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Relative Contributions of Mercury Bioavailability and Microbial Growth Rate on Net Methylmercury Production by Anaerobic Mixed Cultures

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The environmental production of the neurotoxin methylmercury occurs through anaerobic microorganisms such as sulfate-reducers. Rates of mercury methylation are known to be influenced by the productivity of these microbes as well as the bioavailability of inorganic Hg for these organisms. However, the conditions that guide the importance of one factor relative to the other are not well defined. This paper describes experiments with mixed cultures of methylating microorganisms that indicate the existence of a threshold in activity of methylating microorganisms (e.g., sulfate reduction rate) where net methylmercury production is sensitive to either microbial growth or to Hg speciation and bioavailability. At environmental sites of interest, knowledge of this threshold in microbial activity could be useful for remediation purposes by allowing a prioritization of actions that can best minimize in-situ production of methylmercury.

Relative Contributions of Mercury Bioavailability and Microbial Growth Rate on Net Methylmercury Production by Anaerobic Mixed Cultures

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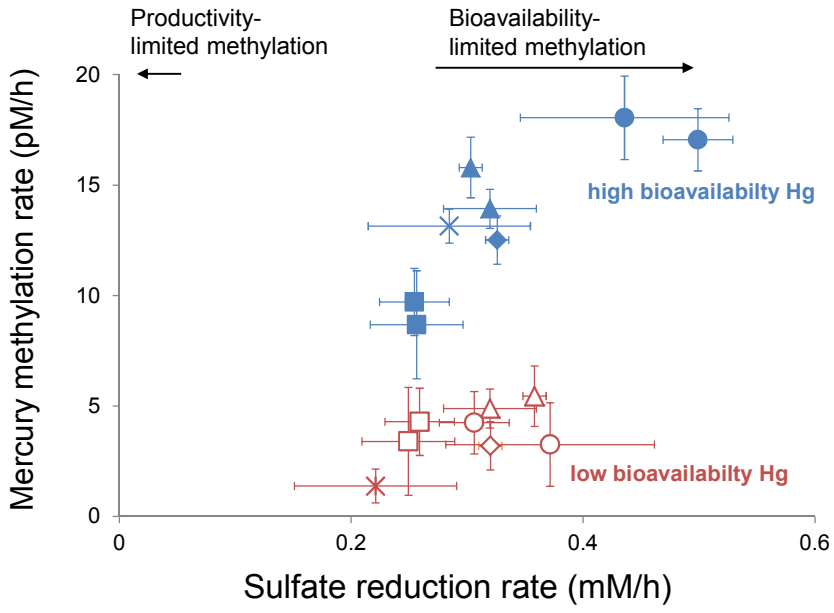
Abstract

Monomethylmercury (MeHg) is produced in many aquatic environments by anaerobic microorganism that take up inorganic forms of Hg(II) and methylate it. Net methylation of Hg(II) appears to be correlated with factors that affect the activity of the anaerobic microbial community and factors that increase the bioavailability of Hg(II) to these organisms. However, the relative importance of one versus the other is difficult to elucidate even though this information can greatly assist remediation efforts and risk assessments. Here, we investigated the effects of Hg speciation (dissolved Hg and nanoparticulate HgS) and microbial activity on the net production of MeHg using two mixed microbial cultures that were enriched from marine sediments under sulfate reducing conditions. The cultures were amended with dissolved Hg (added as a dissolved nitrate salt) and nanoparticulate HgS and grown under multiple carbon substrate concentrations. The results indicated that net mercury methylation was the highest for cultures incubated in the greatest carbon substrate concentration (60 mM) compared to incubations with less carbon (0.6 and 6 mM), regardless of the form of mercury amended. Net MeHg production in cultures exposed to HgS nanoparticles was significantly slower than in cultures exposed to dissolved Hg; however, the difference diminished with slower growing cultures with low carbon addition (0.6 mM). The net Hg methylation rate was found to correlate with sulfate reduction rate in cultures exposed to dissolved Hg, while methylation rate was roughly constant for cultures exposed to nanoparticulate HgS. These results indicated a potential threshold of microbial productivity: below this point net MeHg production was limited by microbial activity, regardless of Hg bioavailability. Above this threshold of productivity, Hg speciation became a contributing factor towards net MeHg production.

Keywords: mercury methylation potential, bioavailability

Graphical Abstract



Introduction

Monomethylmercury (MeHg) is a neurotoxic and highly bioaccumulative compound that poses human health risks via dietary fish consumption[1, 2]. In the aquatic environment, mercury occurs primarily as inorganic species, including many forms of dissolved and particulate inorganic divalent mercury (Hg(II))[3]. A process essential to the bioaccumulation of mercury is the production of MeHg by anaerobic microorganisms in aquatic ecosystems.

Recent research on microbial methylation of mercury has focused on two factors that govern this process: 1) The identification of the methylating microorganisms and the biochemical pathway of methylation; and 2) The chemical speciation of mercury in anaerobic settings and bioavailability of Hg(II) for cellular uptake by methylating microorganisms [4-6]. In each case, the environmental factors that contribute to MeHg production have been studied in depth. For example, conditions that include anaerobic zones, relatively high amounts of total or dissolved organic carbon, and additions of sulfate to freshwater system are all known to increase the productivity of methylating microorganisms, and subsequently MeHg concentrations[7-12]. Likewise, a fraction of the Hg(II) in anaerobic settings is bioavailable to methylating organisms, and it may include dissolved Hg-ligand species (e.g. dissolved Hg-sulfides) as well as Hg associated with particles[3]. In anaerobic settings, Hg(II) tends to be primarily associated with particulate phases[13, 14], including crystalline HgS mineral phases such as metacinnabar, amorphous or nanostructured HgS, and adsorbed forms of Hg(II) (e.g., to metal sulfide particles and particulate organic matter)[3]. These various particulate forms of Hg offer a spectrum of reactivity and bioavailability to methylating organisms due to differences in rates of dissolution and desorption, differences in the ability to accumulate at cell interfaces, and for nanoparticles, a potential ability to cross cell membranes[15-18].

The relative contribution of microbial productivity and Hg bioavailability for net MeHg production rates is not well established and is the focus of this study. Previous research on this topic focused only on one factor such as sulfate reduction rates or the speciation of inorganic Hg for controlling net MeHg production rates.[3, 10, 19-22] Research that can control for both factors would provide greater insight to the processes that influence mercury methylation potential in the environment. For example, our previous work with sediment microcosms [23] indicated that in some conditions low nutrient availability can limit the productivity of methylating microbes and subsequently limit MeHg production rates. In other conditions where methylating

microorganisms are active, the speciation and bioavailability of Hg may be more important for controlling MeHg production. The sediment microcosms described in our previous work [23] did not test many variables in microbial growth rates. Thus, specific conditions in which one factor dominates over the other needs to be studied in greater depth.

