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Identical Hg Isotope Mass Dependent Fractionation Signature during Methylation by Sulfate-Reducing Bacteria in Sulfate and Sulfate-Free Environment

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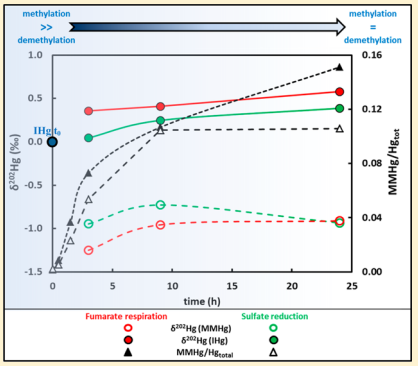
Supporting Information

ABSTRACT: Inorganic mercury (iHg) methylation in aquatic environments is the first step leading to monomethylmercury (MMHg) bioaccumulation in food webs and might play a role in the Hg isotopic composition measured in sediments and organisms. Methylation by sulfate reducing bacteria (SRB) under sulfate-reducing conditions is probably one of the most important sources of MMHg in natural aquatic environments, but its influence on natural Hg isotopic composition remains to be ascertained. In this context, the methylating SRB *Desulfovibrio dechloracetivorans* (strain BerOc1) was incubated under sulfate reducing and fumarate respiration conditions (SR and FR, respectively) to determine Hg species specific (MMHg and IHg) isotopic composition associated with methylation and demethylation kinetics. Our results clearly establish Hg isotope mass-dependent fractionation (MDF) during biotic methylation (−1.20 to +0.58‰ for δ^{202}Hg), but insignificant mass-independent fractionation (MIF) (−0.12 to +0.15‰ for Δ^{201}Hg). During the 24h of the time-course experiments Hg isotopic composition in the produced MMHg becomes significantly lighter than the residual IHg after 1.5h and shows similar δ^{202}Hg values under both FR and SR conditions at the end of the experiments. This suggests a unique pathway responsible for the MDF of Hg isotopes during methylation by this strain regardless the metabolism of the cells. After 9 h of experiment, significant simultaneous demethylation is occurring in the culture and demethylates preferentially the lighter Hg isotopes of MMHg. Therefore, depending on their methylation/demethylation capacities, SRB communities in natural sulfate reducing conditions likely have a significant and specific influence on the Hg isotope composition of MMHg (MDF) in sediments and aquatic organisms.



1. INTRODUCTION

Mercury (Hg) is a global pollutant that has a complex biogeochemical cycle within earth compartments.[1] Methylation of inorganic mercury (IHg) in aquatic ecosystems is a critical step, since it leads to the formation of methylmercury (MMHg) easily accumulated and biomagnified in food webs.[2] Microbial activities play a crucial role in the transformations of Hg species in the environment such as methylation/demethylation and reduction/oxidation reactions.[3] Among sulfate-reducing bacteria (SRB), which have been identified as the principal IHg methylators in anoxic sediments,[4] representatives of the genus *Desulfovibrio* have been extensively studied for their methylation/demethylation capacity.[5−8] Recently *Desulfovibrio desulfuricans* ND132 was selected as a model organism,[9,10] leading to the identification of two specific genes *hgcA* and *hgcB* determined to be mandatory for Hg methylation in all the bacterium.[11] In-situ and pure culture experiments show that MMHg formation rate depends on several parameters such as environmental/physiological conditions,[12,13] bacterial strain,[6,14,15] growth phase[16,17] and Hg bioavailability.[8] Investigations of bacterial methylation are hampered by difficulties in constraining/monitoring the physiological changes that affect the biochemical pathways of Hg methylation.[6,15,18] Thus, relating bacterial communities and activities to the MMHg budget in aquatic ecosystems is still an important challenge.

The natural Hg stable isotope variations are a powerful tool to trace Hg sources[19,20] and its species transformations.[21−23] Previous studies reported that aquatic organisms were enriched in heavier Hg isotopes relative to the sediment which is assumed to be the source of the Hg they accumulate.[24,25] This probably results from the combined effects of of (1) IHg adsorption−desorption to particles[26,27] and dark/biotic reduction,[23,28] followed by (2) the methylation of bioavailable IHg,[29]

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the MMHg photochemical[21] and bacterial/dark demethylation[30,31] once it is released in the water column, and (3) its trophic transfer.[32,33] A recent review[34] highlighted the multiple steps potentially affecting microbial isotope fractionation of Hg at the cellular-level as well as the challenge associated with differentiating between Hg isotope fractionation from microbial and dark abiotic processes in the absence of MIF. Whereas photochemical processes can induce significant mass-independent fractionation (MIF) of Hg isotopes during Hg species conversion,[21,35,36] Hg transformations mediated by microorganisms such as methylation,[29] demethylation,[31] and reduction[28,37] were identified to produce exclusively Hg mass dependent fractionation (MDF). A significant MDF and no MIF was also observed during the experimental abiotic methylation of IHg via a natural biogenic methyl donor.[38] Hence, the interpretation of Hg isotopic signatures in environmental samples requires a careful approach. Additional laboratory studies by using Hg isotopically enriched tracers are crucial to evaluate the potential Hg isotope fractionation during specific Hg transformation pathways.

