Electronic Supplementary Material (ESI) for Environmental Science: Processes & Impacts.

Supporting Information

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

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Materials and Methods

Preparation of HgS nanoparticles. The dissolved mercury stock solution was prepared by dissolving 36.12 mg mL^{-1} $Hg(NO_3)_2$ in a solution of 0.1 N HNO_3. A sodium sulfide stock solution was prepared by dissolving freshly washed and dried crystals of $Na_2S{\cdot}9H_2O$ in N_2-purged water. Na_2S stocks were utilized within 20 h of preparation. HgS nanoparticles were synthesized by adding $Hg(NO_3)_2$ and Na_2S to a concentration of 50 µM each in a buffer solution containing 10 mg-C L^{-1} Suwannee River humic acid (International Humic Substances Society), 0.1 M $NaNO_3$, and 4 mM sodium 4-(2-hydroxyethyl) piperazine-1-ethanesulfonate (adjusted to pH 7.5). The buffer solution was filtered with a 0.2 µm syringe nylon filter (VWR) prior to the addition of Hg and sulfide. The HgS stock solution was aged for 16 h at room temperature prior to use in the biological methylation experiments. Our previous work has shown that this preparation method results in metacinnabar-like nanoparticles with an approximate diameter of 3-5 nm for the primary particles (based on electron microscopy [1]). The nanoparticle stock solution that was synthesized aged for 16 hours for this study consisted of aggregates with an average hydrodynamic diameter of 25.8 ± 2.9 nm (based on light-intensity weighted dynamic light scattering).

Preparation of enriched mercury isotope solutions. The stock solution of ^{199}Hg was prepared by dissolving ^{199}HgO powder in 1% concentrated HNO_3.The $Me^{201}Hg$ stock was synthesized according to Sturup *et al.* [2]. The exact concentration of the synthetized $Me^{201}Hg$ was 98.7% as determined by isotope dilution ICP-MS analysis.

Sulfate reducing medium preparation. Sediment enrichments were grown in a sulfate reducing medium (without Hg) modified from a previous method [3]. In summary, this media contained: 20 mM Na_2SO_4, 4 mM sodium pyruvate and 2 mM lactic acid (60% wt/vol) as the organic carbon substrate, salts (170 mM NaCl, 1.4 mM NaH_2PO_4, 19 mM NH_4Cl, 6.7 mM KCl, 1.5 mM $MgCl_2$, and 1.5 mM $CaCl_2$), 0.2% yeast extract (wt/vol), 25 nM selenate, 25 nM tungstate, 4.4 µM $FeCl_2$, 10 mM MOPS (morpholinepropoanesulfonic acid) buffer, trace metals [3] and vitamins [3] at pH 7.2. For the mixed culture experiments, the same recipe was used except that the concentrations of the carbon substrates varied between experiments. These concentrations were 0.6 mM, 6 mM and 60mM C-substrate, with the same 2:1 molar ratio of pyruvate:lactic acid.

The culture medium was autoclaved for 30 min at 120 ^oC and cooled in an anaerobic chamber (Coy Labs) with a 90% N_2, 5% CO_2, and 5% H_2 atmosphere. After cooling to room temperature, the medium was amended with 100 µM Ti-NTA (titanium(III)nitrilotriacetate) as the reductant and resazurin (0.002% wt/vol) was used as a redox indicator. Ti-NTA and resazurin were filter-sterilized and added into medium tubes just before mixing with sediment or inoculation with the enriched culture.

DNA extraction. Total genomic DNA from the cultures was isolated and purified following a previously described method [4], in which 1.0 mL of a sample was added to a glass bead tube with 0.1 mm beads, mixed with extraction buffer and subjected to bead beating at 4000 rpm in a Mini-Beadbeater-24 (Biospec Products). The obtained supernatant was purified from other organic and inorganic materials and eluted to 50 µL volume followed by an additional purification step with Genomic DNA Clean & Concentrator (Zymo Research). Three replicates per tested sample were used for processing and analysis. Concentrated DNA was eluted to the final volume of 10 µL. The extracted DNA was examined on 1.0%(wt/vol) agarose gels at 75 V for 1 h in 0.5% TAE buffer solution (20 mM Tris, 10 mM acetate, 0.5 mM EDTA; pH 8.0) after staining with ethidium bromide. Images were obtained using the Gel Doc 2000 system (Bio-Rad). The quality of DNA was estimated by measuring A_{260}/A_{280} and A_{230}/A_{260} ratios using a ND-1000 Spectrophotometer (NanoDrop Products). DNA pellets were stored at -20 ^oC for no more than two weeks before further processing and analysis.