The goal of this study was to compare the relative contributions of microbial productivity and Hg speciation for net MeHg production in mixed cultures of anaerobic microorganisms. Anaerobic sulfate-reducing cultures were enriched from two sediment samples obtained from a marine location. The growth of the organisms was controlled by varying the concentrations of organic substrates, while the bioavailability of added Hg was controlled by amending the cultures with either dissolved Hg or nanoparticulate HgS. The rates of net MeHg production were then compared to growth rates of the organisms, sulfate reduction rates, and gene abundance for sulfate reducers.

Materials and Methods

Sediments for microbial enrichments. Sediment samples were obtained from two locations (MS-1 and MS-2) of the same marine water body in August 2012. The precise location of the site cannot be disclosed due to contractual agreements with the site managers. The water depth at the site was approximately 55 to 75 m, while the surface and bottom temperatures averaged 29.5^{o}C and 27^{o}C, respectively. At both sampling locations, dissolved oxygen was not detectable beneath the sediment surface layer, and dissolved sulfide was also not detectable (<1 μM), indicating anoxic but not extremely reducing conditions (per communication with site managers). The production of methylmercury in sediments at this site is a concern due to the presence of Hg-bearing particles in the area. The top layer of the sediment (approximately 5 cm) was collected in triplicates by Van Veen-type grab samplers, packed into acid-cleaned polyethylene jars with Teflon-lined caps and immediately frozen at -20^{o}C, and transported to the lab at Duke University for further analysis.

At the lab a subset of each sample was thawed and characterized for texture (i.e., grain size), total Hg and MeHg content, and organic carbon content. Sediment moisture content was determined by drying samples at 110^{o}C for 24 hours; pH was determined in a slurry comprising of 5 parts distilled water and 1 part sediment. Sediment texture was measured by dispersing the sediments in an aqueous solution of 2.5 N hexametaphosphate ($(NaPO_3)_6$) and mixing. The

relative size fractions were quantified by the rate of gravitational settling of particles [24-26]. Pore water was extracted from the sediment by centrifuging the sediments under aerobic conditions at 3000 *g* for 20 min. The supernatant of this centrifuged sample, designated as "pore water" in this study, was analyzed for sulfate concentration.

Preparation of anaerobic mixed cultures for Hg methylation. The inocula for the mixed cultures were prepared by enriching sediment slurries for anaerobic microorganisms under sulfate reducing conditions. These enrichments were prepared by placing 20 g of homogenized wet sediments from sites MS-1 or MS-2 into serum bottles containing 200 mL of sterile growth medium developed for sulfate-reducing cultures (see references [27], [28] and the SI). The serum bottles were sealed and incubated in the dark at room temperature (20 to 22 ^{o}C) for 7 days. After this time the bottles were mixed end-over-end, large sediment particles were allowed to settle, and aliquots of the overlying water served as inocula for the Hg methylation experiments. Enrichments of anaerobic microbial consortia from MS-1 and MS-2 were each performed on two different occasions. All handling of the sediments, enrichments and the subsequent mixed culture experiments were conducted in an anaerobic chamber (Coy Labs) with an oxygen-free gas mixture (90% N_2, 5% CO_2, and 5% H_2).

The Hg methylation experiments were conducted in 30 mL anaerobic culture tubes containing sterile sulfate reducing medium and inoculated with the supernatant of the enriched sediment slurries. In the first round of experiments, the carbon substrate (C-substrate) concentrations were varied to the following: 0.6 mM, 6 mM, and 60 mM, each comprising a mixture of 2 parts sodium pyruvate and 1 part lactic acid (mole basis). In the second round of mixed culture experiments, five different carbon concentrations were tested (0.6 mM, 0.8 mM, 1 mM, 6 mM and 60 mM with the same ratio of pyruvate to lactic acid as in the first experiment). For each of the carbon concentrations, 20 mL of medium was dispensed into the tubes and 2% (vol/vol) of the inoculum was transferred.

Immediately after inoculation of the culture media with variable C-substrate concentrations, the cultures were amended with either dissolved Hg or nanoparticulate HgS from their respective stock solutions to a final Hg concentration of 5 nM. The preparation of the nanoparticulate HgS stock solutions was followed according to previous work [23] and is summarized in the SI. The nanoparticles comprised of metacinnabar-like structure with primary

particle diameter of 3-5 nm [23] and were aggregated to an average hydrodynamic diameter of 25.8 nm ± 2.9 nm (based on light-intensity weighted dynamic light scattering).

Four sets of controls with the culture media containing 60 mM C-substrate were incubated under the same conditions as the test samples. These included: (1) A blank containing the culture medium, microbial inoculum from either MS-1 or MS-2 slurries, and no added mercury (i.e., Hg blank); (2) An abiotic control consisting of the same culture medium amended with dissolved Hg or nanoparticulate HgS but no microorganisms; (3) A killed control with the culture medium inoculated with microorganisms, amended with dissolved Hg or nanoparticulate HgS, and spiked with 0.4% (vol/vol) concentrated hydrochloric acid (HCl) (trace metal grade); (4) A molybdate control consisting of the inoculated culture medium amended with dissolved Hg or HgS nanoparticles, and 20 mM sodium molybdate, a specific inhibitor of sulfate reduction[29, 30]. All culture tubes were capped with gas-tight seals and placed into the anaerobic jar with GasPak 150 system (Fisher Scientific) to maintain strict anaerobic conditions. The mixed cultures were incubated in the dark on a platform shaker (Eppendorf) at 25 rpm for 64 hours at 30^{o}C.

At each time point, replicate culture tubes (n = 2 or 3) were sacrificed and subsampled for chemical and biological analyses. Prior to liquid sample collection, the tubes were vortexed, and 1 mL of gas was collected from the headspace using a gas-tight syringe. The gaseous mercury content (e.g., Hg^0) in the samples was analyzed by injecting the sample into a gas-tight vial filled with ultrapure water (Barnstead Nanopure, >18 MΩ-cm) containing 2% (vol /vol) BrCl. These samples were then stored for at least 3 days at room temperature prior to total mercury analysis. After the collection of the headspace, liquid aliquots were withdrawn for measurements of optical density at 660 nm (OD_{660nm}), DNA, total mercury, acid volatile sulfide and sulfate (SO_4^2). The remainder of the culture was preserved for MeHg analysis by adding 0.4% (vol/vol) concentrated hydrochloric acid (HCl) (trace metal grade) and stored at 4^{o}C until analysis.

Samples at 10, 48 and 64 hours of incubation were subject to the DNA extraction followed by the quantitative PCR analysis with primers targeting sulfate reducing microorganisms. The details of this method are described in the SI section. The relative abundance of sulfite reductase gene was calculated from the copy numbers of total bacteria in the sample and correlated to the net mercury methylation production.