In this context, the present work focuses on kinetic and isotopic studies of IHg methylation by pure cultures of SRB recognized for their Hg methylating/demethylating potential under anaerobic conditions.[15] Either sulfidogenic or non-sulfidogenic growth conditions, allow Hg methylation by several SRB.[6,13] Both conditions are relevant in natural ecosystems depending on sulfate availability and were compared to assess potential differences in the kinetics of Hg transformation and/or stable isotope fractionation. Among the parameters potentially affecting the rates of transformation and the Hg isotope fractionation during its methylation by SRB, we focused essentially on (i) the unaddressed question of the isotope fractionation of Hg methylated by SRB under sulfate respiration, (ii) the potential influence of the energetically different fumarate (FR) and sulfate respiration (SR) processes on the Hg isotopic composition in MMHg produced by SRB in environments rich or depleted in sulfate, and (iii) the role of the simultaneous biotic demethylation of MMHg.

2. EXPERIMENTAL PROCEDURES

Bacterial strains and culture conditions have been described elsewhere[15] and are detailed in the Supporting Information (SI). Twenty four hour incubations with enriched tracers (^{199}IHg and ^{201}MMHg) were performed to assess gross IHg methylation and MMHg demethylation, and 24h incubations with IHg NIST3133 were performed separately to determinate the isotopic composition of Hg species during bacterial methylation/demethylation.

2.1. Determination of Methylation/Demethylation Kinetics During Hg Incubation with Microorganisms. For incubations with enriched tracers, 8 mL aliquots of the bacterial cultures were transferred into a 10 mL glass vial in which 100 ng g^{-1}of ^{199}IHg and 1.5 ng g^{-1}of ^{201}MMHg were added. Then, vials were immediately sealed with PTFE-coated butyl rubber stoppers, flushed with N_2 gas to maintain anoxic conditions and incubated at 30 °C in the dark. Hg incubations were performed under high cell density and its stability was monitored by regularly measurements of the optical density ($OD_{600\ nm}$). Single assay cultures vials were stopped at selected times (0, 0.5, 2, 8, and 24h) by addition of 4 mL 6 M HNO_3 after removing 4 mL of the culture and stored at 4 °C before microwave extraction. Additional experiments with different biogenic and/or complexing agents added to the incubation medium (L-cysteine, methylcobalamin, chloride) are described in SI Table S.1. Gross IHg methylation (k_m) and gross MMHg demethylation (k_d) rate constants were calculated assuming a pseudo first-order reversible reaction and applying a nonlinear fitting kinetic model[39] (equations S.1. and S.2. in the SI). Cultures with *Desulfovibrio alaskensis* strain G200, a non-methylating SRB, were also exposed to the same amount of IHg and MMHg and were considered as a biotic control. Incubations for each experimental condition were conducted in triplicates.

Incubations with NIST3133 were performed under similar conditions than the ones for enriched tracers described above. However, isotopically enriched tracers were in this case replaced by the addition of 100 ng g^{-1} of natural IHg standard NIST3133. Triplicates of cultures were stopped and analyzed at times 0, 1.5, 3, 9, and 24h.

2.2. Hg Speciation Analysis and Quantification. Bacterial cultures were directly digested in the incubation vial with an analytical microwave and analyzed by GC-ICP-MS as detailed elsewhere.[14,15] Hg species were propylated by using $NaBPr_4$ and extracted in isooctane by manual shaking during 5 min. Extracts were stored at −20 °C before analysis by GC-ICP-MS (Trace GC, Thermo Fisher coupled to ICPMS (Xseries 2, Thermo Fisher). Quantification of Hg species was carried out by reverse isotope dilution analysis (when the incubation was performed with isotopically enriched tracers) by adding the adequate amount of natural IHg and MMHg standards. In this case, the experimental data was mathematically processed by applying isotope pattern deconvolution approaches previously developed in our laboratory and detailed in previous works.[15,40−42] This technique allows the quantification of both Hg species concentrations and transformation factors (i.e., methylation and demethylation) affecting the two isotopic tracers during the analytical procedure. Hg species quantification on the cultures incubated with natural standards was performed by classic isotopic dilution technique.[41]

In a series of very recent studies, the unexpected capacities of SRBs to simultaneously oxidize dissolved elemental mercury (Hg^0) to IHg with subsequent methylation and to reduce IHg to Hg^0 was evidenced.[43−45] However, as satisfactory mass balance based on MMHg and IHg species were obtained in this study for incubations of BerOc1 with either natural or enriched isotopes, we can infer the presence of two unique pools of mercury (MMHg and IHg) for this strain under our experimental conditions. Hg species recoveries for the different experiments are discussed in details in the SI and all data is presented in Tables S2 and S3.

2.3. Hg Complexation in Solution. The chemical composition of each defined medium for the different experiments is described in details in the SI. The speciation and anionic coordination of the Hg species in solution (with OH, Cl, S, cysteine, methylcobalamin) was modeled using the visual MINTEQ ver.3.0 software as previously used.[38] The major IHg and MMHg complexes obtained under the different conditions of incubation are reported in SI Table S4. It should be noticed that the binding of Hg species to complex biomolecules is not included in the model due to the large number of potentials Hg-bioligands.[46,47] Only the comparison of experiments conducted with Cys and MeCo has been here used as a proxy for the role of organic binding ligands on Hg methylation/demethylation kinetics.

