*Article*

# Evidence of Mercury Methylation and Demethylation by the Estuarine Microbial Communities Obtained in Stable Hg Isotope Studies

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**Abstract:** Microbial activity is a critical factor controlling methylmercury formation in aquatic environments. Microbial communities were isolated from sediments of two highly mercury-polluted areas of the Tagus Estuary (Barreiro and Cala do Norte) and differentiated according to their dependence on oxygen into three groups: aerobic, anaerobic, and sulphate-reducing microbial communities. Their potential to methylate mercury and demethylate methylmercury was evaluated through incubation with isotope-enriched Hg species ($^{199}$HgCl and $CH_3^{201}$HgCl). The results showed that the isolated microbial communities are actively involved in methylation and demethylation processes. The production of $CH_3^{199}$Hg was positively correlated with sulphate-reducing microbial communities, methylating up to 0.07% of the added $^{199}$Hg within 48 h of incubation. A high rate of $CH_3^{201}$Hg degradation was observed and >20% of $CH_3^{201}$Hg was transformed. Mercury removal of inorganic forms was also observed. The results prove the simultaneous occurrence of microbial methylation and demethylation processes and indicate that microorganisms are mainly responsible for methylmercury formation and accumulation in the polluted Tagus Estuary.

**Keywords:** bacteria; SRB; estuaries; methylmercury; mercury; methylation; demethylation; biogeochemistry; estuarine chemistry; mercury isotopes

## 1. Introduction

Methylmercury (MeHg) is one of the most toxic forms of mercury [1] and has been extensively studied for its neurotoxic effects such as blindness, loss of balance, and in severe cases, death [2]. Humans are mainly exposed to MeHg via the consumption of fish and marine mammals [3]. The release of mercurial compounds by industrial activities has been the cause of two large epidemic disasters related to the consumption of contaminated fish in Japan (Minamata Bay and Agano River) [3].

The Tagus Estuary (Portugal), the object of this study, experienced high levels of mercury contamination as a result of past industrial activity mainly related to pyrite processing and chloralkali production [4–6]. In particular, the two areas included in this study in the North (Cala do Norte) and the South (Barreiro) are severely contaminated, with reported levels for total mercury of up to 11 and 33 mg/kg and for MeHg of up to 28 and 47 μg/kg, respectively [7].

MeHg is formed in the environment when the oxidized mercury species react with a methyl group [8]. Methylation of mercury can occur under aerobic and anaerobic conditions by abiotic or biotic mechanisms. The abiotic transmethylation reactions include the transfer of a methyl group by the action of ultraviolet radiation or by reaction with humic and fulvic acids [9]. However, in aquatic environments, the biomethylation of mercuric mercury is the major pathway responsible for high concentrations of MeHg [10]. The biomethylation of mercury has been associated with a wide array of organisms with different metabolic pathways and taxa [11]. It was first described by Jensen and Jernelov in 1969 [12], who at the time assumed that the methyl group was transferred to $Hg^{2+}$ by the carbon monoxide dehydrogenase (CODH) pathway in sulphate-reducing bacteria (SRB) [8]. Therefore, the production of MeHg was primarily associated with SRB [13]. Later, iron-reducing bacteria [14] and methanogens [15] were also associated with MeHg production in anoxic environments. Recently, a genetic basis for mercury methylation was provided through the identification of two genes (*hgcA* and *hgcB*) in mercury-methylating bacteria [16].

Numerous microorganisms have been also reported to convert MeHg into less toxic forms [17] by cleaving the carbon-mercury bond ($Hg–CH_3$) [18]. Both oxidative and reductive demethylation pathways have been reported. Oxidative demethylation is mediated by anaerobic bacteria and probably related to carbon metabolism (C1), with release of $CO_2$ and $Hg^{2+}$ [1]. Reductive demethylation leads to the formation of $Hg^0$ and $CH_4$ as end products and is usually genetically encoded by a cluster of genes organized in the *mer* operon [8]. The encoded enzymes break the covalent Hg–C bond (organomercurial lyase) and reduce $Hg^{2+}$ to $Hg^0$ (mercuric reductase) [19–21]. However, abiotic MeHg degradation is also possible, such as the photodegradation of MeHg mediated by the action of ultraviolet light [8].

The concentration and bioaccumulation of MeHg in aquatic environments depends on the balance between both methylation and demethylation processes. In the aquatic environment, these two processes are typically microbially mediated and occur mostly in sediments [10,22]. The rate of these processes is a function of the microbial activity and the concentration of bioavailable mercury species. Thus, the integrated study of these two simultaneous processes is important to understand the dynamics of production and degradation of MeHg, which is critical for future remediation management in contaminated environments.

These processes have been investigated using sediments and pure cultures of isolated microorganisms under anaerobic and aerobic conditions [14,15,22–25]. The objective of the present work was to investigate communal activities of bacteria and establish their contribution for the processes of mercury methylation and demethylation by using isotope-enriched Hg species and inductively coupled plasma mass spectrometry (ICP-MS) analysis.

## 2. Materials and Methods

### 2.1. Studied Areas and Sampling

Two areas of the Tagus Estuary were sampled: Barreiro—38°40′45.40″ N; 9°3′1.70″ W and Cala do Norte—38°51′21.21″ N; 9°3′40.51″ W. Sediment samples were collected during spring. Sediment cores of approximately 24 cm in length were collected and rapidly sliced into layers of 3 cm (Figure 1). Samples were stored refrigerated in sealed tubes and transported to the lab for mercury-resistant microbial community isolation.

### 2.2. Microbial Communities' Isolation

Inoculums were prepared through the dilution of sediment samples with 20 mL of distilled and sterile water. After vigorous shaking, 5 mL was taken from each suspension and added to a new tube, creating a mixture of the 24-cm sediment core (Figure 1A). The mixture was shaken and after centrifugation at 5000 rpm for 1 min (4 °C), 2–5 mL of supernatant was inoculated into liquid media containing 2 μg/mL $Hg^{2+}$. Figure 1A schematizes the techniques used for the isolation of different Hg-resistant microbial communities: the aerobic microbial community (AMC), anaerobic