Chemical analyses. The concentration of total mercury in liquid samples was quantified by stannous chloride reduction, gold amalgamation, and cold vapor atomic fluorescence spectrometry (CVAFS) [31]. For total mercury concentration in sediments, the samples were first digested in a mixture of 8 mL concentrated HCl and 2 mL concentrated HNO_3 and heated to 90°C for 5 h. After the digestion step, the solution was cooled and diluted for analysis by CVAFS.

Methylmercury concentration in liquid samples was quantified by distillation, aqueous phase ethylation, gas chromatographic separation, pyrolysis and inductively coupled plasma mass spectrometry[32]. The efficiency of the distillation step was quantified by spiking each sample with $Me^{201}Hg$ (50 pg mercury) as an internal standard. The recovery of the $Me^{201}Hg$ spike was used to correct for the MeHg concentration in each sample[33].

Methylmercury in the original MS-1 and MS-2 sediment samples was extracted by acid-dichloromethane leaching and aqueous back extraction prior to analysis[34]. Sediment MeHg concentrations were corrected for extraction efficiency using a MeHg standard (Brooks Rand) and reported on dry sediment weight basis.

Total organic carbon (TOC) in sediments was quantified by the difference of total carbon content and inorganic carbon content of dried sediment samples (i.e. heated to 100°C). Inorganic carbon content corresponded to the total carbon quantified in dried samples that were further heated to 400°C for 12 h. All carbon contents were determined using a TOC-V CPH total organic carbon analyzer (Shimadzu).

Sulfate concentration was determined by ion chromatography (Dionex ICS-2000, Sunnyvale, CA) using an AS18 analytical column, ASRS 300 suppressor and KOH eluent generator. Samples for acid volatile sulfide (AVS) quantification were preserved with $ZnSO_4$ and KOH (final concentrations of 20 mM and 4 mM, respectively) and stored at 4˚ C until analysis. For analysis, each sample was placed into a closed glass reactor with 1 N HCl, purged with ultra high purity N_2 for 30 min into a 10% v/v NaOH solution, and analyzed for sulfide concentration via colorimtery.[35, 36]

Data Analyses. Linear-least squares regressions were performed for time-dependent MeHg and sulfate concentration data from the cultures. The slope values for these regressions were used to estimate rates of net MeHg production and sulfate reduction. The regression parameters (slope

and intercept) were calculated and reported with their respective standard errors. Correlations were also performed to compare the sulfate-reducing community (expressed as sulfate reduction rate or gene abundance) with mercury methylation (expressed as net rate of production or % of total Hg as MeHg). Single-factor analysis of variance (ANOVA) was used to compare rates between culture treatments (e.g., sulfate reduction rates in dissolved Hg amendments versus nano HgS amendments). Significant differences between treatment types were designated for comparisons yielding p-values less than 0.05. All statistical analyses were performed with the Analysis ToolPak in Microsoft Excel.

Results and Discussion

Characteristics of sediments for the enrichments. The analysis of relevant chemical characteristics of the MS-1and MS-2 sediments used for the enrichments (Table 1) indicated that sulfate concentrations in the porewaters were approximately 2700 - 2900 mg L^{-1}. Total Hg concentrations in MS-1 and MS-2 sediments were 12.2 ± 5.9 mg kg^{-1} and 16.6 ± 2.8 mg kg^{-1}, respectively, and reflected elevated mercury concentrations relative to the median crustal earth concentration (approximately 0.05 mg kg^{-1} [37]). Methylmercury content in sediments was also high (Table 1). However, the MeHg represented only a small percentage of the total Hg (0.001-0.002%). Total organic carbon (TOC) content was greater for the MS-1 sediment than for MS-2, but overall. was relatively low for both sediments (31.8 ± 0.8 mg kg^{-1} and 4.5 ± 0.6 mg kg^{-1} for MS-1 and MS-2, respectively). Thus, the activity of the native microbial community was expected to be low.

Growth rates of mixed cultures under variable carbon substrate concentration and type of added Hg. The growth rates of the mixed cultures depended on the concentration of C-substrate present in the culture media and was unaffected by the type of mercury added. In all cases, similar growth patterns were observed, with faster growth kinetics for cultures incubated at the greatest concentrations of C-substrate (Figure 1). Examination of the growth curves revealed an initial 2 h to 5 h lag phase followed by a rapid 10-hour increase in cell density, corresponding to a specific growth rate μ of 0.064 to 0.070 h^{-1}. This fast growth was followed by significantly slower growth or no additional growth.

Net production of MeHg in mixed cultures. Net MeHg production depended on the type of mercury added (i.e., dissolved Hg and nanoparticulate HgS) and the amount of C-substrate present in the growth media (Figure 2). Both mixed cultures exposed to dissolved Hg demonstrated net increases of MeHg concentrations during the exponential and stationary phases of growth (from approximately 5 h to 64 h incubation time). In the mixed cultures inoculated from the MS-1 sediment enrichment (Figure 2A), 10.9%, 22.6%, and 29.8% of the added dissolved mercury spike (5 nM total) was converted to MeHg at the end of the experiment for mixed cultures incubated in 0.6 mM, 6 mM and 60 mM C-substrate, respectively. For the mixed cultures derived from the MS-2 sediment enrichment (Figure 2C), relatively less of the added dissolved Hg was converted to MeHg (1.4 to 5.3% as MeHg). This difference between the mixed cultures is likely due to differences in composition of microbial populations in the MS-1 and MS-2 derived cultures.

For mixed cultures that received HgS nanoparticles (Figures 2B and 2D), MeHg concentrations in most cultures increased during the first 5 h to 24 hours and slowed after this point to the end of the experiment. In MS-1 mixed cultures with HgS nanoparticles added, 3.9 to 12.7% of initial Hg spike was converted to MeHg at the end of the experiment, while in the MS-2 mixed cultures, 0.3 to 2.1% of initial Hg was methylated. These percent methylated values were 3 to 4 times lower than in respective cultures with dissolved Hg added and the same C-substrate concentration. Moreover, these differences between the dissolved Hg and nanoparticulate HgS amendments were not caused by variable bacterial growth rates, as cell growth was the same regardless of the type of added Hg (Figure 1). Rather, the difference was likely due to the limited bioavailability of Hg originating from HgS nanoparticles relative to dissolved Hg.

The mercury added to the culture flasks was, for the most part, fully recovered in the culture media during the entire experiment. In both MS-1 and MS-2 mixed cultures amended with either dissolved Hg or nanoparticulate HgS, at least 85% of the added Hg was quantified in the culture media (Figure S1). Approximately 15 to 25% of the added mercury sorbed to the container while less than 1.0% was recovered in the headspace of the culture vials. We also hypothesize that the speciation of the Hg changed during the course of the incubation. For example, the production of sulfide (Figure S2) from the sulfate reducing community likely changed the speciation of Hg in the cultures and the relative partitioning of Hg between

dissolved and particulate phases. Nevertheless, the initial form of Hg added to the cultures (i.e. dissolved Hg or nanoparticulate HgS) played an important role in determining net methylation rates.