2.4. Measurement of Hg Species-Specific Isotopic Ratios. The determination of Hg species (MMHg and IHg)

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Table 1. Fractions and Isotopic Ratios of Hg Species during the Time-Course Experiment after Incubation of 100 ng g^{-1} IHg NIST 3133 (or 100 ng.g^{-1} MMHg STREM) with Strains BerOc-1 and G200 under Various Growing Conditions (Fumarate Respiration and Sulfate Reduction (BerOc-1) and Pyruvate Fermentation (G200)

					$\delta^{202}Hg_{IHg}$ (‰)		$\Delta^{201}Hg_{IHg}$ (‰)		$\Delta^{200}Hg_{IHg}$ (‰)		$\delta^{202}Hg_{MMHg}$ (‰)		$\Delta^{201}Hg_{MMHg}$ (‰)		$\Delta^{200}Hg^{MMHg}$ (‰)	
incubation conditions IHg/MMHg	strain	time (h)	f IHg	f MMHg	mean	2 SD	mean	2 SD	mean	2 SD	mean	2 SD	mean	2 SD	mean	2 SD
Sulfate-Free Metabolism																
IHg	BerOc-1	0	1.00	0.00	0.00	0.20	0.06	0.06	−0.02	0.05	n/a		n/a		n/a	
IHg	BerOc-1	0.5	0.99	0.01	−0.16	0.30	0.00	0.09	0.01	0.07	n/a		n/a		n/a	
IHg	BerOc-1	1.5	0.96	0.04	−0.36	0.21	0.06	0.07	0.02	0.05	−1.20	0.40	0.11	0.20	−0.07	0.07
IHg	BerOc-1	3	0.92	0.07	0.36	0.32	0.00	0.03	0.01	0.02	−1.25	0.15	−0.10	0.06	−0.06	0.04
IHg	BerOc-1	9	0.88	0.11	0.41	0.20	−0.01	0.10	0.05	0.14	−0.96	0.05	−0.12	0.18	−0.12	0.07
IHg	BerOc-1	24	0.83	0.15	0.58	0.23	0.01	0.13	0.09	0.14	−0.91	0.29	−0.08	0.26	−0.03	0.11
IHg	G200	0	1.00	0.00	0.00	0.16	−0.04	0.10	−0.01	0.15	n/a		n/a		n/a	
IHg	G200	24	1.00	0.00	0.16	0.28	0.05	0.13	0.04	0.15	n/a		n/a		n/a	
MMHg	G200	0	1.00	0.00	n/a		n/a		n/a		0.00		−0.01		−0.15	
MMHg	G200	24	0.04	0.96	n/a		n/a		n/a		0.16	0.33	−0.15	0.22	−0.05	0.13
Sulfate-Reduction																
IHg	BerOc-1	0	1.00	0.00	0.00	0.31	−0.10	0.17	−0.03	0.06	n/;a		n/a		n/a	
IHg	BerOc-1	0.5	0.99	0.01	−0.04	0.20	0.02	0.03	0.10	0.08	n/a		n/a		n/a	
IHg	BerOc-1	1.5	0.98	0.02	0.26	0.17	0.15	0.00	0.04	0.01	−1.04	0.15	0.15	0.08	−0.08	0.05
IHg	BerOc-1	3	0.94	0.05	0.05	0.24	0.15	0.13	−0.01	0.14	−0.95	0.16	0.00	0.16	0.00	0.13
IHg	BerOc-1	9	0.90	0.10	0.25	0.24	0.04	0.07	0.00	0.03	−0.73	0.11	−0.03	0.07	−0.10	0.12
IHg	BerOc-1	24	0.88	0.11	0.39	0.17	0.05	0.12	0.01	0.02	−0.93	0.10	0.12	0.08	−0.11	0.00
IHg	G200	0	1.00	0.00	0.00	0.27	0.02	0.06	0.00	0.12	n/a		n/a		n/a	
IHg	G200	24	1.00	0.00	0.00	0.25	0.03	0.18	−0.03	0.09	n/a		n/a		n/a	
MMHg	G200	0	1.00	0.00	n/a		n/a		n/a		0.00	0.48	−0.10	0.14	−0.04	0.11
MMHg	G200	24	0.12	0.88	0.26	0.47	0.86	0.21	−0.17	0.17	−0.28	0.24	−0.23	0.10	−0.09	0.19

isotopic ratios has been carried out by coupling a gas chromatograph (Focus GC, Thermo Fisher) with multi-collector ICPMS (NuPlasma, Nu Instrument).[48] This analytical approach allows the simultaneous determination of isotope ratios of Hg species in the same sample[29,48−50] and has been applied in a preliminary work on Hg isotopic fractionation during microbial methylation under fermentative condition.[29] Sample uptake and derivatization prior to injection to GC-MC-ICPMS followed the same protocol used for Hg species quantification by GC-ICP-MS, although the volume of sample derivatized was higher (5 mL) in order to have a transient signal adequate for precise and accurate isotopic ratios measurements (top of the peak intensity from 0.5 to 5 V). The linear regression method was adopted for better accuracy and precision.[48] Each sample was bracketed with NIST 3133 to express results as per mil (‰) delta notation (δ) using the following equations:

$$\delta^{xxx}Hg_{sample} = [(^{xxx/198}Hg_{sample}/^{xxx/198}Hg_{NIST3133}) - 1] \times 1000 \quad (1)$$