PCR amplification. The DNA was used to analyze the abundance of the dissimilatory sulfite reductase *dsr*A gene and 16S rDNA gene. Gene sequences were amplified by polymerase chain reaction (PCR) using universal 16S rDNA primers [5] and DSR-1F+ (5'-ACSCACTGGAAGCACGCCGG-3') and DSR-R (5'-GTGGMRCCGTGCAKRTTGG-3) primers [6]. PCR amplification was performed on StepOne Real-Time PCR System (Applied Biosystems) using SYBR Green Supermix with ROX (Bio-Rad Laboratories). Amplification of the extracted DNA[6] was performed in a 25 µL final volume with 12.5 µL Master Mix QIAGEN (QIAGEN), $MgCl_2$ (final concentration of 1.75 mM), and 1 µL (final concentration of

300 nM) of primer DSR-1F+ (5'-ACSCACTGGAAGCACGCCGG-3') and DSR-R (5'-GTGGMRCCGTGCAKRTTGG-3). The following PCR conditions were used: (1) 15 min at 95 °C; (2) 35 cycles, with 1 cycle consisting of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s; and (3) a final extension at 72 °C for 7 min. Amplification of 1114-F (5'-CGGCAACGAGCGCAACCC-3') and 1275-R (5'-CCATTGTAGCACGTGTGTAGCC-3') primer pair was performed in a 25 μL final volume with 12.5 μL Master Mix QIAGEN (Qiagen), $MgCl_2$ (final concentration of 1.5 mM), and 1 μL (final concentration of 400 nM) of primers. The PCR procedure consisted of 15 min of initial denaturation at 95 °C, followed by 35 cycles denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. All DNA samples were amplified in triplicates. The dissociation curve was used to detect the presence of primer dimers or non-specific amplification products in the PCR reactions.

Genomic DNA of *E.coli* strain K12 and *Desulfobulbus propionicus* 1pr3 (ATCC 33891), isolated using PureLink Genomic DNA extraction kit (Invitrogen), were used as positive controls for the presence of total bacteria and sulfate reducing microorganisms, respectively. Samples with Milli-Q water were utilized as negative controls. Standard curves were generated with PCR products, purified with QIAquick PCR Purification Kit (Qiagen), of the individual genes targeted against the fluorescence corresponding to initial DNA concentration. The relative abundance of *dsrA* was calculated from the copy number of total 16S rDNA gene within the same sample. It is important to note that this calculation assumes that the copy number of the 16S rDNA and *ds*rA genes per genome is the same [7]. It has been reported that different bacterial groups may have 1 to 15 copies of the 16S rDNA gene per genome [7]; thus the calculations performed indicates only the relative abundance and not the absolute quantity of sulfate reducing bacteria in the sample [8].

Table S1. Linear regression parameters for plots of net MeHg concentration (pM) versus time (h) (shown in Figure 2). The regressions were performed for data points in the 10 to 64 hour time frame for mixed microbial cultures inoculated from enrichments of MS-1 and MS-2 sediments, grown under different C-substrate concentrations, and amended with either dissolved Hg or nanoparticulate HgS.

	0.6 mM C-substrate		6 mM C-substrate		60 mM C-substrate	
	dissolved Hg	nano HgS	dissolved Hg	nano HgS	dissolved Hg	nano HgS
MS-1 mixed culture						
Slope (pM MeHg h^{-1})	8.67±2.43	3.39±0.93	13.9±0.88	4.88±0.57	18.1±1.9	3.25±1.39
Intercept	52.3±102	5.67±39.3	253±37	215±24	398±79	451±58
R^2	0.86	0.87	0.99	0.97	0.98	0.73
p value	0.071	0.068	0.004	0.013	0.011	0.14
MS-2 mixed culture						
Slope (pM MeHg h^{-1})	1.14±0.07	0.168±0.036	2.06±0.35	0.525±0.240	4.10±0.53	1.74±0.17
Intercept	0.12±2.8	2.57±1.51	-2.79±14.9	7.91±10.1	17.3±22.4	-3.66±7.00
R^2	0.99	0.92	0.94	0.70	0.97	0.98
p value	0.003	0.042	0.029	0.16	0.016	0.009

Table S2. Linear regression parameters for plots of sulfate concentration (mM) versus time (h) (shown in Figure 2). The regressions were performed for data points in the 10 to 64 hour time frame for mixed microbial cultures inoculated from enrichments of MS-1 and MS-2 sediments, grown under different C-substrate concentrations, and amended with either dissolved Hg or nanoparticulate HgS.