microbial community (AnMC), and sulphate-reducing microbial community (SO$_4$-RMC), which are also described below.
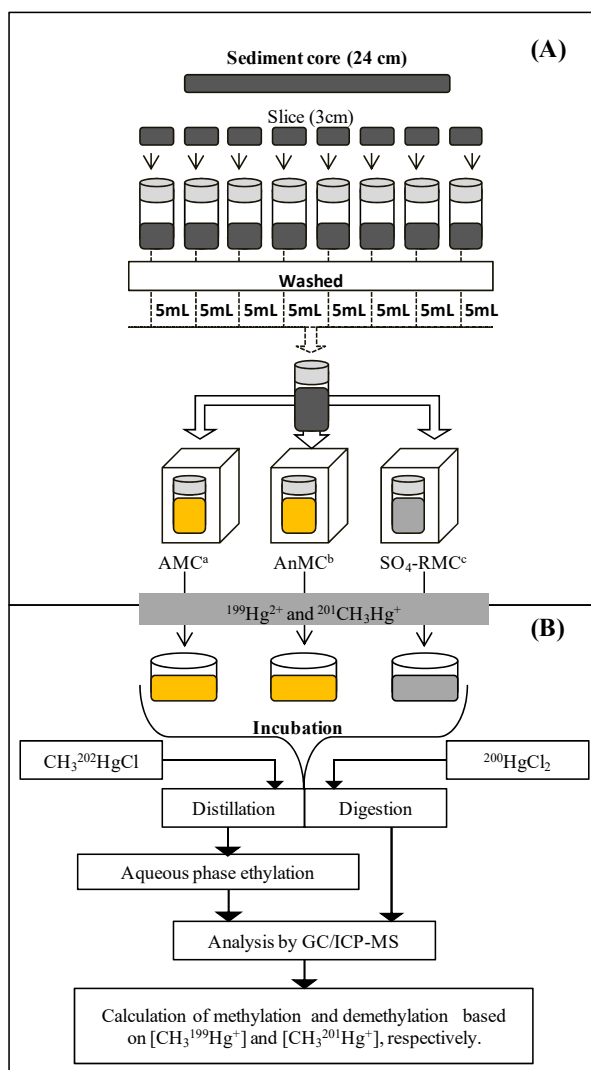


**Figure 1.** Illustration of the process for the isolation of microbial communities (**A**) and subsequent incubation with isotope-enriched Hg species to evaluate microbial potential to methylate and demethylate mercury (**B**). CH$_3$$^{201}$Hg degradation and CH$_3$$^{199}$Hg production were monitored using gas chromatography and inductively coupled plasma mass spectrometry (GC/ICP-MS) analysis.

*Aerobic community*: To isolate the AMC, 2 mL of washed sediment supernatant was inoculated in 20 mL of Mueller–Hinton (MH) broth and incubated under aerobic conditions. After 24 h of growing at 37 °C, bacterial growth was visible. From this bacterial suspension, 2 mL of the inoculums were transferred to a new MH broth containing 2 μg/mL Hg$^{2+}$ and incubated in aerobic conditions.

*Anaerobic communities*: To isolate the AnMC and SO$_4$-RMC, 5 mL of the supernatant was inoculated in serum bottles (Belco Glass Inc., Vineland, NJ, USA) containing 50 mL of MH broth and *Postgate* C medium, respectively. (*Postgate* C contains sulphate that can be reduced to sulphide, forming a black precipitate that indicates SRB growth [7]). Media were prepared under nonsterile conditions and added to N$_2$-gassed serum bottles and closed with rubber stoppers with a crimped metal seal, after which the bottled media was autoclaved. To avoid O$_2$ contamination, all inoculations were performed using anaerobic techniques under N$_2$ flux in an anaerobic glove box. After 3 days of growing at 37 °C, bacterial growth was visible, and 5 mL of the inoculum was transferred to new bottled medium supplemented with 2 μg/mL Hg$^{2+}$.

All three communities were stored in the respective media (MH broth or *Postgate* C) plus 15% of glycerol containing 2 μg/mL $Hg^{2+}$ at −80 °C.

## 2.3. Determination of Mercury Resistance

Mercury resistance levels of each microbial community were determined, as described previously for individual bacteria [26] for mercuric mercury ($HgCl_2$) ((Sigma-Aldrich, St. Louis, MO, USA Portugal),) and MeHg ($CH_3HgCl$) (Sigma-Aldrich, St. Louis, MO, USA), using nominal concentrations ranging from 0.01 to 1003 μg/mL $Hg^{2+}$ and 0.01 to 100 μg/mL $CH_3Hg^+$. Determinations of mercury resistance were carried out in duplicate at each concentration tested. After incubation at 37 °C for 24 h in the dark and under aerobic and anaerobic (anaerobic jars with AnaeroGen sachet (Oxoid, Basingstoke, UK)) conditions, bacterial growth was monitored. The mercury resistance was registered as the lowest concentration of test compounds without visible growth. All data points represent the mean ± standard deviation (STD) of 2 independent determinations (each one also performed in duplicate).

## 2.4. Mercury Methylation and Demethylation Evaluation

Methylation and demethylation potential were evaluated simultaneously for the three isolated microbial communities as illustrated in Figure 1B. A spike solution containing isotope-enriched $^{199}HgCl_2$ and $CH_3^{201}HgCl$ in a proportion of approximately 100:1 was prepared (see below 2.5 and added to the growth media, where the microbial communities (AMC, AnMC, and $SO_4$-MC) were placed. After incubation, MeHg analysis was performed as described below.

## 2.5. Preparation of the Spike Solution

A stock solution of $^{199}HgCl_2$ (880 μg/mL $^{199}Hg$) was obtained by dissolving $^{199}Hg$-enriched (91.95% purity) HgO (Oak Ridge National Laboratories) in 1 mL of hydrochloric acid (10 mM). $CH_3^{201}HgCl$ (96.17% purity) used for the demethylation assay was synthesized from HgO (Oak Ridge National Laboratories, Oak Ridge, EUA) using the methylcobalamin method. The spike solution was prepared by adding 60 μL of $^{199}Hg$ stock and 15 μL of $CH_3^{201}HgCl$ (80 μg/mL) to deionized water (final volume of 5 mL). Thus, the spike solution contained 0.205 μg/mL of $CH_3^{201}Hg$ and 10.56 μg/mL of $^{199}Hg$ and was used for the subsequent methylation and demethylation assays.

## 2.6. Microbial Community Incubation with Mercury Isotopes

Mercury spike solution was added to overnight culture suspensions (adjusted to $10^6$ colony-forming units (CFU)/mL) to achieve 0.106 μg/mL of $^{199}Hg$ and 0.002 μg/mL of $CH_3^{201}Hg$. The microbial community suspensions and controls (MH broth and *Postgate* C medium plus spike solution) were incubated at 37 °C under aerobic conditions for AMC and anaerobic conditions for AnMC and $SO_4$-RMC. Anaerobic conditions were achieved using serum bottles prepared as described above (Section 2.1 of Material and Methods). Samples were taken after 6 and 28 h of microbial growth, and in the case of $SO_4$-RMC, also at 48 h. After each experimental endpoint, the optical density was measured using absorption spectrophotometry (595 nm) and the microbial suspension was filtered using syringe 0.4-μm filters (Acrodisc, Sigma Aldrich, Merck KGaA, Darmstadt, Germany) to separate the supernatant for further methylmercury analysis. Two independent experiments were carried out for each experimental condition.

