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Effect of Inorganic and Organic Ligands on the Bioavailability of Methylmercury as Determined by Using a *mer-lux* Bioreporter

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A *mer-lux* bioreporter was constructed to assess the bioavailability of methylmercury [$CH_3Hg(II)$] in *Escherichia coli*. The bioreporter was shown to be sensitive, with a detection limit of 2.5 nM $CH_3Hg(II)$, and was used to investigate the effects of chlorides, humic acids, and thiols on the bioavailability of $CH_3Hg(II)$ in *E. coli*. It was found that increasing the concentration of chlorides resulted in an increase in $CH_3Hg(II)$ bioavailability, suggesting that there was passive diffusion of the neutral complex (CH_3HgCl^0). Humic acids were found to reduce the bioavailability of $CH_3Hg(II)$ in varying degrees. Complexation with cysteine resulted in increased bioavailability of $CH_3Hg(II)$, while assays with equivalent concentrations of methionine and leucine had little or no effect on bioavailability. The mechanism of uptake of the mercurial-cysteine complexes is likely not passive diffusion but could result from the activities of a cysteine transport system. The bioavailability of $CH_3Hg(II)$ decreased with increasing glutathione concentrations.

Methylmercury [$CH_3Hg(II)$] is extremely toxic to humans and wildlife. In this paper, Hg(II) and $CH_3Hg(II)$ refer to all the possible species of inorganic mercury and methylmercury, respectively, while Hg^{2+} and CH_3Hg^+ refer to the divalent mercuric ion and the methylmercuric ion, respectively. Despite an awareness of the toxicity of $CH_3Hg(II)$, there is little detailed information on how its speciation in the oceans and estuaries affects its bioavailability to microbes. Bioavailability of $CH_3Hg(II)$ depends on its speciation in the medium, which is influenced by the binding constant and the quantity of dissolved anions and ligands present, such as chlorides, sulfides, and natural dissolved organic matter (NDOM), in pore water and the water column. In estuaries and oceans, CH_3Hg^+ forms a neutral complex with Cl^- (CH_3HgCl^0), which dominates the speciation in the absence of sulfides and elevated levels of organic ligands. CH_3Hg^+ has also been shown to bind strongly to NDOM (1, 14, 15), which is a complex mixture of organic compounds found in environmental systems that are, or have decomposed from, material originating from marine and terrestrial cells of plants, animals, and microbes (13, 34). Although Hg^{2+} and CH_3Hg^+ have high binding constants with thiols, such as cysteine and glutathione (24, 25), there are only a few studies detailing the effects of these thiols, as a subgroup of dissolved organic matter (DOM), in the water column and sediments on the bioavailability of Hg(II) and $CH_3Hg(II)$ (12, 32). Concentrations of glutathione in Galveston Bay, TX, determined by high-performance liquid chromatography (HPLC) were reported to be in the range of 18 to 79 nM in June 2003 (12). Studies in other coastal and open-ocean environments have also found nanomolar levels of thiols (6, 17). As thiols have very high binding constants with Hg^{2+} and CH_3Hg^+, nanomolar levels of dissolved thiols are relatively substantial quantities and are sufficient to outcompete other potential inorganic anions (e.g., Cl^- and OH^-) that form complexes with Hg^{2+} and CH_3Hg^+. Hence, the impact of thiols on the bioavailability of both Hg(II) and $CH_3Hg(II)$ ought to be studied. Cysteine is an amino acid, while glutathione is a small peptide. Both compounds are made and used physiologically by bacteria, and they are polar and charged, depending on the pH, as they have carboxyl, amine, and thiol groups.

Although it has been postulated that uptake of $CH_3Hg(II)$ into microbes occurs via the process of passive diffusion of the neutrally charged $CH_3Hg(II)$ complexes, such as CH_3HgCl^0 and CH_3HgOH^0 (18), there has been scant experimental evidence to support this hypothesis for complex environmental solutions. Additionally, there is some evidence in the literature that $CH_3Hg(II)$ can cross membranes, either when present in solution as a charged complex (22) or when bound to larger organic ligands, likely through active-transport mechanisms (16, 26a). The mechanism behind such uptake has not been investigated in any detail, and there is little evidence for uptake under conditions representative of the natural environment. One possible way to test the uptake pathways is to culture bacteria or phytoplankton with increasing chloride content in a minimal medium while keeping the concentration of $CH_3Hg(II)$ constant and subsequently measuring the uptake of $CH_3Hg(II)$ complexes (18). If passive diffusion is a mechanism of uptake, or if uptake depends on the presence of the neutral complex only, there would be an increase in $CH_3Hg(II)$ uptake with an increase in chloride content because of the increased formation of the neutral complex, CH_3HgCl^0. Also, if passive diffusion is a mechanism of $CH_3Hg(II)$ uptake, then increased formation of bulky or charged complexes, such as CH_3Hg-NDOM, should lead to a decrease in uptake. Due to the polarity and bulkiness of metal-thiol complexes, they are not likely to passively diffuse into bacteria and have been shown to be taken up with the assistance of transport proteins (33). It has also been shown that when CH_3Hg^+ is bound to cysteine, the complex can be transported across the blood brain barrier in mammals by the use of neutral amino acid transporters known as LAT1 and LAT2 (29) and across the intestinal membranes of fish (16). However, it is not clear whether this mechanism is applicable to Hg(II) and $CH_3Hg(II)$ uptake in bacteria. Demonstration of uptake in the