	0.6 mM C-substrate		6 mM C-substrate		60 mM C-substrate	
	dissolved Hg	nano HgS	dissolved Hg	nano HgS	dissolved Hg	nano HgS
MS-1 mixed culture						
Slope (mM SO_4^{2-} h^{-1})	-0.256±0.036	-0.211±0.035	-0.273±0.036	-0.258±0.046	-0.436±0.088	-0.274±0.068
Intercept	28.3±1.5	27.2±1.48	23.9±1.5	27.8±1.9	28.3±3.7	26.5±2.86
R^2	0.97	0.95	0.97	0.94	0.92	0.89
p value	0.019	0.027	0.017	0.030	0.038	0.056
MS-2 mixed culture						
Slope (mM SO_4^{2-} h^{-1})	-0.162±0.026	-0.102±0.005	-0.119±0.014	-0.0827±0.0222	-0.140±0.004	-0.121±0.004
Intercept	25.0±1.08	25.2±0.2	23.3±0.6	23.4±0.9	18.9±0.2	22.6±0.2
R^2	0.95	0.996	0.97	0.87	0.998	0.997
p value	0.024	0.002	0.014	0.065	0.0009	0.001

Table S3. Linear regression parameters for correlations shown in Figure 3, which are plots of MeHg concentration and growth (OD_{660}) of mixed microbial cultures inoculated from enrichments of MS-1 and MS-2 sediments, grown under different C-substrate concentrations, and amended with either dissolved Hg or nanoparticulate HgS.

	0.6 mM C-substrate		6 mM C-substrate		60 mM C-substrate	
	dissolved Hg	nano HgS	dissolved Hg	nano HgS	dissolved Hg	nano HgS
MS-1 mixed culture						
Slope (pM MeHg OD_{660}^{-1})	3170±1310	1180±490	2330±140	759±152	2120±137	364±184
Intercept	-386±318	-160±123	-253±63	71.7±67.4	-568±102	295±143
R^2	0.75	0.75	0.99	0.93	0.70	0.66
p value	0.14	0.14	0.004	0.038	0.004	0.19
MS-2 mixed culture						
Slope (pM MeHg OD_{660}^{-1})	608±70	85.0±38.7	1790±450	411±131	1410±660	358±241
Intercept	-252±34	-33.0±18.9	-939±256	-204±74	-939±517	-420±176
R^2	0.97	0.71	0.89	0.83	0.70	0.79
p value	0.013	0.16	0.058	0.088	0.17	0.11