In the Hg blank, killed controls, and abiotic controls, the measured MeHg concentrations were less than 4.1 pM for all time points (data not shown). These concentrations were much smaller than the MeHg concentrations observed at the end of the 64-h incubations for all the live cultures amended with either form of mercury (Figure 2).

Consumption of sulfate and abundance of sulfite reductase genes. The activity of SRB was evaluated by monitoring sulfate concentrations during the incubation and quantifying the relative abundance of sulfite reductase genes in the cultures. In all mixed cultures, sulfate concentrations followed an approximately linear decrease with time (Figure 2). The greatest decrease in sulfate concentration was observed for cultures incubated with 60 mM C-substrate, an observation consistent with the expected greater microbial activity at higher substrate concentration.

The addition of sodium molybdate resulted in almost complete inhibition of sulfate consumption and MeHg production (Figure S3), consistent with previous studies.[29, 30] The concentrations of MeHg at the end of the incubation in these cultures ranged from 0.06 to 0.16% of initial mercury spike, thus indicating that the predominant mercury methylators in these mixed cultures were associated with the sulfate reducing community (to be expected given the enrichment conditions).

The abundance of the sulfite reductase *dsrA* gene normalized to the abundance of the 16S rDNA gene increased with incubation time (Figure S4), as expected for cultures grown under sulfate reducing conditions. The relative amount of *dsrA* was greatest for mixed cultures incubated with 60 mM C-substrate (19% for MS-1 and 10% for MS-2 culture). For context, the abundances of *dsrA* in the surface of marine sediments can vary from 2 to 30%.[38, 39] The abundance of *dsrA* gene in the MS-1 mixed cultures appeared to increase with each time point during incubation (Figures S4A and S4B). However, for the MS-2 culture, the abundance of *dsrA* gene was relatively lower and did not increase appreciably during the first 48 h of the incubation (Figures S4C and S4D). A slight increase was observed at the 64 h time point, when the relative *dsrA* gene abundance was 1% to 9%. These values, however, are less than the

relative *dsrA* gene abundances observed in the MS-1 mixed cultures, consistent with differences in growth rates and MeHg concentrations between the two mixed cultures.

Correlations between microbial growth, sulfate reduction, and net MeHg production. The relationships between microbial growth, sulfate reduction, and net MeHg production were compared for time points between 10 and 64 hours in the incubation. These time points were selected because the largest changes in MeHg concentrations were observed during this time frame for the mixed cultures (Figure 2). Cell density of most cultures showed a strong linear relation to the MeHg concentrations (Figure 3), similar to other studies [27, 40]. An exception to this trend was observed for the MS-2 mixed culture with dissolved Hg added, where net MeHg concentrations increased while cell growth was minimal (Figure 3C).

Additional comparisons were made between the rates of net MeHg production and sulfate consumption for the time periods of most active net Hg methylation (Figure 4). These rates were calculated from the slopes of linear regressions for the 10 h to 64 h data points shown on Figure 2, and also for a second round of enrichment experiments performed with five different C-substrate concentrations (time-course data not shown). While we recognize that some of the concentrations values were not linear with time, we used this approach to provide an estimate that could be used for comparison purposes. The parameters for the regressions are shown in Tables S1 and S2.

When all sulfate reduction rates were compared for the same cultures, the rates did not change with the type of Hg added (Figure 4). For example in the MS-1 cultures, sulfate reduction rates were 0.25 – 0.5 mM h^{-1} in the dissolved Hg amendments (Figure 4A) and 0.21 – 0.37 mM h^{-1} in the nanoparticulate HgS amendments (Figure 4B). These rates were not significantly different ($p = 0.36$ for single factor ANOVA). Likewise in the MS-2 cultures, the sulfate reduction rates were 0.15 – 0.25 mM h^{-1} in the dissolved Hg amendments and 0.12- 0.34 mM h^{-1} in the nanoparticulate HgS amendments ($p = 0.34$) (Figure 4C and 4D). We also note that these rates are similar to sulfate reduction rates observed in saline settings that are rich in organic matter and highly productive ecosystems (up to 1.5 mM h^{-1}). [10, 41]

The rates of net methylation were for the most part faster in cultures that received dissolved Hg than those that received nanoparticulate HgS. For example in the MS-1 cultures that received dissolved Hg, net MeHg production rates were 8.7 – 18.1 pM h^{-1} (Figure 4A), while

the cultures receiving nanoparticulate HgS had rates of 1.4 – 5.4 pM MeHg h^{-1} (Figure 4B). Similar trends were observed for the MS-2 cultures, although the difference was smaller (1.1 – 4.4 pM h^{-1} in dissolved Hg amendments; 0.17 – 1.7 pM h^{-1} in the nanoparticle HgS amendments, Figure 4C and 4D). For context, the rates of net MeHg production shown in Figure 4 were similar to or greater than rates observed previously in sediment slurries amended with the same amount of mercury (0.5 – 3 pM h^{-1}).[23]

The difference in methylation rates between dissolved Hg and nanoparticulate HgS amendments is consistent with expectations that dissolved Hg is more bioavailable to the microorganisms than nanoparticulate HgS. However, the difference diminished at low sulfate reduction rates. In other words, the slope of the regression line in Figure 4A is greater than the slope of the regression for Figure 4B . A comparison of slope values for Figures 4C and 4D was not warranted, as both values were not significantly different from zero ($p > 0.05$).

The contribution of the sulfate reducers in the mixed cultures is further highlighted in Figure 5, where the percentage of methylated mercury is plotted as a function of the relative abundance of *dsrA* genes. Regression analysis indicated significant correlations ($p < 0.05$, n=9) between these two parameters for the mixed culture from MS-1 (Figures 5A and 5B).The correlations indicated that cultures spiked with dissolved Hg showed a steeper positive trend (slope = 1.4 (% as MeHg)×(% dsrA abundance)$^{-1}$, Figure 5A) than the nanoparticulate HgS amended ones (slope = 0.65 (% as MeHg)×(% dsrA abundance)$^{-1}$, Figure 5B). Similarly, for MS-2 cultures, there was a steeper positive trend in amendments with dissolved Hg (slope = 0.67, Figure 5C) relative to the culture amended with nanoparticulate HgS (slope = 0.13, Figure 5D). The slope values for the MS-2 culture were smaller than the MS-1 culture due to the smaller abundance of *dsrA* genes in MS-2 (<6%) relative to the MS-1 (5-20%).