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TABLE 1 Primers and sequences used in the study

Primer set	Sequence[a]
merbEXF1N	AGAGggatccAGATGAAGCTCGCCCCATATATTTTAG
merbEXR1	GAGAtctagaTCACGGTGTCCTAGATGACATG
merBF1	ACTTCTCACTTCGGTCAATCG
merBR1	CGCACCAGGCATACAGAC

[a] The lowercase letters are the inserted sequences containing restriction enzyme sites (ggatcc, BamHI; tctaga, XbaI); the GAGA sequence protects the restriction enzyme sites.

presence of such molecules would indicate that uptake involves a facilitated or passive mechanism.

In a previous study, Selinofova et al. (28) designed a plasmid bioreporter (pRB28) to detect Hg(II), in which *merR* and the *mer* promoter/operator regions were fused with the promoterless *luciferase* (*lux*) plasmid. In order to effectively determine organomercurial compounds using a *mer-lux* plasmid bioreporter, it is necessary to convert organomercurials to Hg^{2+}. Endo et al. (8) incorporated the gene *merB* into a similar design of a *mer-lux* plasmid bioreporter (pHYB3Lux) and showed that the detection limit of this new bioreporter for phenylmercury acetate ranged from 50 nM to 5 μM. MerB catalyzes the breakdown of phenylmercury and $CH_3Hg(II)$ to Hg^{2+} (2, 26), which binds to MerR, thereby switching on the *lux* system. In this study, we attempted to develop a similar biosensor/bioreporter that can quantify the amount of $CH_3Hg(II)$ that has entered a cell from the medium and to use the biosensor to investigate the impacts of chloride and different forms of NDOM, such as thiols and humic acids, on the bioavailability of $CH_3Hg(II)$ in bacterial cells. The bioreporter plasmid pRB28 was modified by incorporating *merB* into pRB28 under the control of the *mer* promoter/operator region to facilitate effective measurement of $CH_3Hg(II)$ in the cell.

MATERIALS AND METHODS

Construction of the bioreporter (pDES1). To prepare the plasmid vector pRB28 for cloning, *Escherichia coli* cells harboring the vector were grown overnight (O/N) on a Luria-Bertani (LB) agar plate. All LB plates and broth were made according to the method of Miller (19), and the antibiotic used for selection was ampicillin (100 μg ml^{-1}). Growth of cells in plates and broth was performed at 37°C O/N. A single colony was selected and used to inoculate a 50-ml sterile centrifuge tube with 15 ml LB medium at 37°C with shaking at 270 rpm. Plasmid isolation was performed with a Midiprep kit (Invitrogen). The plasmid (pRB28) was then digested with the restriction enzymes BamHI and XbaI, and the terminal phosphate groups of the digested DNA were removed with shrimp alkaline phosphatase (SAP). Enzyme digestion was followed by incubation for 15 min at 65°C. The digested plasmid was purified with Zymo DNA Clean and Concentrator (Zymo Research) and stored at 4°C.

The full-length sequence of organomercurial lyase (*merB*) was PCR amplified from the *E. coli* plasmid pQZB1 (23). PCR was performed using the reported method (36) with the primer set merbEXF1N-merbEXR1 (Table 1), where the lowercase letters are the inserted sequences containing restriction enzyme sites (BamHI and XbaI) and the GAGA sequence protects the restriction enzyme sites. The PCR fragment was cloned, and the resultant colonies were randomly picked and sequenced as described by Zhang et al. (36). A clone (pBL1) with the correct *merB* sequence was double digested with BamHI and XbaI, and the inserted DNA was gel purified as described by Zhang and Lin (37). Using a 3:1 insert/vector molar ratio, the insert was ligated to the digested and SAP-treated pRB28 vector mentioned above using DNA ligase (Invitrogen). Ligation was carried out at 15°C for 16 h, and the solution was then transformed into Stratagene's XL-gold cells {Tet^r Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173* *endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F′ *proAB lacI*^{q}*Z*ΔM15 Tn*10* (Tet^r) Amy Cam^r]} using the manufacturer's protocol. Several resultant colonies were picked and digested with BamHI-XbaI to check if the plasmid contained *merB*; the plasmid DNA was then sequenced using primers merBF1 and merBR1 to ensure the accuracy of the induced *merB*. A recombinant plasmid containing the correct *merB* sequence was named pDES1 (Fig. 1), and the *E. coli* strain harboring this plasmid was designated NMZA1.