## 2.7. Analysis of MeHg

The analysis of MeHg was performed via distillation/ethylation. MeHg was extracted from supernatant samples using water vapor distillation. Supernatant aliquots (250 μL) were transferred into Teflon distillation vials containing 10 mL deionized water, 200 μL KCl (20% *v/v*), and 500 μL $H_2SO_4$ (9 M). The samples were distilled under a nitrogen gas flow of 80 mL/min at 135 °C. The distillate was collected into Teflon distillation vials containing 5 mL of deionized water. After collection of approximately 90% of the distillate, the process was stopped. Blanks were prepared following the

same procedure. Total MeHg was measured on a Tekran 2700 MeHg Auto Analysis System, using EPA method 1630 developed by US Environmental Protection Agency.

The concentration of isotopes ($CH_3{}^{201}Hg$ and $CH_3{}^{199}Hg$) was quantified after gas chromatographic separation using inductively coupled plasma mass spectrometry (X-Series II ICP-MS, Thermo Fisher Scientific Inc., Waltham, MA, USA) by species-specific isotope dilution, using $CH_3{}^{202}HgCl$ as an internal standard, which was added to each sample before the distillation (Figure 1B). The measurement procedure and the scheme to calculate the tracer concentrations are described in detail elsewhere [27]. The following isotopes of Hg were measured: $^{199}Hg$ (Hg methylation), $^{201}Hg$ (MeHg demethylation), $^{202}Hg$ (internal standard), and $^{200}Hg$ (representing ambient MeHg). For each batch of samples, distillation blanks were analyzed for quality control purposes and found to be negligible ($0.9 \pm 0.9$ pg per distillation), resulting in typical detection limits of 0.011 ng/mL. MeHg recovery was typically >80%.

## 2.8. Analysis of Total Hg

Total mercury was determined in digested samples of supernatants using cold-vapor atomic fluorescence spectrometry (CV-AFS). Filtered samples were treated overnight with an oxidant agent (0.5% of 0.2 N bromine monochloride solution, BrCl) plus 0.5% HCl to convert all mercury into its ionic form. The digestion was stopped with the addition of $NaH_2OH{\cdot}HCl$ (20 μL to 40 mL). To correct for procedural losses, $^{200}HgCl$ was added to the samples as an internal standard prior to the digestion (Figure 1B).

The concentration of Hg isotopes in the digest was quantified using continuous-flow cold-vapor ICP/MS analysis (X-Series II ICP-MS, Thermo Fisher Scientific Inc.). The acidified sample was continuously mixed with a solution of stannous chloride 3% (*w*/*v*) in 10% HCl (*v*/*v*) by means of a peristaltic pump. The formed mercury vapor was separated from the liquid using an in-house-made gas–liquid separator and the elemental mercury was swept into the plasma of the ICP/MS. The following isotopes of mercury were measured and quantified using isotope dilution calculation: $^{199}Hg$ (from $^{199}HgCl$ added for methylation essay), $^{201}Hg$ (from $CH_3{}^{201}Hg$ added for demethylation essay), $^{200}Hg$ (internal standard), and $^{202}Hg$ (to calculate ambient total mercury). For each batch of samples, digestion blanks were analyzed for quality control purposes and found to be negligible ($0.15 \pm 0.1$ ng/mL), resulting in typical detection limits of 0.48 ng/mL for a 50-mL sample.

## 2.9. Determination of Methylation and Demethylation Rates

The formation of MeHg was evaluated by measuring the amount of MeHg production ($CH_3{}^{199}Hg$) from the inorganic spike ($^{199}Hg$), and the rate was calculated as: Methylation (%) = $([CH_3{}^{199}Hg]_{Final} \times 100)/[^{199}HgT]_{Initial}$. The percentage of demethylated $CH_3{}^{201}Hg$ was calculated as follows: Demethylation (%) = $([CH_3{}^{201}Hg_{Initial} - CH_3{}^{201}Hg_{Final}] \times 100)/[CH_3{}^{201}HgT]_{Initial}$.

## 2.10. Evaluation of Microbial Hg-Reduction Potential

Mercury reduction and subsequent volatilization of $Hg^0$ was verified according to the protocol described by François et al. [28], with some modifications. To the overnight microbial community adjusted to $10^6$ CFU/mL with MH in a 12-well microplate, $HgCl_2$ solution was added to achieve 2 μg/mL $Hg^{2+}$. A sensitive X-ray film layer was inserted in the microplate, followed by incubation at 37 °C in the dark for 48 h. The $Hg^0$ volatilization was observed through the foggy areas on the X-ray film, due to the reduction of $Ag^+$ by mercury vapor ($Hg^0$). The optical density was measured at 595 nm (Hitachi spectrophotometer), and the cells were harvested by centrifugation at $15,300 \times g$ for 5 min with the supernatant and cell pellet separated for total mercury (HgT) analysis. Harvested cells were washed with sterile deionized water and weighed.

Determination of HgT was performed by pyrolytic reduction and atomic absorption spectrometry using a LECO AMA-254 gold amalgamator [29]. The experiment was performed in duplicate with an uninoculated control run in the same conditions. The percentage of reduction was calculated as: Reduction (%) = $(HgT_{Initial} - (HgT_{Supernatant} + HgT_{Cell\ pellet}))/(HgT_{Initial}) \times 100$.

## 3. Results

### 3.1. Microbial Community Characterization

Sediments from two mercury-polluted areas of the Tagus Estuary Barreiro and Cala do Norte were sampled (Figure 1) to isolate three microbial communities exhibiting mercury resistance: the aerobic microbial community (AMC), anaerobic microbial community (AnMC), and sulphate-reducing microbial community ($SO_4$-RMC). The AMC was a group of microorganisms capable of growing in a typical microbiological medium (MH) in the presence of oxygen, while the AnMC and $SO_4$-RMC were groups of microorganisms capable of growing in the absence of oxygen. The difference between the AnMC and $SO_4$-RMC is the medium used: while the AnMC was grown in a typical microbiological medium (MH), $SO_4$-RMC were grown in a selective medium for SRB (*Postgate* C). Mercury resistance levels found for these communities were higher for $SO_4$-RMC (Table 1).