The trends shown in Figures 4 and 5 suggest that if the experiments were performed at low microbial growth rates (i.e., sulfate reduction rates much less than 0.1 mM h^{-1}), net MeHg production would likely be similar regardless of the type of Hg added. The results of this study did not have sufficiently low sulfate reduction rates to fully demonstrate this phenomenon. However in our previous experiments with sediment slurry microcosms[23], cultures with low sulfate reduction rates (ca. 5×10^{-3} mM h^{-1}) had produced MeHg at the same net rate even though they received forms of Hg with different bioavailability (i.e., dissolved Hg and nanoparticulate HgS). In this case the low productivity of the microbial community, as indicated by low sulfate

reduction rates, was the limiting factor for controlling net MeHg production. In contrast, at high sulfate reduction rates (such as those shown in Figure 4) the bioavailability of Hg was important for controlling net MeHg production. The notion of growth-limited versus bioavailability-limited mercury methylation would imply the existence of a threshold sulfate reduction rate. However, the quantification of this threshold cannot be firmly established with this data set due to relatively large uncertainties of the regression parameters (i.e. slope and intercept) for the data in Figure 4. Further work should include a broader range of microbial growth rates, particularly at low growth conditions.

Net methylation potential was greater in MS-1 cultures than in MS-2 cultures. The mixed cultures derived from MS-1 had a net production rates of MeHg (Figure 4A and 4B) that were 2 to 10 times faster than the culture enriched from MS-2 (Figure 4C and 4D), regardless of the form of mercury supplied. Likewise, sulfate reduction rates for the MS-1 cultures were generally faster than rates for the MS-2 cultures.

The differences between these two mixed cultures are not known, but could be explained by a number of factors. For example, the MS-1 and MS-2 cultures originated from two different original sediment samples. Thus, the enrichments produced two distinct microbial communities that likely differed in abundance of methylating and demethylating microorganisms. While the data indicated that sulfate reducing microorganisms were the major organisms in both cultures and the principal methylators of Hg (as indicated by the molybdate controls and the positive relationships between sulfate reduction, C-substrate concentration, and MeHg concentrations), not all sulfate reducers are capable of MeHg production[28]. Thus, the relative abundance of methylators was likely greater for MS-1 cultures than for MS-2 cultures. As there are many possible explanations for such functional differences between the mixed cultures, further analysis is needed for the overall microbial community composition and biomolecular signatures specific to the Hg methylation phenotype.

Environmental implications. Our results show that net mercury methylation is related to the bioavailability of mercury and microbial growth, as demonstrated in a previous study[3]. The new finding here is the possible existence of a threshold in activity of methylating microorganisms

(i.e., sulfate reduction rate in our experiments) where net MeHg production is sensitive to either microbial growth or to Hg speciation and bioavailability. At environmental sites of interest, knowledge of this threshold in microbial activity could be useful for remediation purposes by allowing a prioritization of factors that influence net MeHg production. For example, at field sites of relatively low activity for methylating microorganisms, remedial actions that attempt to reduce Hg bioavailability may not be effective. Rather, the remediation strategy should focus on those measures that suppress the activity of the methylators (e.g., aeration, preventive measures for eutrophication). In contrast, for sites above the threshold of microbial activity, actions could aim to reduce both microbial activity of methylators and the bioavailability of Hg.

The results of this research also demonstrated that microbial growth and carbon substrate concentration played an important role in net MeHg production. Despite the similar growth yields over the 64 h incubation time, MS-1 and MS-2 mixed bacterial cultures methylated mercury at different rates and yields. Given the higher percentage of SRB found in MS-1 culture and a rapid steady increase in *dsrA* gene copy numbers parallel to the increase in net MeHg production, we may assume that this culture consisted of either a greater abundance of Hg methylators or microorganisms with higher rates of methylation . Likewise, the MS-2 culture could have comprised of more MeHg degrading microbes. Phylogenetic analysis of both cultures could assist in the understanding of this phenomenon.

In the mixed culture experiments for this study, sulfate reduction rates were used as a proxy for the activity of methylators since the experiment was designed to enrich for sulfate reducing microorganisms. This research also showed that relative gene abundance for the sulfate reducers could be an indicator of activity of the methylating community. However, in the natural environment, other anaerobic microorganisms including iron reducers and methanogens are known to methylate Hg[42-44]. Future work is needed to include a broader diversity of methylating microorganisms. This work would require a more appropriate parameter to signify the productivity of all methylating microorganisms and perhaps should quantify gene expression in addition to gene abundance. The recent discovery of a gene cluster directly linked to Hg methylation[42, 45] provides a promising path forward in this respect.

Overall this study demonstrated that differentiation of key factors affecting mercury methylation can be achieved by designing experiments that simultaneously control for the initial mercury speciation and microbial activity. Such an approach may provide a basis for the

development of methods to quantify mercury methylation potential and conceptual models that quantitatively link mercury methylation potential to specific changes in geochemical and biological conditions in the field.

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Supporting Information

SI section includes detailed methods for the preparation of HgS nanoparticles and mercury enriched isotope solutions, sulfate reducing medium composition, chemical analyses, quantification of total 16S rDNA and *dsrA* gene fragments, linear regression parameters for correlations between net MeHg concentration and growth (OD_{660}), mercury mass balance, methylmercury production in molybdate amended controls, abundance of *dsrA* genes relative to 16S rDNA and abundance of *dsrA* genes versus C-substrate concentration.

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Figure Captions

Figure 1. Growth of microorganism, as shown by optical density (OD) at 660 nm in mixed anaerobic cultures extracted from MS-1 (A,B) and MS-2 (C,D) sediments, grown under different C-substrate concentrations (0.6 mM, 6 mM and 60 mM), and amended with either dissolved Hg (A,C) or nanoparticulate HgS (B,D). The error bars represent 1 s.d. for triplicate biological samples.

Figure 2. Net production of methylmercury (filled symbols) and reduction of sulfate (open symbols) in mixed anaerobic cultures from MS-1 (A,B) and MS-2 (C,D) sediments, grown under different C-substrate concentrations, and amended with either 5 nM dissolved Hg (A,C) or 5 nM nanoparticulate HgS (B,D). The error bars represent 1 s.d. for triplicate biological samples. Linear regressions were performed for the 10 - 64 h data points; the regression parameters are listed in Table S2.

