental microcosms. This result is not consistent with the observed relationship between MeHg and S, as MPA can also methylate Hg, although they contributed only a relatively small portion of the Hg methylation that occurred (Fig. 3). Gradients in whole-community respiration among sites may

have contributed to the relationship between potential net MeHg and final CH_4 concentration in uninhibited sediment. This relationship should not be interpreted to suggest that methanogens are dominant methylators of Hg in these sediments. Thus, concentrations of electron donors and acceptors likely influence SRB activity (Lovley and Klug 1986), and the generality of SRB as the primary methylators of Hg (Gilmour et al. 1992) bears investigation in other stream types.

Microbes besides SRB and MPA may have contributed the second highest source of MeHg. Assuming that MeHg produced in the control represented 100% of the methylating potential, other microbes potentially accounted for ~ 18% of the overall Hg methylation (Fig. 3). Microbes that would have been active in this community may have used a variety of terminal electron acceptors, including iron, benzoate and fumarate reduction (Gilmour et al. 2013). Among these, FeRB are known to coexist with SRB in river sediment (Yu et al. 2012). However, Reedy Fork Creek did not show an increase in Hg methylation in the MPA and SRB inhibited treatments (Fig. 3). Lower Fe concentrations at Reedy Fork Creek may have reduced the importance of FeRB in Hg methylation.

The lack of statistical difference between SRB-inhibited and MPA and SRB co-inhibited treatments suggests that MPA were not important methylators of Hg in this study. Although MPA are known to produce MeHg, only members of the class Methanomicrobia that possess the *hgcAB* gene cluster are known to perform Hg methylation (Gilmour et al. 2013); distribution of the *hgcAB* gene cluster in the MPA community was beyond the scope of this study. It is possible that other methylating microbes could have been released from competition when both SRB and MPA were inhibited, accounting for the lack of observed differences in Hg methylation between those treatments. Minimal MeHg production in the chloramphenicol microbial inhibition treatment illustrates that microbes rather than abiotic processes were the primary producers of MeHg in the sediment, consistent with many previous studies in sediment (Gilmour et al. 1992; Fleming et al. 2006; Harmon et al. 2007) and periphyton (Cleckner et al. 1999; Desrosiers et al. 2006; Hamelin et al. 2011). Because Hg can be methylated abiotically (Celo et al. 2006), the abiotic pathway may account for the trace amount of methylation (formed as $Me^{200}Hg$) observed in the chloramphenicol treatment. Alternatively, the added chloramphenicol may not have completely inhibited all microbial methylation activities.

The positive relationship between CH_4 and % $Me^{200}Hg$ (Fig. 4) seemingly contradicts results from the findings of our experiments, implying MPA were methylating Hg(II) in excess of what was observed in MPA active treatments. Factors promoting the methylation of mercury such as substrate availability, including acetate, could have simultaneously stimulated CH_4 production. Rather than suggesting that methanogens were producing MeHg, this could be an artifact of both processes correlating with factors concerning anaerobic metabolism. Further, CH_4 is also a product of degradation of MeHg, which may be microbially mediated by the *merB* gene (Schaefer et al. 2004). Accordingly, a portion of the CH_4 produced in the control treatment may have been the product of microbial demethylation, resulting in the observed relationship between % $Me^{200}Hg$ and CH_4 concentration observed in the control. The importance of MPA methylation in systems with biofilms, such as periphyton (Hamelin et al. 2011), and roots of macrophytes (Correia et al. 2013) does not seem to apply to these stream sediments. Rather, these results provide evidence that in the stream sediments we investigated SRB are the major methylators of

Hg(II), much like in estuarine (Duran et al. 2008; Gilmour et al. 2011) and marine sediments (King et al. 2001).

Conclusions

Our results contribute to the growing literature on CH_4 production in stream sediments (Stanley et al. 2016), and provide some new insights into anaerobic microbial ecology in streams. As discussed above, SRB appear to have suppressed methanogenic activity in treatments where SRB were active, suggesting that SRB may be exhibiting control of CH_4 production in stream sediments, partially mitigating CH_4 emissions from streams. We did not explicitly examine the role of sulfate concentration in limiting SRB activity, although we did examine methane efflux at a limited range of sulfate concentrations and found no evidence of sulfate limitation. However, the dynamics between SRB and MPA at lower sulfate concentrations than those observed in our study likely warrant further investigation. Additional work is needed to understand other factors controlling spatial and temporal variability of CH_4 production in streams. Because static microcosms were used primarily due to safety and expense considerations with Hg studies, variability in flowing water and stream sediments were not preserved in this study. Further research using artificial and natural streams is needed to investigate the interaction of SRB and MPA under more natural and dynamic conditions. However, SRB clearly regulated the potential for CH_4 emissions in our study. Thus, SRB appear to provide an ecosystem service by mitigating the potential for CH_4 emission from streams. Most the MeHg production appeared to be mediated by SRB even though methanogenic conditions were present, potentially reflecting low rates of MeHg production by MPA (Gilmour et al. 2013). MeHg producing microbes other than SRB or MPA had a role in the production in MeHg in sediments (Fig. 3), although that role was much lower than that of SRB. However, if sulfate availability is highly limited in natural conditions then these groups could become an important source of MeHg.

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