Deviation from the mass dependent fractionation line for the isotopes ^{199}Hg,^{200}Hg and ^{201}Hg is expressed using capital delta notation (Δ), according to the equations[51] provided in the SI. Uncertainties on the Hg delta values reported in this manuscript are expressed as the 2SD of replicate measurements on replicates of incubations (n = 2−6 isotopic ratio measurements for 1 data point in figures and Table 1, see SI Table S5). External standards (IHg UM-Almadén, MMHg STREM) with previously reported isotopic composition were used for method validation. Details of GC-MC-ICPMS parameters and analytical setup can be found elsewhere.[29,50] Long-term reproducibility of UM-Almadén (2 weeks of measurements, n = 44, 2 SD) is given as δ^{202}Hg value which was −0.52 ± 0.18‰ (see details for other IRs and short-term precision in the SI) which is in agreement with previously published values for this standard.[52] Periodic isotope cross contamination with isotope 199 (30% of the total number of samples) was observed from unintentional contact with samples containing large amount of ^{199}IHg enriched tracer in our laboratory (sample preparation and reagents were separated in dedicated rooms).[29,38] However, this did not lead to significant contamination for the other isotopes (see Discussion and SI Tables S5, S6, and S7 in the SI). To avoid a lack of data points for the interpretation of the MIF, the δ^{199}Hg data were thus discarded, and δ^{201}Hg data were used instead to follow potential MIF on odd Hg isotopes. All single measurements for Hg species isotope ratios are reported in SI Table S5. Hg isotopes recoveries for Hg species in the different experiments are discussed in the SI and presented in Table S7.

3. RESULTS AND DISCUSSION

3.1. Methylation and Demethylation Yields and Kinetics Under SR and FR. Methylation yields show significant differences depending on the tested conditions (Figure 1 and Table 2). Under FR, the incubation of ^{199}IHg at high cell density (10^7 to 10^8 cells mL^{-1}, $OD_{600\ nm}$ = 0.20) led to 15.9 ± 0.6% of net methylation, whereas only 2.7 ± 0.1% of the ^{199}IHg added was methylated after 24 h when cell density was 10-times lower ($OD_{600\ nm}$ = 0.02) and no significant methylation was observed when cell density was 100-times lower ($OD_{600\ nm}$ <0.01). Thus, a lower cell density for strain

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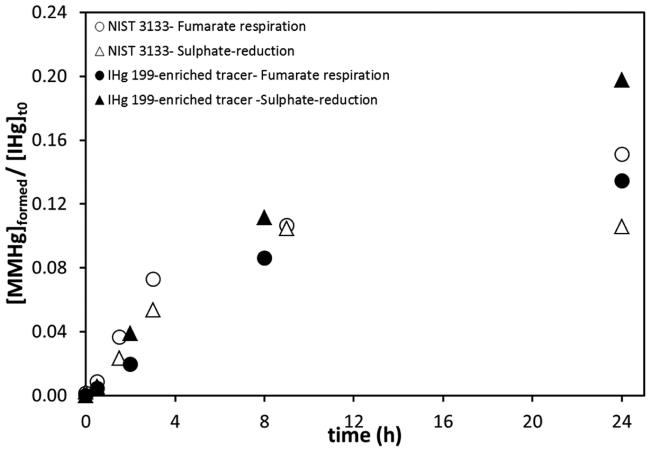

Figure 1. Fraction of MMHg formed as a function of time for incubations of 100 ng.g^{-1} enriched tracer ^{199}IHg (filled symbols) or 100 ng.g^{-1} natural NIST 3133 IHg (open symbols) with *Desulfovibrio dechoroacetivorans* strain BerOc-1 at maximum cells density during both fumarate and sulfate respiration conditions. Error bars on the replicates measurements (not shown) were lower than 5% of the average measurements.

BerOc-1 led to a decrease of MMHg production, as previously reported for other bacterial strains.[8,14,53]

Similar to FR, under SR and after 24 h of incubation at high cell density, the maximum of net methylation yield was 19.8 ± 0.7% (n = 3). Both, SR and FR incubations of strain BerOc-1 with 100 ng g^{-1} IHg exhibited higher rate constants for demethylation than for methylation ($k_d > k_m$) with k_d values 3–10 times higher than k_m ones, as recently reported for fumarate respiration conditions.[46] Gross methylation calculated from 100 ng g^{-1} initial IHg was within the same order of magnitude whatever the physiologies (i.e., SR and FR) or the presence of methylcobalamin or L-Cysteine. However, the addition of high level of chloride dramatically decreased the yield of methylation (0.6%, see SI) under both SR and FR conditions. Among experiments at maximum cell density and without high chloride concentration, we report values of Hg methylation and demethylation rate constants which are within the same order of magnitude than previously reported ones with pure strain cultures of the genus *Desulfobulbus* and *Desulfovibrio* (range 4–80 × 10^{-3}h^{-1}).[15,53]

Significant differences were observed for the transformation rate constants under FR depending on the Hg species added (i.e., ^{199}IHg or ^{201}MMHg), exhibiting k_m/k_d ratio lower and higher than 1, respectively for ^{199}IHg and ^{201}MMHg (Table 2). Recent studies suggest that the produced MMHg is released in the extracellular medium,[8,9,46] questioning if the demethylation pathway occurs: in the cell, in the outer membrane, or in the extracellular medium. Despite large differences between k_m^{199} and k_m^{201}, k_d^{199} and k_d^{201} exhibited only a 2-fold difference, as already observed,[15] and this may be attributed to a demethylation process not limited by the uptake of initial MMHg (in contrast with IHg).[54]