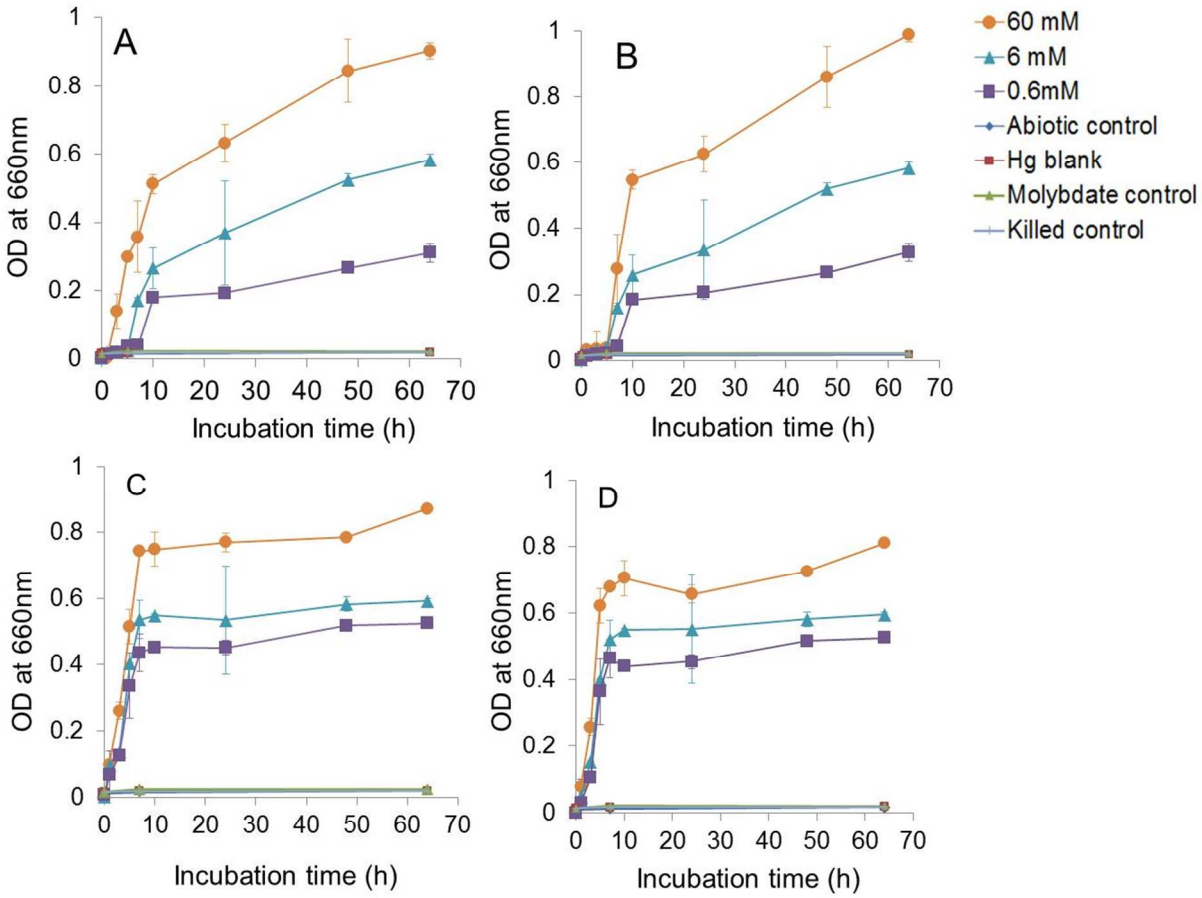
Figure 3. Relationship between methylmercury concentration and cell density of mixed microbial cultures enriched from MS-1 (A, B) and MS-2 (C, D) sediments grown under different C-substrate concentrations, and amended with either dissolved Hg (A, C) or nanoparticulate HgS (B, D). The error bars represent 1 s.d. for triplicate biological samples. Linear regression parameters are listed in Table S3.

Figure 4. Net MeHg production rate as a function of sulfate reduction rate calculated between 10 and 64 hours for mixed microbial cultures grown at different C-substrate concentrations. Mixed cultures were enriched from MS-1 (A, B) and MS-2 (C, D) sediments and were amended with either dissolved Hg (A, C) or nanoparticulate HgS (B, D). The error bars represent 1 s.d. for triplicate samples in the test groups. Some errors bars are smaller than the data points. Solid black lines correspond to linear least squares regression of the data.

Figure 5. Net methylation of mercury (as % of total Hg) as a function of *dsrA* gene relative abundance for the 10 to 64 h incubation time points for mixed microbial cultures grown at different C-substrate concentrations. Mixed cultures were inoculated from enrichments of MS-1 (A, B) and MS-2 (C, D) sediments and were amended with either dissolved Hg (A, C) or nanoparticulate HgS (B, D). Solid black lines correspond to linear least squares regression of the data.

Table 1. Characteristics of original sediments used in enrichment experiments. Concentrations represent the mean ± standard deviation of triplicate samples. Sediment concentrations are reported on a dry weight basis.

Parameters	Sediment sample ID	
	MS-1	MS-2
Sediment texture	Clay loam	Sandy loam
Clay (%) (< 2 μm)	63	9
Silt (%) (2-50 μm)	28	10
Sand (%) (> 50 μm)	9	81
pH (pore water)	7.6	8.0
Total mercury in sediment (mg kg^{-1})	12.2±5.9	16.6±2.8
Methyl mercury in sediment (ng kg^{-1})	145±25.6	381±40.8
TOC in sediment (mg kg^{-1})	31.8±0.8	4.5± 0.6
Sulfate (pore water, mg L^{-1})	2934±273	2749±418
Wet-to-dry mass ratio	1.34	1.28