**Table 1.** Mercury ($HgCl_2$ and $CH_3HgCl$) resistance levels of the microbial communities isolated from two mercury-contaminated areas of Tagus estuary—at Cala do Norte and Barreiro.

| | $HgCl_2$ (µg/mL) | | | $CH_3HgCl$ (µg/mL) | | |
|---|---|---|---|---|---|---|
| | AMC | AnMC | $SO_4$-RMC | AMC | AnMC | $SO_4$-RMC |
| **Cala do Norte** | 10 | 8 | 50–100 | 2.5 | 0.5 | 2.5 |
| **Barreiro** | 13 | 50 | 50–100 | 2.5 | 10 | 2.5 |

**Notes:** AMC: aerobic microbial community, AnMC: anaerobic microbial community, and $SO_4$-RMC: sulfate-reducing microbial community.

### 3.2. Mercury Content after Incubation

Figure 2 shows the percentage of $CH_3{}^{201}Hg$ and total $^{199}Hg$ (% of initial) after 6 h and 28 h of incubation in the presence of bacteria. $CH_3{}^{201}Hg$ decreased over time, with 10–69% remaining after 28 h. Among the three evaluated microbial communities, the highest demethylation rates were registered in AMC samples of isolated communities from Cala do Norte (Figure 2). Total $^{199}Hg$ also decreased over time (Figure 2).
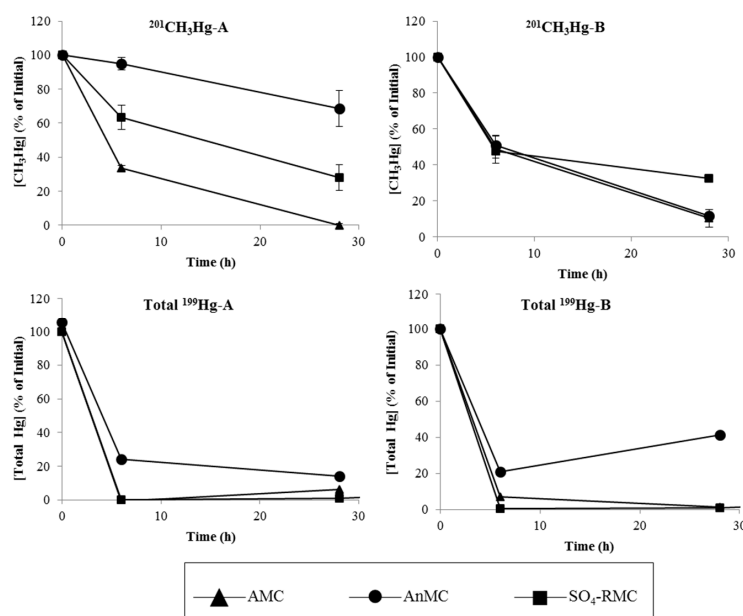


**Figure 2.** $CH_3{}^{201}Hg$ and total (all forms) $^{199}Hg$ content in supernatant samples after 6 and 28 h of incubation of three different microbial communities of aerobic microbes (AMC), anaerobic microbes (AnMC), and sulphate-reducing microbes ($SO_4$-RMC) after initial addition of 2.05 ng/mL of $CH_3{}^{201}Hg^+$ and 105.6 ng/mL of $^{199}Hg^{2+}$. The three microbial communities were isolated from two areas of the Tagus Estuary in Cala do Norte (**A**) and Barreiro (**B**).

### 3.3. MeHg Formation

Figure 3 shows MeHg concentrations in liquid media samples, discriminating the methylated $CH_3{}^{199}Hg$ from $^{199}HgT$. In $SO_4$-RMC media, between 0.02% and 0.07% of the initial $^{199}Hg$ (0.02–0.07 ng/mL) was methylated. Methylation was also observed for the AnMC (0.01% of the initial $^{199}Hg$) (Figure 3).
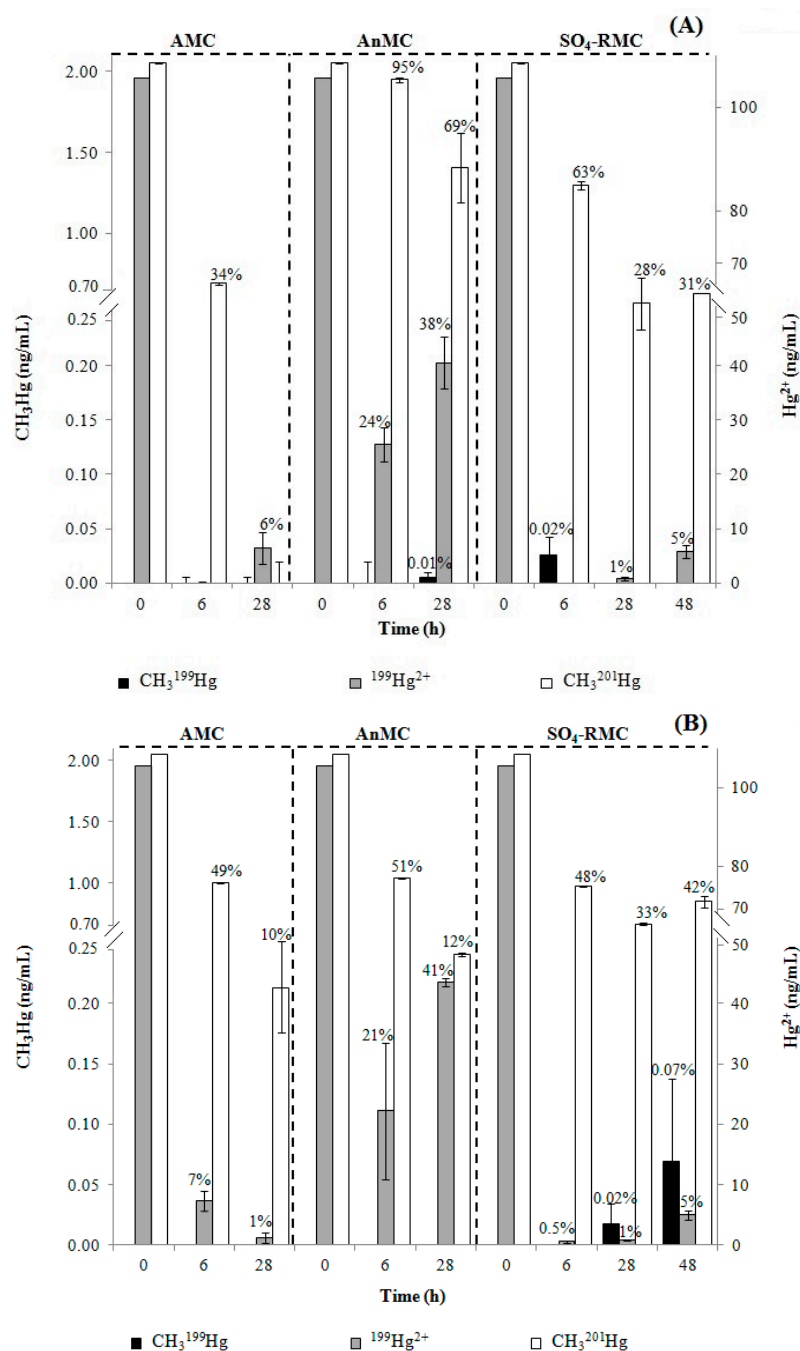