We observed lower MMHg production per cell under SR than under FR after 24h. It can be attributed to the lower bioavailability of IHg for the cells under SR conditions because of the potential formation of insoluble Hg sulfide complexes.[9,13] According to the complexation model of IHg in solution estimated under SR (SI Table S4), the predominance of the negatively charged $HgHS^{2-}$ inorganic complex should limit Hg uptake while a significant fraction of neutral complexes (i.e., $Hg(SH)_2$ about 16%) might favor efficient cell uptake.[55,56] The same range of neutral complexes is observed under FR (i.e., $HgCl_2$ about 22%). Thus, the similar proportion of neutral, bioavailable forms of inorganic Hg might explain why comparable rates and yields of IHg methylation are obtained under both SR and FR (Table 2). Based on recent studies, it would also make sense that the decline of the observed methylation yield per cell is due to a lower IHg sorption on the cellular thiols at the cell surface[45] compared to potential ligand–metal based competitive adsorption in solution mediated by extracellular polymeric substances (EPS) produced by the cells and which concentration should be higher at higher cell density.[45,57]

Both methylation and demethylation might also be limited by the transport of Hg species across the diffusion boundary layer and the association/dissociation rate of the Hg complex in the reaction layer.[58] Hence, inorganic and organic Hg species complexation in bulk solution, in the diffusion layer and in the reaction layer surrounding the external membranes of micro-

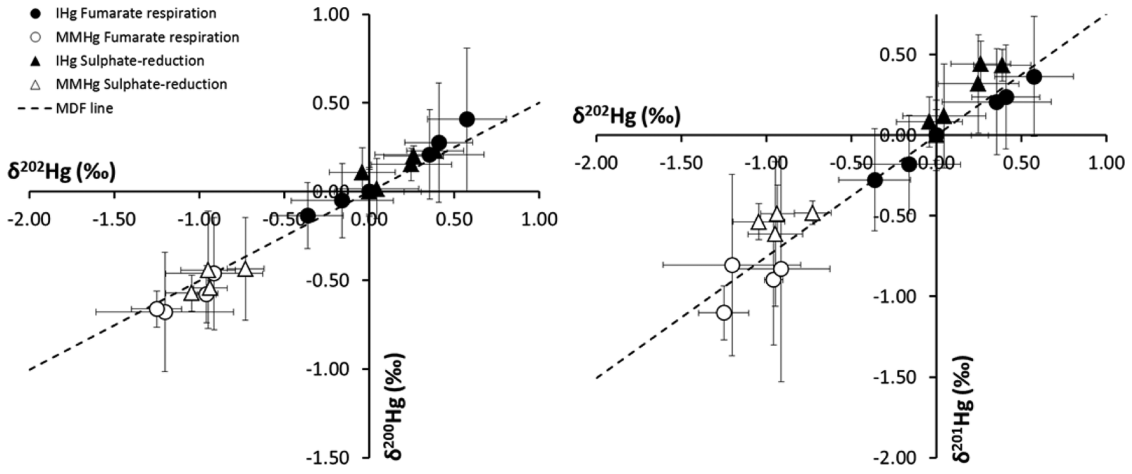

Figure 2. Tri-isotopic diagrams (δ^{202}Hg vs δ^{200}Hg, left; δ^{202}Hg vs δ^{201}Hg, right, delta values in ‰) displaying mass dependent fractionation of IHg and MMHg during methylation/demethylation by *Desulfovibrio dechloroacetivorans* strain BerOc-1 under fumarate and sulfate respiration conditions. The dashed line represents the theoretical mass dependent fractionation line. Error bars represent the 2 SD uncertainties on procedural replicates measurements of samples by GC-MC-ICPMS.

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organisms, are likely to influence Hg bioavailability for active or passive cell uptake.[8,54,59] It can be considered as a first limiting step for the subsequent methylation and demethylation reaction, but unfortunately our model for Hg species complexation in solution only takes into account inorganic complexes. This does not allow to conclude for the role of organic ligands binding Hg species since, for example, a recent study suggests that the MMHg released outside the strain BerOc-1 cells under fumarate respiration is associated with high molecular weight biomolecules.[46] Evidently, it might influence MMHg demethylation by limiting or enhancing the active transport to the cell through the membrane depending on the characteristics of these ligands.[8]

However, the results obtained with stable enriched tracer ^{199}IHg show that neither the type of electron acceptor (i.e., sulfate or fumarate) nor the presence of methylcobalamin or cysteine were significant parameters affecting the methylation potential of strain BerOc-1. This suggests that the gene regulated enzymatic pathway of methylation might be the rate limiting step for IHg methylation in strain BerOc-1.

3.2. Hg Species-Specific Isotope Fractionation. *3.2.1. Unique Hg Stable Isotope MDF and No MIF Associated with the Hg-Methylation Biosynthetic Pathway.* Under both FR and SR conditions, the incubation of strain BerOc-1 with IHg (NIST 3133 standard) showed significant isotope fractionation of Hg species during the time-course of the experiment (Table 1), with the Hg isotopic composition of each Hg species being specific and opposite between IHg and MMHg. All δ values were on the theoretical mass dependent fractionation line within the 2SD analytical error (Figure 2), showing no significant MIF ($\Delta^{201}Hg_{IHg}$ = 0.04 ± 0.07 ‰ (n = 12, 1 SD), $\Delta^{201}Hg_{MMHg}$ = 0.01 ± 0.11‰ (n = 8, 1 SD)). Methylation of IHg by strain BerOc-1, as expected from kinetic fractionation law, produced MDF of Hg isotopes that leads to the enrichment of MMHg in lighter isotopes relative to the initial IHg (NIST3133) ($\delta^{202}Hg_{MMHg}$ = −0.91 ± 0.29‰ and −0.93 ± 0.10‰ after 24 h for FR and SR, respectively). In contrast, residual IHg was enriched in heavier isotopes ($\delta^{202}Hg_{IHg}$ = 0.58 ± 0.23‰ and 0.39 ± 0.17‰ after 24 h under FR and SR conditions, respectively) (Figure 3).