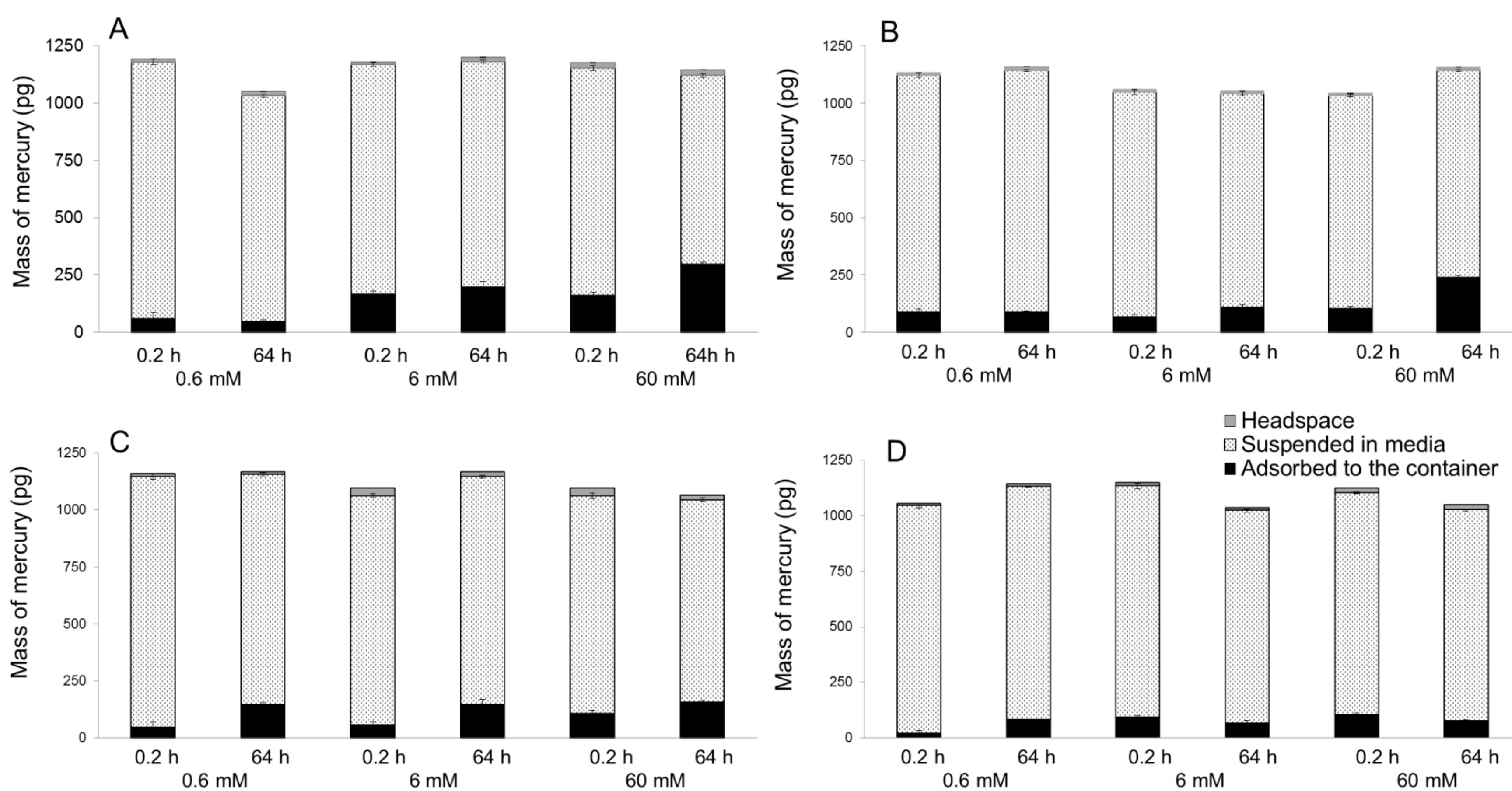

Figure S1. Measurements of mercury in the culture medium, headspace and the container in each mixed culture. The mixed cultures were inoculated from MS-1 (A,B) and MS-2 (C,D) sediments, grown under different C-substrate concentrations while amended with 5 nM of either dissolved Hg (A,C) or nanoparticulate HgS (B,D) (corresponding to a total of 1000 pg of Hg in each culture tube). Mass balance measurements were performed for the <0.2 h and 64h incubation time points. Total recoveries of the added Hg were between 90% and 118% for all tested samples.