**Figure 3.** Representation of isotope concentrations in the supernatant of media containing different microbial communities. The results show the formation of $CH_3{}^{199}Hg$ from the initial 105.6 ng/mL $^{199}Hg^{2+}$ added and the decrease of the initial 2.05 ng/mL of $CH_3{}^{201}Hg$ with incubation time (6, 28, and 48 h). Three different microbial communities, an aerobic microbial community (AMC), anaerobic microbial community (AnMC), and sulphate-reducing microbial community ($SO_4$-RMC), were isolated from two mercury-contaminated areas of the Tagus estuary—Cala do Norte (**A**) and Barreiro (**B**).

The highest percentage of methylation was registered in the media containing $SO_4$-RMC from Barreiro (Figure 3B).

### 3.4. MeHg Degradation

The $CH_3{}^{201}Hg$ concentration decreased during incubation (Figures 2–4). This decrease was more accentuated in media containing the AMC from Cala do Norte, where only 0% to 10% of the initial $CH_3{}^{201}Hg$ added to media remained after 28 h.
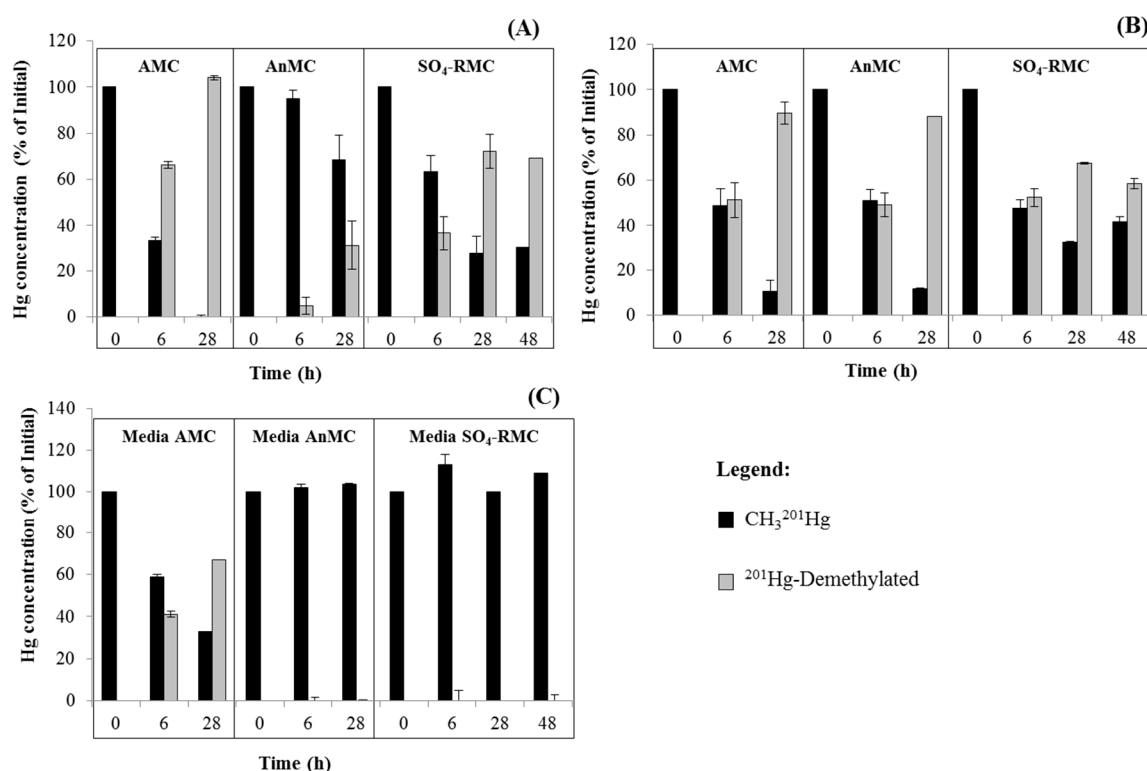


**Figure 4.** Concentration of $CH_3{}^{201}Hg$ after 6, 28, and 48 h of incubation with the three microbial communities of aerobic microbes (AMC), anaerobic microbes (AnMC), and sulphate-reducing microbes ($SO_4$-RMC) isolated from Cala do Norte (**A**) and Barreiro (**B**). The control media were also evaluated (**C**).

Figure 4 shows the percentage of $^{201}Hg$ demethylated over time. In the noninoculated control, a decrease in $CH_3{}^{201}Hg$ concentration was also observed for the aerobic control (AMC) as a consequence of abiotic demethylation, albeit to a lesser extent compared to the inoculated homologous sample (Figure 4C). No decrease in $CH_3{}^{201}Hg$ in the control media of AnMC or $SO_4$-RMC was observed (Figure 4C).

### 3.5. Hg$^{2+}$ Reduction and Hg$^0$ Volatilization

Figure 5 shows that both aerobic and anaerobic communities were able to remove mercury from liquid media containing $HgCl_2$ by cell uptake and also by the reduction of $Hg^{2+}$ with subsequent volatilization of $Hg^0$. The percentage of reduced $Hg^{2+}$ was 40 and 49% for aerobes and 16 and 37% for anaerobes from Cala do Norte and Barreiro, respectively (Figure 5).
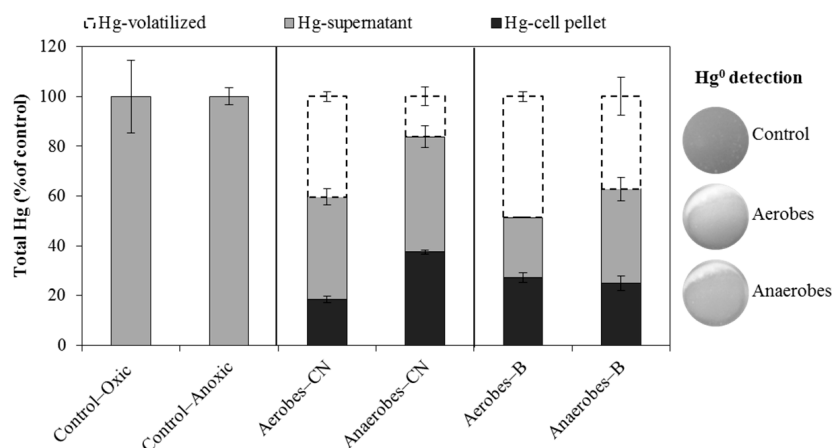
**Figure 5.** Final balance of total mercury after incubation of aerobic and anaerobic microbial communities isolated from two areas of the Tagus estuary (CN—Cala do Norte and B—Barreiro) with $HgCl_2$ for 48 h. The graphic representation shows the percentage of $Hg^0$ volatilized and total mercury remaining in the cell pellet and supernatant. $Hg^0$ volatilization was detected by the foggy area resulting from the reaction between $Hg^0$ and Ag using an X-ray film.