We first observed that Hg MDF extent during Hg methylation by SRB *Desulfovibrio dechloroacetivorans* strain BerOc-1 is not significantly dependent on the bacterial metabolism, since delta values of both Hg species under SR and FR conditions after 24h were not different considering the analytical uncertainties (Table 1 and Figure 2). Earliest measurement of MMHg isotopic composition (after 1.5h of incubation) also showed significantly negative values for $\delta^{202}Hg_{MMHg}$ under both FR and SR conditions (−1.20 ± 0.40‰ and −1.04 ± 0.15‰, respectively). Unfortunately, it was not possible to measure isotopic ratios of MMHg before 1.5h because since MMHg concentrations were lower than required for GC-MC-ICPMS analysis. Under both FR and SR, $\delta^{202}Hg_{IHg}$ and $\delta^{202}Hg_{MMHg}$ did not show significant variations after 9 h of experiment (Table 1 and Figure 3). Nevertheless, the yield of net methylation after 24 h is significantly higher under FR than SR conditions (15.1% and 10.6%, respectively, Table 2). This suggests that neither the net methylation yield nor the growing cells metabolism seems to significantly affect the resulting Hg species isotopic composition after 24h.

Kinetic isotope fractionation factors $\alpha_{202/198}$ have been calculated assuming Rayleigh distillation equation (eq S.8 of the SI) on the remaining fraction of Hg[28,29,31,37] (here IHg) to characterize the Hg isotopic fractionation during methylation of IHg by SRB. Taken independently, FR and SR conditions displayed $\alpha_{202/198}$ values (1.0044 ± 0.0011 (n = 6, 2 SD) and 1.0025 ± 0.0011 (n = 6, 2 SD), respectively) that were not significantly different considering the associated uncertainty (SI Table S8 and Figure S1). However, we report here only an "apparent" kinetic fractionation factors since the opposite reactions of methylation/demethylation (discussed later in the manuscript) potentially associated with different MMHg and IHg complexes can affect the observed Hg isotopic signatures of Hg species. Therefore, it does not allow to assess or model accurate fractionation factor values with Rayleigh equations in such complex biological media impacted by multiple transformations, where in addition, the product of the reaction (here MMHg) can be converted back to the product of the reaction.[38]

For all the tested conditions, no significant MIF was observed (Figure 2). This is in agreement with previous microbial studies which focused on Hg species transformation pathways in the dark such as IHg methylation,[29] MMHg demethylation,[31] or IHg reduction.[28,37] In opposition, for studies on photoinduced Hg transformation pathways, such as photoreduction of both IHg[21,22,36] and MMHg[21,35] or photochemical methylation of IHg,[60] both MDF and MIF have been reported. Hence, the absence of Hg stable isotope MIF during microbial activity, and especially during methylation/demethylation, has two major implications. First, it makes difficult to differentiate such biotic pathway with dark abiotic pathways for which no significant MIF was also observed.[38] Second, it clearly supports the idea that significant odd Hg isotopes anomalies (MIF) in natural samples most probably track the photochemical conversion of MMHg and other Hg species rather than their microbial transformation.[21,22]

3.2.2. Influence of Bacterial Cell Density and Methylation Rate on Hg Species-Specific Isotope Fractionation. Cell density during the experiments with strain BerOc-1 was kept relatively constant (about 10^8 cell mL^{-1}) from an inoculum of exponentially grown cells prior to 100 ng g^{-1} IHg exposure. Our experiments were conducted in the same incubation medium (see SI) as in experiments conducted with a different strain, *Desulfobulbus propionicus*.[29] The cell density in the mentioned experiments was initially inferior and it was

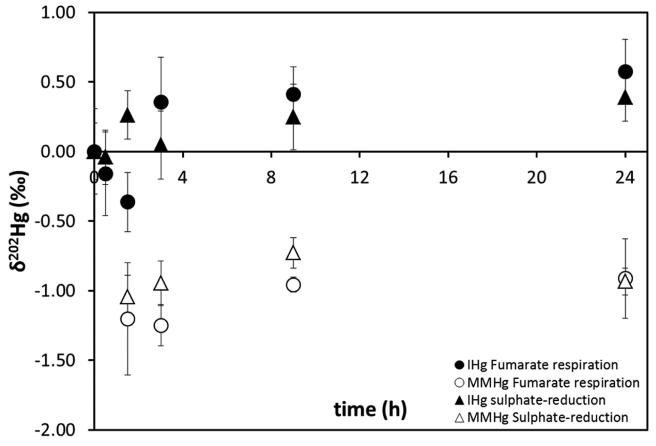

Figure 3. Evolution of Hg species (IHg and MMHg) specific isotope composition (as δ^{202}Hg) during 24h of incubation of 100 ng.g^{-1} NIST3133 with *Desulfovibrio dechloroacetivorans* strain BerOc-1 under fumarate and sulfate respiration conditions.