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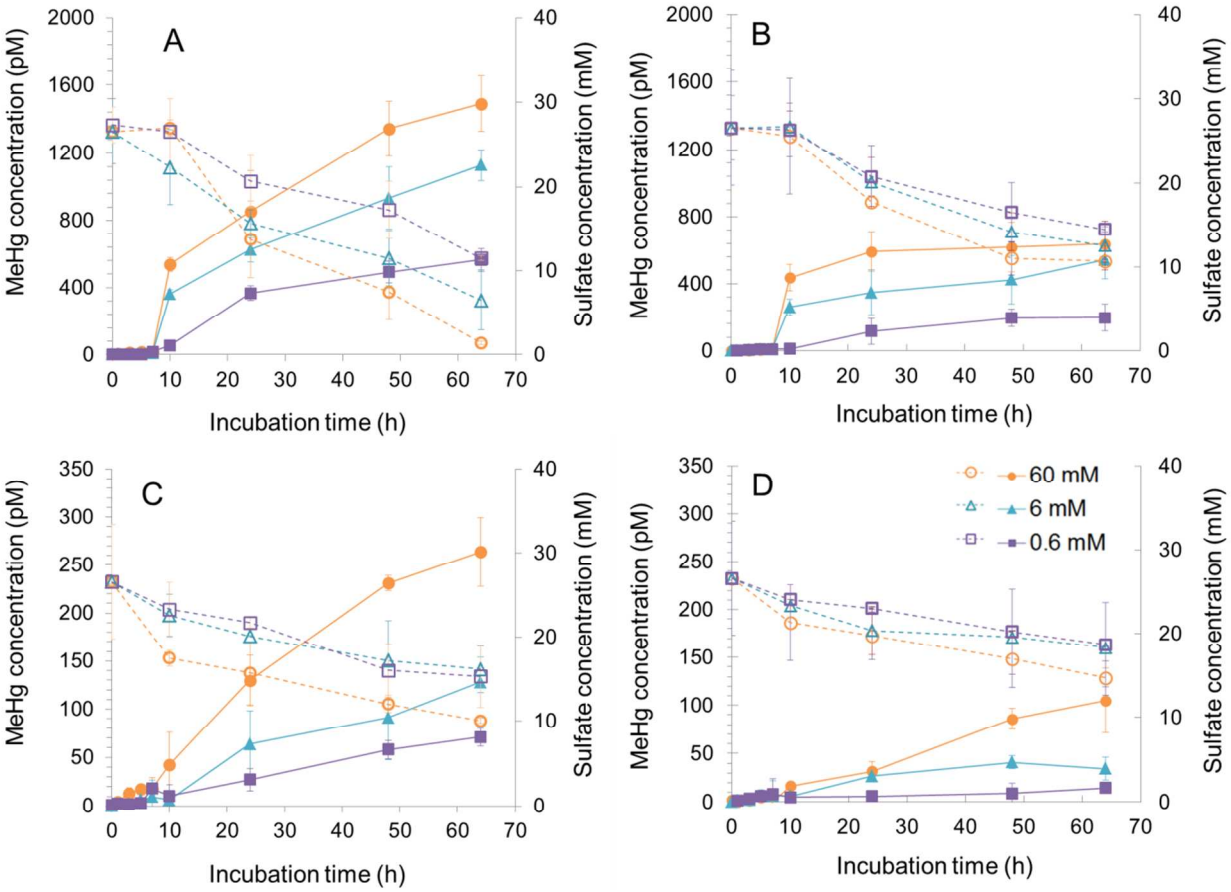

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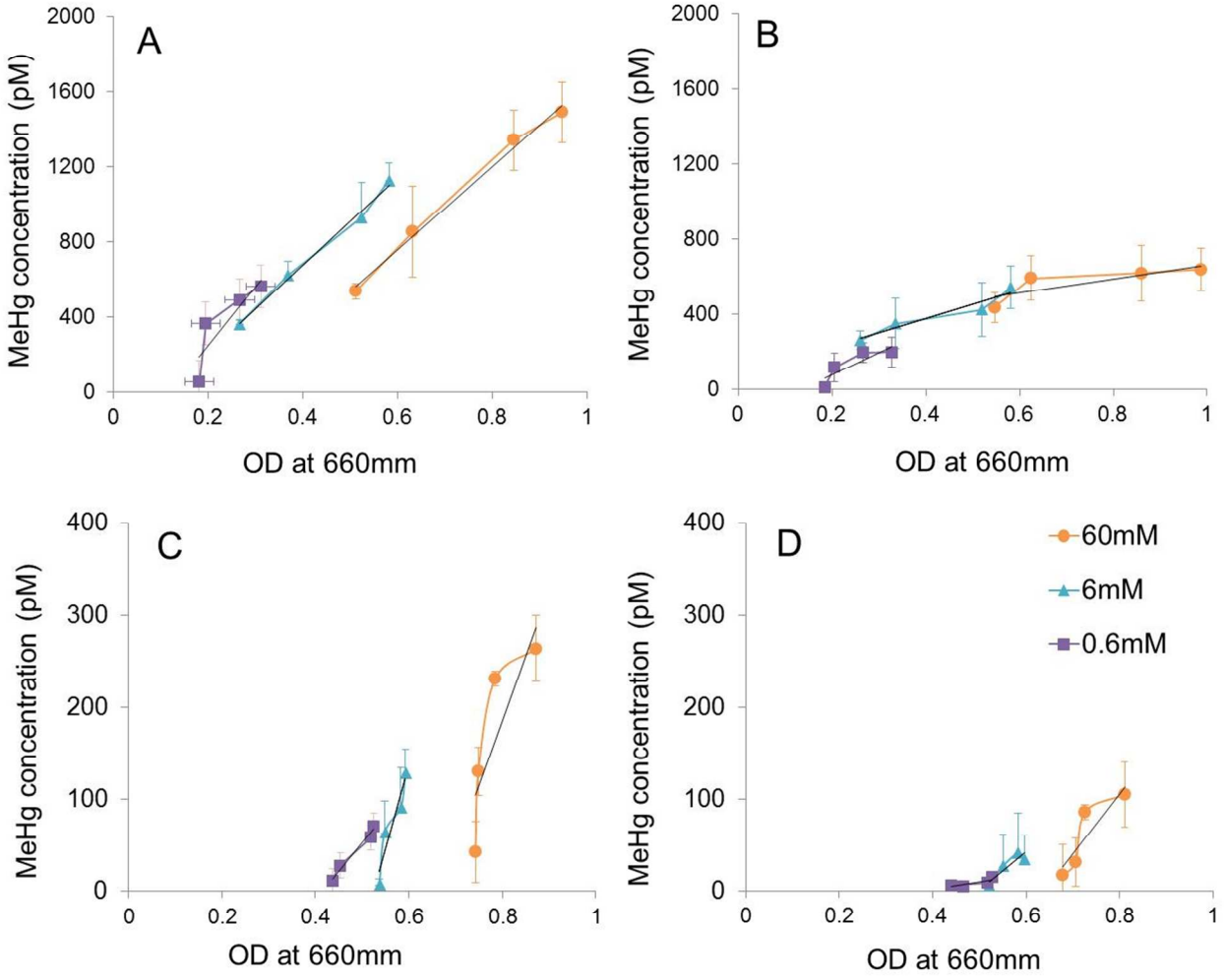