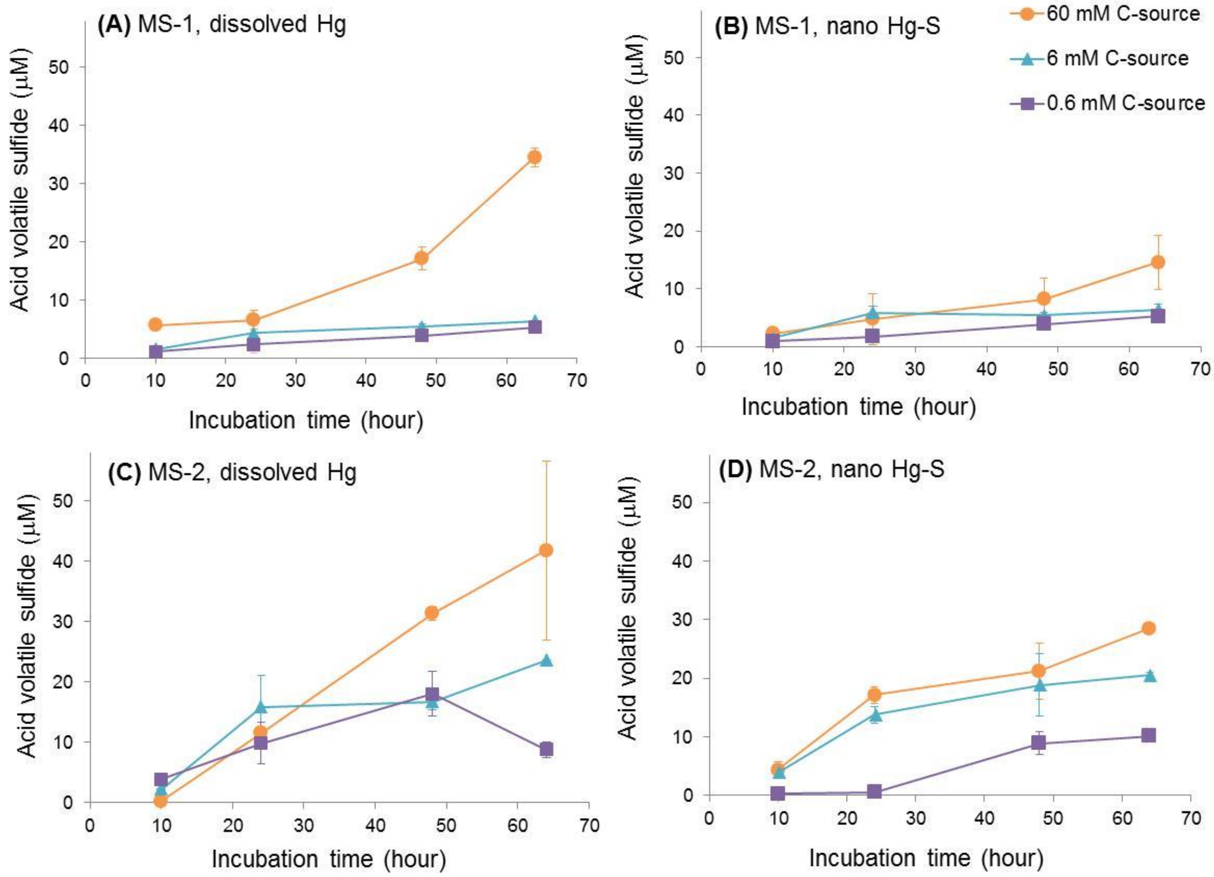

Figure S2. Production of acid volatile sulfide (AVS) in MS-1 and MS-2 mixed cultures amended with either dissolved Hg or nanoparticulate HgS.