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Table 2. Yields of Net Methylation (%), Rate Constants of Methylation (k_m) and Demethylation (k_d) of Hg under Different Incubation Conditions of *Desulfovibrio dechloroacetivorans* Strain BerOc-1 with 800 ng of ^{199}IHg or Natural NIST3133 Hg (i.e. 100 ng g^{-1} IHg in 8 mL of solution) after Exponential Growth and at 30°C in the Dark

incubation conditions		enriched tracer (^{199}Hg(II)) or NIST3133 Hg(II)	net methylation (%) after 24h	2SD	k_m (0.10^{-3} h^{-1})	SE	k_d (0.10^{-3} h^{-1})	SE	k_m/k_d	SE
Fumarate respiration										
max. cells density	(≈10^8 cells/mL)	enriched tracer	15.9	0.4	14.8	2.4	84.4	13.6	0.18	0.04
	(≈10^8 cells/mL)	NIST 3133	15.1	1.3	27.3	4.2	145.4	22.6	0.19	0.04
	10-times diluted	enriched tracer	2.7	0.1	n/a	n/a	n/a	n/a	n/a	n/a
	100-times diluted	enriched tracer	0	0	n/a	n/a	n/a	n/a	n/a	n/a
Sulfate-reduction										
max. cells density	(≈10^8 cells/mL)	enriched tracer	19.8	0.5	19.4	1.5	66.1	5.1	0.29	0.03
	(≈10^8 cells/mL)	NIST 3133	10.6	0.8	25.6	5.0	205.1	39.7	0.12	0.03

incubated with lower amounts of IHg (50 μg mL^{-1}). The methylation rate was also significantly higher in our study (0.6% MMHg produced per hour during 24 h vs 0.06% MMHg produced per hour during 140 h). However, the Hg isotope fractionation of Hg compounds in our experiments is comparable to the one observed in experiments with *Desulfobulbus propionicus*. Hg(II) reduction and MMHg demethylation by Hg resistant micro-organisms[28,31,37] showed higher net reaction yields (higher than 50%) and a wide range of Hg initial concentrations (from 30 to 600 ng g^{-1}) was used, and always slightly lower fractionation factors were observed (Table S8). Our observations compared with available data on Hg isotope fractionation during microbial reactions ([34] and references herein) suggests that variations in cell density, growth phase, Hg compounds concentration and transformations rates do not appear to significantly impact the Hg isotope fractionation during such transformations.

It remains ambiguous to estimate the contribution of each step of the Hg methylation route on the Hg isotope fractionation. It has been suggested that the cellular pathways of transformation are primarily responsible for the observed isotopic fractionation as long as the uptake rate is significantly higher than the biotransformation rate.[28,34] The energy-dependent IHg uptake could be mediated by unspecific metal membrane divalent cation transporters, which is supported by the inhibition of IHg methylation by zinc and cadmium.[61] Even if that happened, this resulted in no modification of the isotopic fractionation of MMHg. It suggests a common route under SR or FR conditions because the complexation model in solution used in this study reveals that, under both conditions, a comparable fraction of neutral Hg compounds is available (about 20%, see SI Table S4). Therefore, a difference in IHg passive uptake and subsequent bioavailabilty for methylation between SR and FR conditions is unlikely. We conclude that both the same strain and same amounts of bioavailable IHg complexes for *D. dechloroacetivorans* strain BerOc-1 under both SR and FR lead to similar MMHg formation rate, resulting in identical MDF signature.

3.2.3. Role of the Biological Demethylation. Microbial demethylation preferentially transforms the lighter Hg isotopes of the MMHg[31] molecules, which may considerably influence the Hg species isotope composition when significant amounts of MMHg accumulate in the medium.[46] It was suggested for *Desulfobulbus propionicus*[29] that the simultaneous demethylation is likely to suppress the kinetic fractionation effect of the methylation step after significant release of MMHg in the medium. In such case, IHg in the culture medium would be a mixed pool of residual "heavy" IHg from methylation and "lighter" IHg from demethylation. This statement is strongly supported by our results since no significant changes of the Hg isotopic composition of MMHg occur after 3h and until the end of the experiment (Table 1). This corresponds the decrease of the net methylation of IHg when the ratio $[MMHg]_{formed}/[IHg]_{t0}$ starts to follow a logarithmic evolution with time due to pseudo first-order reversible reactions (Figure 1). As reported above, for strain BerOc-1 the rate constant for demethylation is about 10 times higher than the methylation one. Hence, we can reasonably assume that demethylation impacts the observed Hg isotopic composition of IHg and MMHg species in the late measurements of the time-course experiments. This further lead to the measured steady-state between IHg and MMHg isotopic composition after 24h.

As observed in unidirectional experiments,[28,31] the suppression of Hg isotopes fractionation at the end of our experiments could also be related to the decrease of bioavailable IHg and of its uptake rate. Complementary experiments (see SI) showed that the presence of thiol ligand (L-cysteine in this study) did not affect IHg methylation yield and/or rate constant by BerOc-1, even if it promotes Hg uptake for other sulfate and iron reducing bacteria strains.[8,62] Thus, for strain BerOc-1 passive diffusion of neutral Hg complexes, like others SRB strains,[13] seems to be more likely than an active transport across the membrane as previously suggested in section 3.2.2. A decrease of the available pool of neutral IHg species for methylation would result in the suppression of the measured fractionation only if heavier Hg isotopes from IHg remaining in the cell (residual from methylation) can be more easily methylated than the ones from new IHg influx. Even if part of the heavier Hg isotopes from residual IHg is methylated together with IHg uptaken from the medium, they account for about 10% of the total IHg after 9 h. Since same proportion of neutral Hg species are available between t0 and t24h for both SR and FR (SI Table S4), we can assume that significant amount remain in the medium. As a consequence, this evolution of IHg speciation in the culture medium should not result in any decrease of IHg chemical availability and uptake by the cells. We conclude that a decrease of substrate bioavailability should play only a minor role in the observed suppression of Hg isotope fractionation under our experimental conditions, and that demethylation is the main process responsible for this observation.