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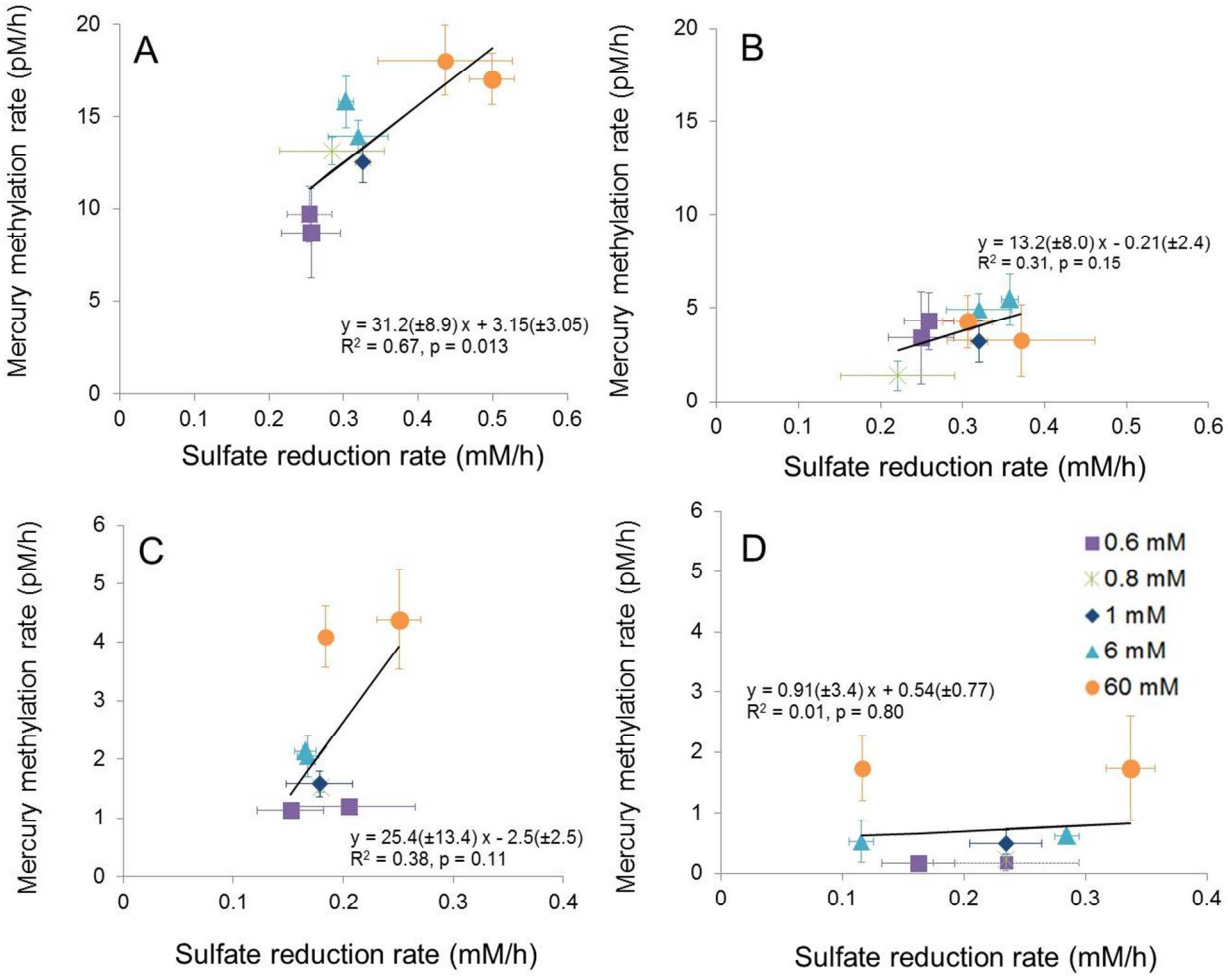

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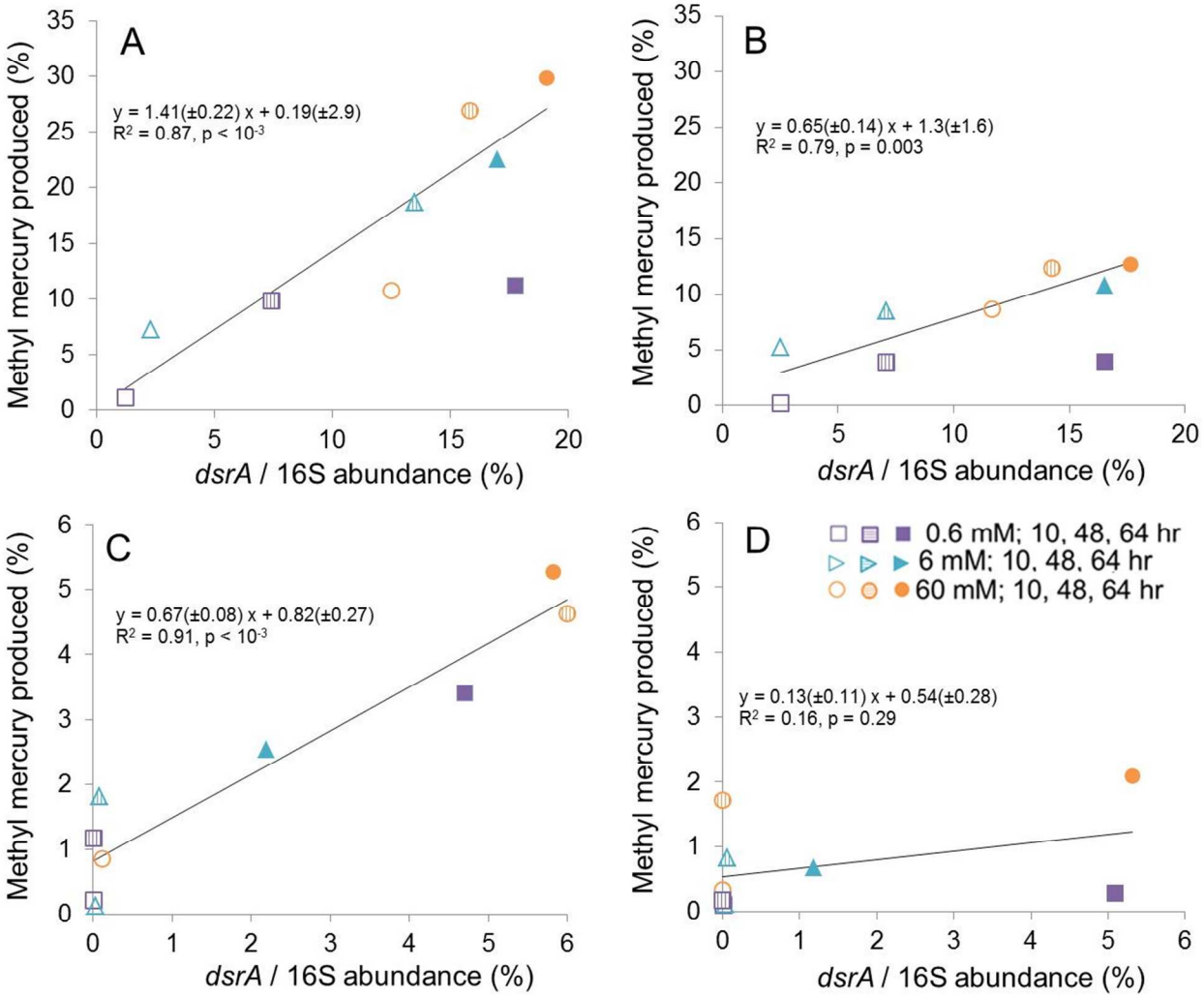

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