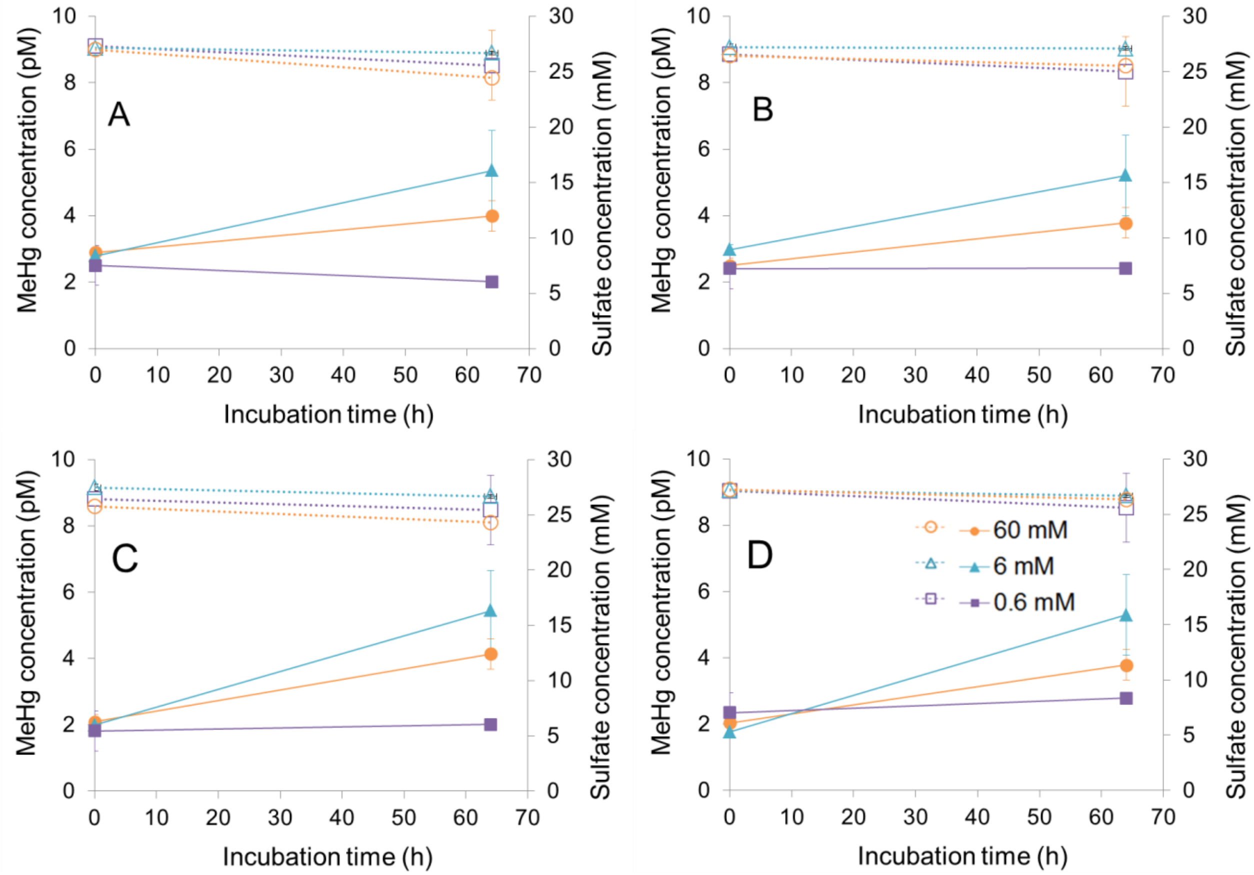

Figure S3. Net production of methylmercury (filled symbols) and reduction of sulfate (open symbols) in mixed anaerobic cultures enriched from MS-1 (A,B) and MS-2 (C,D) sediments, amended with 20 mM sodium molybdate, grown with different C-substrate concentrations, and amended with either dissolved Hg (A, C) or nanoparticulate HgS (B, D). The error bars represent 1 s.d. for triplicate biological samples.