3.2.4. Role of Bacterial Methylation Pathway(s) on Hg Isotope Fractionation. The study of S, O, and C isotope fractionation suggests that isotope fractionation during SR

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strongly depends on the enzymatic regulation in the cell and on the involved enzymatic pathway.[67] In the same way, it is possible that different enzymatic or nonenzymatic pathways responsible of Hg transformation by microorganisms produce different Hg isotope fractionations. The extent of carbon isotope fractionation during SRB activity seems to be independent of the growth phase and mostly dependent on the growth rate.[67] A recent work also demonstrated that the availability and the nature of the electron donor may influence the fractionation of S isotopes during SR by a marine *Desulfovibrio* sp.[68] A study on microbial Cr(VI) reduction showed that the isotopic fractionation factor of Cr isotopes was dependent on the electron donor concentration (lactate).[69] Thus, the magnitude of elemental (i.e., C, S, Cr, Hg) stable isotope fractionation by SRB may be related to the metabolic process in which the studied element is involved.

Enzymatic catalysis has been evidenced for the IHg methylation via Acetyl-CoA synthase pathway in *Desulfovibrio desulfuricans* LS,[63,64] meanwhile in several strains of *Desulfovibrio* and *Desulfobulbus* it seems to be independent from this pathways.[53] *Desulfovibrio desulfuricans* strain ND132, phylogenetically close to BerOc-1, is able to significantly methylate Hg without any Hg resistance.[9] However, a new study identified two genes (*hgcA* and *hgcB*) present in all methylating bacteria (but not in nonmethylators) with sequenced genomes.[11] Similarly, numerous MMHg and/or IHg resistant bacterial strains also use specific enzymes (from *mer* genes) to catalyze IHg reduction or MMHg degradation.[31,37,65,66] It was suggested that, for those Hg resistant strains, MMHg demethylation by organomercurial lyase (*MerB*) and reduction of IHg by mercuric reductase (*MerA*) were the primary step causing Hg stable isotope fractionation.[28,31]

Considering that all methylating bacteria harbor the genes encoding the enzymes catalyzing the Hg-methylation reaction[11] and that this enzymatic step can be the primary step causing isotope fractionation, different metabolisms (sulfate-reduction, fumarate respiration, pyruvate fermentation) or different strain/populations of methylating bacteria would result in the same extent of Hg isotopes fractionation during methylation. Indeed, the same magnitude for Hg isotope fractionation was reported during Hg methylation by distinct species of the *Desulfovibrionaceae* family under both FR and SR (our study) and by *D. propionicus* (*Desulfobulbaceae* family) under fermentation.[29] This is also consistent with the fact that different enzyme-specific transformations pathways, that is, MMHg demethylation, IHg reduction, and IHg methylation, seem to display slightly different kinetic Hg isotope fractionation factors (1.0004, 1.0012−1.0026, and 1.0025−1.0044, respectively) as summarized in SI Table S8 from available literature. We conclude that the unique enzymatic process of methylation encoded by specific genes, recently discovered and common to all tested methylators,[11] can be the primary responsible for the observed Hg isotope fractionation between IHg and the product MMHg regardless of the bacterial metabolism or the strain involved.

Among Hg methylation studies, with SRB cultures (this study and the one with incubation of Hg with *Desulfobulbus propionicus*[29]) or under abiotic conditions involving methylcobalamin,[38] it has been demonstrated a similar pattern of Hg isotope fractionation with the production of MMHg enriched in lighter Hg isotopes. Since the methylcobalamin-like intermediate is also requested for IHg methylation by microorganisms,[11] we suggest that the methylcarbanion transfer can be responsible for the formation of lighter MMHg. When microorganisms are involved, the enzymatic regulation leading to the release of this intermediate could be the limiting step for the reaction of methylation.

3.3. Environmental Implications for Hg Isotope Tracing. We demonstrate here that the Hg methylation by SRB in sulfate-reducing environments (anoxic waters, sediments, and biofilms) can provide, to the water column or benthic organisms, a MMHg presenting a lighter Hg isotopic composition than the IHg initially deposited and occurring in the system, with no modification of the MIF signature. This effect can probably be enhanced if any molecular or turbulent diffusion of MMHg from the pore waters to the water column is taking place.

Regarding further MMHg bioaccumulation in food chains, this process compete with the photodegradation of MMHg for which the residual MMHg in the water column is enriched in heavier isotopes[21] compared to the "native" MMHg from methylation in the sediments or the water column. This is consistent with environmental studies that observed heavier Hg isotopic composition of Hg in organisms living in pelagic or clear shallow water column (i.e., higher photochemical activities) rather than deeper mesopelagic or turbid (i.e., less or no photochemical activities) zones.[20,25,70,71] Since dark microbial Hg demethylation is an important pathways occurring in aquatic environments,[3,72] our study also suggests that this process can locally contribute, with MMHg photoreduction, to the heavier Hg isotopic composition measured in fish muscle when compared to sediments of their environments.[24,25]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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