## 4. Discussion

This study shows the role of different communities of bacteria on the processes of mercury methylation and demethylation. The incubation of these communities with isotope-enriched Hg species ($^{199}HgCl$ and $CH_3{}^{201}HgCl$) highlights their ability to remove both inorganic as well as organomercurial species from the medium (Figure 2). Besides the removal, transformation of inorganic species was also observed, namely their methylation.

Methylation of mercuric mercury (i.e., $CH_3{}^{199}Hg$ production) was observed in media containing anaerobes, especially SRB (Figure 3). Although methylation was observed previously for a few aerobic bacteria isolated from the Tagus estuary [25], the study of the aerobic community did not detect Hg methylation, suggesting that this type of bacteria does not significantly contribute to MeHg formation.

The highest percentage of observed MeHg formation ranged from 0.02% to 0.07% of $^{199}Hg^{2+}$ initially added (105.6 ng/mL) to the media containing SRB from Cala do Norte and Barreiro, respectively (Figure 3). Likewise, other groups [8,30] also reported that methylation is a process promoted by anaerobes and that over 95% of the mercury methylation occurs in anoxic sediments [31], pointing out sulphate reducers as the main methylators [24,32]. Supplemental Table S1 describes all the isolates for the two studied areas identified during our investigations. These results indicate that the observed methylation by the $SO_4$-MRC from Barreiro and Cala do Norte can be related to the presence of *Desulfovibrio desulfuricans*, and for the AnMC from Cala do Norte, can be related to *Clostridium* spp., namely *Clostridium difficile* (Supplemental Table S1). These microorganisms are well known for their methylation potential [31–36]; in particular, *Desulfovibrio desulfuricans* ND132 is well known as a model organism for Hg methylation [37].

Comparing the two sampled areas, data show that the percentage of methylation was higher in Barreiro (0.07%) than in Cala do Norte (0.02%). Possible explanations for this observation may be differences in bacterial species composition related to the higher and longer-term mercury contamination in Barreiro causing a selective pressure for methylators [7,25]. On average, sediments of Cala do Norte have 11.7 µg/g total Hg, including 28.4 ng/g of MeHg, and sediments of Barreiro have 33.2 µg/g total Hg, including 47.2 ng/g of MeHg [7]. Applying the rate of methylation observed in this study (mentioned above) to ambient field concentrations of total Hg, up to 2.34 ng/g day$^{-1}$ and 11.62 ng/g day$^{-1}$ of MeHg could originate from microbial methylation in Cala do Norte and Barreiro, respectively. It is important to note, however, that physicochemical conditions in the estuary may differ from laboratory conditions. Therefore, these estimates are potential formation rates and still need confirmation in the field.

Demethylation was observed in all inoculated media (Figure 4), indicating that demethylation is common between aerobes and anaerobes [17,34,38] and that both biotic and abiotic mechanisms may be involved [23]. Although *mer B* (organomercurial lyase) was not found among our isolates, reduction of $Hg^{2+}$ into $Hg^0$ was detected (Figure 5), which indicates a presence of the *mer* operon amid the isolates; in fact, *mer* A was encompassed by isolates from these areas in our previous study [25]. Thus, we propose that the mechanism for demethylation by microbial communities may also be related to the presence of the *mer* operon. Besides, Figure 3A shows a methylation rate of 0.02% in $SO_4$-RMC media after the first 6 h, while no $CH_3{}^{199}Hg$ was detected after 28 h and 48 h, which could be explained by the simultaneous occurrence of microbial methylation and demethylation processes [33]. Obviously, there is a lot of complexity in this media and certain species may be present, but not sufficiently active, or might be conditioned by the sediment's chemistry and availability of nutrients [39]. Comparing the proportion of mercury methylation and demethylation in the present study, it appears that in estuarine sediments containing $CH_3Hg^+$ and $Hg^{2+}$, microorganisms are responsible for $CH_3Hg^+$ demethylation in both oxic and anoxic sediments and for methylation in anoxic sediments.

The analyses of ${}^{199}HgT$ (Figure 2) revealed that $Hg^{2+}$ is removed from liquid media during the incubation with microbes. Between 59–99% of ${}^{199}HgT$ disappeared after 28 h, which may be explained as a result of cell uptake and reduction of $Hg^{2+}$ to $Hg^0$ and its subsequent volatilization from the medium. Both phenomena, i.e., cell uptake and reduction followed by volatilization, were previously observed among individual microorganisms, such as *Bacillus*, *Vibrio*, *Aeromonas*, *Geobacter*, and *Enterobacteriaceae* [7,25], and here also for aerobic and anaerobic communities (Figure 5) isolated from Tagus estuary sediments.

Figure 6 summarizes all the transformations observed by the three evaluated microbial communities of the Tagus estuary, including the simultaneous occurrence of methylation of mercuric mercury and demethylation of MeHg. MeHg demethylation and reduction of mercuric mercury to elemental mercury constitutes a natural detoxification pathway that mitigates the formation and accumulation of the neurotoxic MeHg and promotes the overall removal of mercury from this aquatic system. On the other hand, newly formed MeHg can bioaccumulate, and subsequent biomagnification in food webs represents a severe risk for human and environmental health.
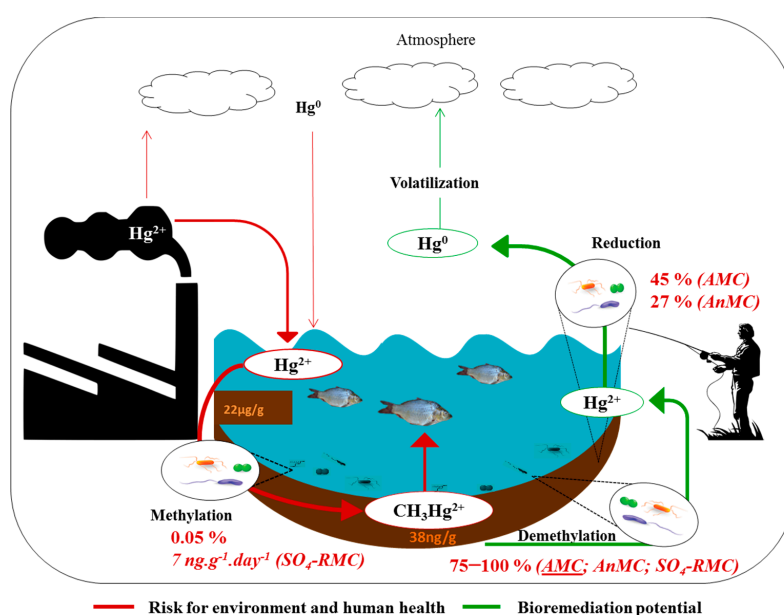