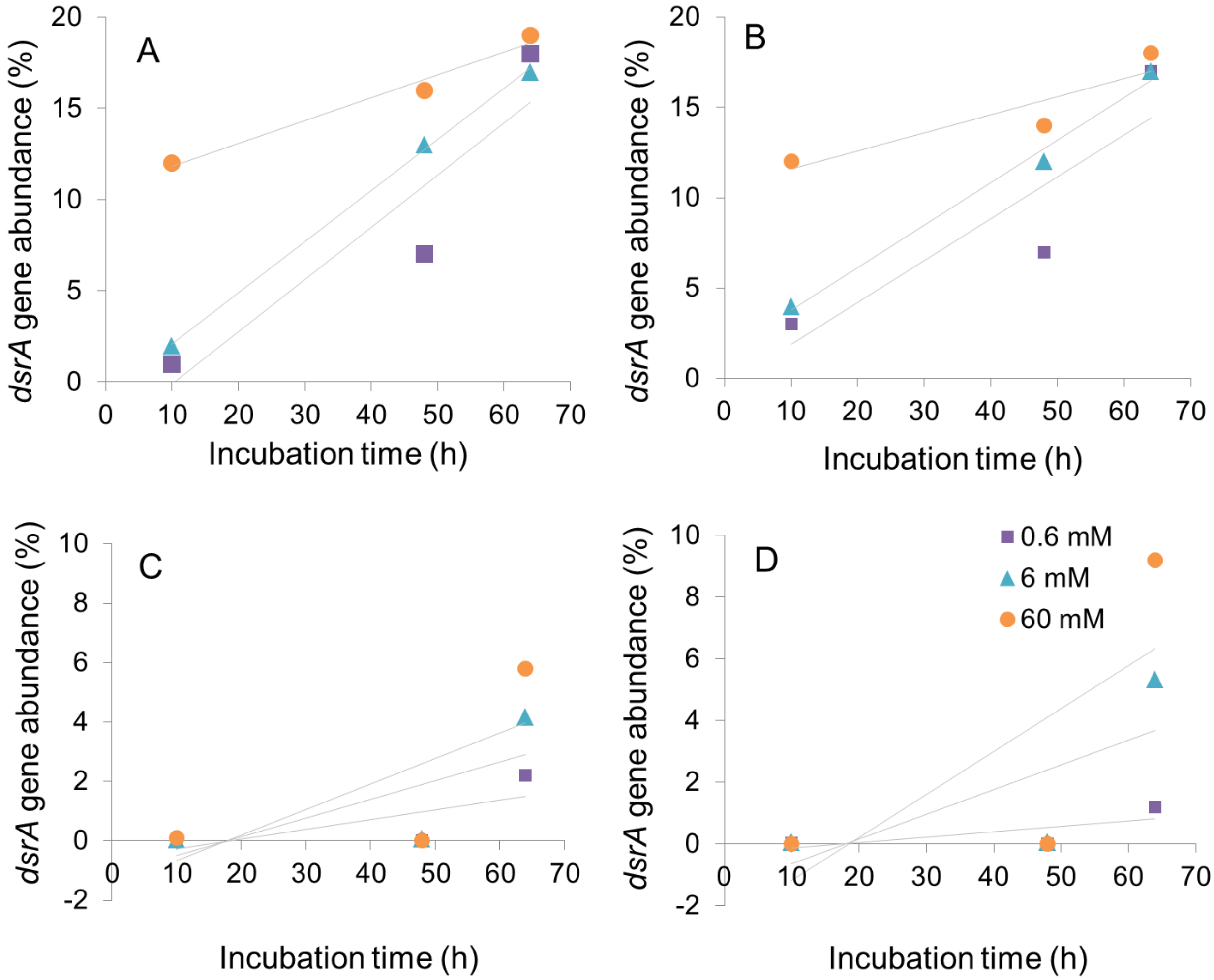

Figure S4. Abundance of *dsrA* genes, relative to 16S rDNA gene in mixed anaerobic cultures enriched from MS-1 (A, B) and MS-2 (C, D) sediments, grown under different C-substrate concentrations, and amended with either dissolved Hg (A, C) or nanoparticulate HgS (B, D).

Supporting Information References

1. Zhang T,Kim B,Levard Cm,Reinsch BC,Lowry GV,Deshusses MA,Hsu-Kim H. 2012. Methylation of mercury by bacteria exposed to dissolved, nanoparticulate, and microparticulate mercuric sulfides. *Environ. Sci. Technol.* 46: 6950-6958.

2. Stürup S,Chen C,Jukosky J,Folt C. 2005. Isotope dilution quantification of 200Hg2+ and CH3201Hg+ enriched species-specific tracers in aquatic systems by cold vapor ICPMS and algebraic de-convoluting. *Int. J. Mass Spectrom.* 242: 225-231.

3. Gilmour CC,Elias DA,Kucken AM,Brown SD,Palumbo AV,Schadt CW,Wall JD. 2011. Sulfate-reducing bacterium *Desulfovibrio desulfuricans* ND132 as a model for understanding bacterial mercury methylation. *Appl. Environ. Microbiol.* 77: 3938-3951.

4. Kucharzyk KH,Hsu-Kim H,Deshusses M. 2014. Modifications of DNA extraction methods using sediments of various origins. . *personal communication.*

5. Denman SE,McSweeney CS. 2006. Development of a real-time PCR assay for monitoring anaerobic fungal and cellulolytic bacterial populations within the rumen. *FEMS Microbiol. Ecol.* 58: 572-582.

6. Kondo R,Nedwell DB,Purdy KJ,Silva SQ. 2004. Detection and enumeration of sulphate-reducing bacteria in estuarine sediments by competitive PCR. *Geomicrobiol. J.* 21: 145-157.

7. Achá D,Hintelmann H,Yee J. 2011. Importance of sulfate reducing bacteria in mercury methylation and demethylation in periphyton from Bolivian Amazon region. *Chemosphere.* 82: 911-916.

8. Lee ZM-P,Bussema C,Schmidt TM. 2009. rrnDB: documenting the number of rRNA and tRNA genes in bacteria and archaea. *Nucleic Acids Res.* 37: D489-D493.