**Figure 6.** Summary of the results showing microbial-mediated transformations of mercury in the Tagus estuary and their context in human and environmental health. Rate conversions are the averaged values of results reported in this paper for Barreiro and Cala do Norte, and mercury concentrations in sediments, previously reported by Figueiredo et al. [7] were also averaged to indicate the sediments' levels.

## 5. Conclusions

The Tagus Estuary, one of the largest estuaries in Europe, has a history of high mercury contamination due to past industrial activities. Mercury-resistant bacteria are the main drivers for critical mercury methylation and demethylation processes. Here, we used for the first time isotope-enriched Hg species ($^{199}HgCl$ and $CH_3{}^{201}HgCl$) for incubations with a diversity of estuarine microbial communities. A significant finding was the existence of concomitant methylation and demethylation processes performed by microbes and the definition of their contribution for the biogeochemical cycle of mercury. Mercury methylation, which represents a risk for environmental and human health, is mainly carried out by SRB in anoxic environments, while significant demethylation was shown for both aerobic and anaerobic microbial communities in anoxic and oxic environments. The latter process may also constitute a potentially important bioremediation pathway. Overall, this study provides important direction for continued investigation of the estuary regarding (i) mercury biogeochemical speciation, (ii) environmental risks, and (iii) wastewater treatment requirements.

## References

1. Barkay, T.; Poulain, A. Mercury (micro) biogeochemistry in polar environments. *FEMS Microbiol. Ecol.* **2007**, *59*, 232–241. [CrossRef] [PubMed]

2. Clarkson, T.W.; Magos, L. The toxicology of mercury and its chemical compounds. *Crit. Rev. Toxicol.* **2006**, *36*, 609–662. [CrossRef] [PubMed]

3. Clarkson, T.W.; Magos, L.; Myers, G.J. Toxicology of mercury–Current exposures and clinical manifestations. *N. Engl. J. Med.* **2003**, *349*, 1731–1737. [CrossRef] [PubMed]

4. Canário, J.; Vale, C.; Caetano, M. Mercury in contaminated sediments and pore waters at a contaminated enriched in sulphate (Tagus Estuary). *Environ. Pollut.* **2003**, *126*, 425–433. [CrossRef]

5. Canário, J.; Vale, C.; Caetano, M. Distribution of monomethylmercury and mercury in surface sediments of the Tagus Estuary (Portugal). *Mar. Pollut. Bull.* **2005**, *50*, 1142–1145. [CrossRef] [PubMed]

6. Figuères, G.; Martin, J.M.; Meybeck, M.; Seyler, P. A comparative study of mercury contamination in the Tagus Estuary (Portugal) and major French Estuaries (Gironde, Loire, Rhône). *Estuar. Coast. Shelf. Sci.* **1985**, *20*, 183–203. [CrossRef]

7. Figueiredo, N.L.; Canário, J.; Duarte, A.; Serralheiro, M.L.; Carvalho, C. Isolation and characterization of mercury-resistant bacteria from sediments of Tagus Estuary (Portugal): Implications for environmental and human health risk assessment. *J. Toxicol. Environ. Health A* **2014**, *77*, 155–168. [CrossRef] [PubMed]

8. Barkay, T.; Wagner-Dobler, I. Challenges and achievements in controlling mercury toxicity in the environment. *Adv. Appl. Microbiol.* **2005**, *57*, 1–40. [CrossRef] [PubMed]

9. Morel, F.M.M.; Kraepiel, A.M.L.; Amyot, M. The chemical cycle and bioaccumulation of mercury. *Annu. Rev. Ecol.* **1998**, *29*, 543–566. [CrossRef]

10. Celo, V.; Lean, D.R.S.; Scott, S.L. Abiotic methylation of mercury in the aquatic environment. *Sci. Total Environ.* **2006**, *368*, 126–137. [CrossRef] [PubMed]

11. Gilmour, C.C.; Podar, M.; Bullock, A.L.; Graham, A.M.; Brown, S.D.; Somenahally, A.C.; Johs, A.; Hurt, R.A., Jr.; Bailey, K.L.; Elias, D.A. Mercury methylation by novel microorganisms from new environments. *Environ. Sci. Technol.* **2013**, *47*, 11810–11820. [CrossRef] [PubMed]

12. Jensen, S.; Jernelöv, A. Biological methylation of mercury in aquatic organisms. *Nature* **1969**, *223*, 753–754. [CrossRef] [PubMed]

13. King, J.K.; Harmon, S.M.; Fu, T.T.; Gladden, J.B. Mercury removal, methylmercury formation, and sulphate-reducing bacteria profiles in wetland mesocosms. *Chemosphere* **2002**, *46*, 859–870. [CrossRef]

14. Kerin, E.J.; Gilmour, C.C.; Roden, E.; Suzuki, M.T.; Coates, J.D.; Mason, R.P. Mercury methylation by dissimilatory iron-reducing bacteria. *Appl. Environ. Microbiol.* **2006**, *72*, 7919–7921. [CrossRef] [PubMed]

15. Hamelin, S.; Amyot, M.; Barkay, T.; Wang, Y.; Planas, D. Methanogens: Principal methylators of mercury in Lake Periphyton. *Environ. Sci. Technol.* **2011**, *45*, 7693–7700. [CrossRef] [PubMed]

16. Parks, J.M.; Johs, A.; Podar, M.; Bridou, R.; Hurt, R.A.; Smith, S.D.; Tomanicek, S.J.; Qian, Y.; Brown, S.D.; Brandt, C.C.; et al. The genetic basis for bacterial mercury methylation. *Science* **2013**, *339*, 1332–1335. [CrossRef] [PubMed]

17. Oremland, R.S.; Culbertson, C.W.; Winfrey, M.R. Methylmercury decomposition in sediments and bacterial cultures: Involvement of methanogens and sulphate reducers in oxidative demethylation. *Appl. Environ. Microbiol.* **1991**, *57*, 130–137. [PubMed]

18. Nascimento, A.M.A.; Chartone-Souza, E. Operon *mer*: Bacterial resistance to mercury and potential for bioremediation of contaminated environments. *Genet. Mol. Res.* **2003**, *2*, 92–101. [PubMed]

19. Parks, J.M.; Guo, H.; Momany, C.; Liang, L.; Miller, S.M.; Summers, A.O.; Smith, J.C. Mechanism of Hg-C protonolysis in the organomercurial lyase MerB. *J. Am. Chem. Soc.* **2009**, *131*, 13278–13285. [CrossRef] [PubMed]

20. Schaefer, J.K.; Yagi, J.; Reinfelder, J.R.; Cardona, T.; Ellickson, K.M.; Tel-Or, S.; Barkay, T. Role of the bacterial organomercury lyase (MerB) in controlling methylmercury accumulation in mercury-contaminated natural waters. *Environ. Sci. Technol.* **2004**, *38*, 4304–4311. [CrossRef] [PubMed]

21. Sinéad, M.; Chadhain, N.; Schaefer, J.K.; Crane, S.; Zylstra, G.J.; Barkay, T. Analysis of mercuric reductase (merA) gene diversity in an anaerobic mercury-contaminated sediment enrichment. *Environ. Microbiol.* **2006**, *8*, 1746–1752. [CrossRef]

22. Hintelmann, H.; Keppel-Jones, K.; Evans, R.D. Constants of mercury methylation and demethylation rates in sediments and comparison of tracer and ambient mercury availability. *Environ. Toxicol. Chem.* **2000**, *19*, 2204–2211. [CrossRef]

23. Martín-DOImedios, R.C.; Tessier, E.; Amouroux, D.; Guyoneaud, R.; Duran, R.; Caumette, P.; Donard, O.F.X. Mercury methylation/demethylation and volatilization pathways in estuarine sediment slurries using species-specific enriched stable isotopes. *Mar. Chem.* **2004**, *9*, 107–123. [CrossRef]

24. Heyes, A.; Mason, R.P.; Kim, E.-H.; Sunderland, E. Mercury methylation in estuaries: Insights from using measuring rates using stable mercury isotopes. *Mar. Chem.* **2006**, *102*, 134–147. [CrossRef]

25. Figueiredo, N.; Canário, J.; O'Driscoll, N.; Duarte, A.; Carvalho, C. Aerobic Mercury-resistant bacteria alter Mercury speciation and retention in the Tagus Estuary (Portugal). *Ecotoxicol. Environ. Saf.* **2016**, *124*, 60–67. [CrossRef] [PubMed]

26. Figueiredo, N.L.; Areias, A.; Mendes, R.; Canário, J.; Duarte, A.; Carvalho, C. Mercury-resistant bacteria from salt marsh of Tagus Estuary: The influence of plants presence and mercury contamination levels. *J. Toxicol. Environ. Health A* **2014**, *77*, 959–971. [CrossRef] [PubMed]

27. Ogrinc, N.; Hintelmann, H.; Eckley, C.; Lojen, S. Biogeochemical Influence on Carbon Isotope Signature in Boreal Lake Sediments. *Hydrobiologia* **2003**, *494*, 207–213. [CrossRef]

28. François, F.; Lombard, C.; Guigner, J.M.; Soreau, P.; Brian-Jaisson, F.; Martino, G.; Vandervennet, M.; Garcia, D.; Molinier, A.L.; Pignol, D.; et al. Isolation and characterization of environmental bacteria capable of extracellular biosorption of mercury. *Appl. Environ. Microbiol.* **2011**, *78*, 1097–1106. [CrossRef] [PubMed]

29. Costley, C.T.; Mossop, K.F.; Dean, J.R.; Garden, L.M.; Marshall, J.; Carroll, J. Determination of mercury in environmental and biological samples using pyrolysis atomic absorption spectrometry with gold amalgamation. *Anal. Chim. Acta* **2000**, *405*, 179–183. [CrossRef]

30. Batten, K.; Scow, K. Sediment Microbial Community Composition and Methylmercury pollution at four mercury mine-impacted sites. *Microb. Ecol.* **2003**, *46*, 29–441. [CrossRef] [PubMed]

31. Compeau, G.C.; Bartha, R. Sulphate-reducing bacteria: Principal methylators of Mercury in anoxic estuarine sediment. *Appl. Environ. Microbiol.* **1985**, *50*, 498–502. [PubMed]

32. King, J.K.; Kostka, J.E.; Frischer, M.E.; Saunders, F.M. Sulphate-reducing bacteria methylate mercury at variable rates in pure culture and in marine sediments. *Appl. Environ. Microbiol.* **2000**, *66*, 2430–2437. [CrossRef] [PubMed]

33. Achá, D.; Pabón, C.A.; Hintelmann, H. Mercury methylation and hydrogen sulfide production among unexpected strains isolated from periphyton of two macrophytes of the Amazon. *FEMS Microbiol. Ecol.* **2012**, *80*, 637–645. [CrossRef] [PubMed]

34. Pak, K.-R.; Bartha, R. Mercury methylation by interspecies hydrogen and acetate transfer between sulfidogens and methanogens. *Appl. Environ. Microbiol.* **1998**, *64*, 1987–1990. [PubMed]

35. Pan-Hou, H.S.; Imura, N. Involvement of mercury methylation in microbial mercury detoxication. *Arch. Microbiol.* **1982**, *131*, 76–177. [CrossRef]

36. Spangler, W.J.; Spigarelli, J.L.; Rose, J.M.; Flippin, R.S.; Miller, H.H. Degradation of methylmercury by bacteria isolated from environmental samples. *Appl. Microbiol.* **1973**, *25*, 488–493. [PubMed]

37. Gilmour, C.C.; Elias, D.A.; Kucken, A.M.; Brown, S.D.; Palumbo, A.V.; Schadt, C.W.; Wall, J.D. Sulfate-reducing bacterium desulfovibrio desulfuricans ND132 as a model for understanding bacterial mercury methylation. *Appl. Environ. Microbiol.* **2011**, 3938–3951. [CrossRef] [PubMed]

38. Baldi, F.; Pepi, M.; Filippelli, M. Methylmercury resistance in *Desulfovibrio desulfuricans* strains in relation to methylmercury degradation. *Appl. Environ. Microbiol.* **1993**, *59*, 2479–2485. [PubMed]

39. Figueiredo, N.L.L.; Canário, J.; Serralheiro, M.L.; Carvalho, C. Optimization of microbial detoxification for a mercury-contaminated environment. *J. Toxicol. Environ. Health A* **2017**, *80*, 788–796. [CrossRef] [PubMed]