Materials. The monomethylmercury chloride (Sigma-Aldrich) used was 99.9% pure. Stock solutions (100 μM) were prepared by dissolving monomethylmercury chloride crystals in deionized water (Millipore), acidified with concentrated HCl to pH 3, and were stored in a 4°C refrigerator. On the day of assay, a fresh dilution was made from the stock solution to obtain a secondary standard of 1.4 μM. Pahokee Peat humic acid and Elliot Soil humic acid were obtained from the International Humic Substances Society (IHSS) in solid form, while a humic acid of unknown origin was obtained from Alfa Aesar. All humic acid solutions were prepared by dissolving weighed amounts in water with some addition of KOH and filtering the solutions with 0.45-μm filters. The medium of choice for all assays was M63 minimal medium (19). M63 consists of KH_2PO_4 (100 mM), $(NH_4)_2SO_4$ (15 mM), $FeSO_4 \cdot 7H_2O$ (1.7 μM), $MgSO_4$ (1 mM), and glycerol (0.2%). No vitamins or yeast was added in order to simplify the speciation of the medium. The pH of the medium was 7. A Packard Tri-Carb 3100 TR Liquid Scintillation Analyzer was used to measure bioluminescence. The machine was set to the single-photon mode. The temperature for the single-photon determination was room temperature, and the average time of measurement was 7 h.

***mer-lux* assays.** A single colony of the *E. coli* strain NMZA1 was grown in LB broth O/N with ampicillin (100 μg ml^{-1}) with shaking at 270 rpm. The LB cultures were centrifuged and resuspended in M63 minimal medium 3 times to ensure that all the LB solution was removed. These cells in M63 medium were diluted to an optical density at 660 nm of 0.6 with M63 and incubated at 37°C for 3 days with daily addition of fresh M63 medium (28). After the 3-day incubation, the cell cultures were further diluted with M63 medium to an optical density at 660 nm of 0.1. To obtain a calibration curve for the sensor, on the day of assay, different concentrations of

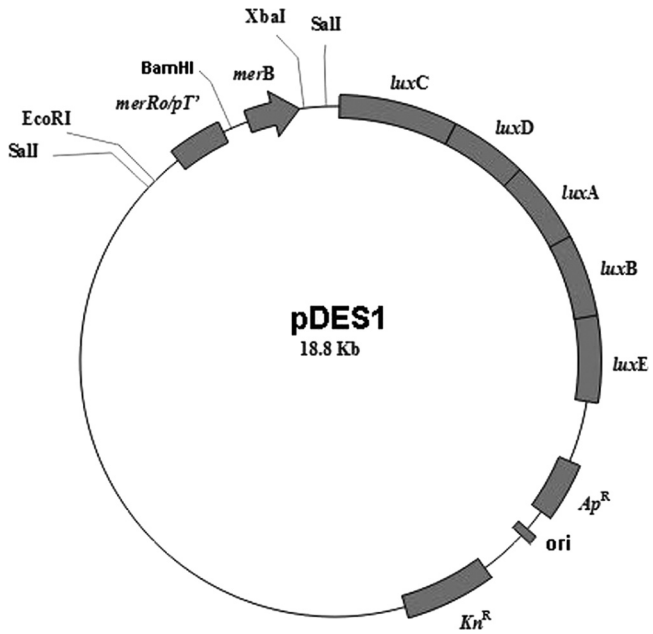

FIG 1 Diagram of the modification of pRB28 by inserting the *merB* gene into the BamHI-XbaI region under the control of the *mer* promoter (*merRo/pT′*, where *o/p* = operator).

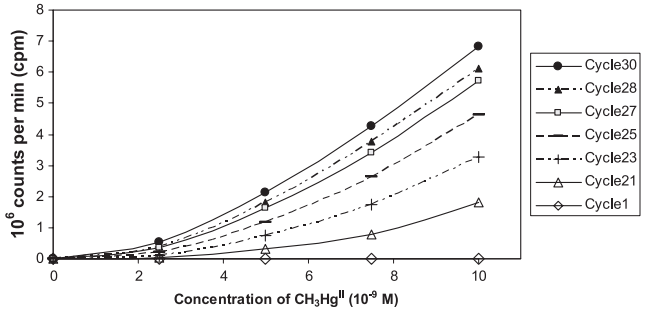

FIG 2 $CH_3Hg(II)$ bioavailability in M63 minimal medium when the $CH_3Hg(II)$ concentration is increased from 0 to 10 nM. The test organism was the *E. coli* strain NMZA1, while the bacterial optical density at 660 nm was 0.05.

$CH_3Hg(II)$ were added to 1.0 ml of M63 medium in acid-washed 20-ml scintillation vials. The vials were shaken in the dark at 60 rpm for 2 h, and 1.0 ml of NMZA1 cells in M63 medium with an optical density of 0.1 was added to 1.0 ml of the solution of M63 containing $CH_3Hg(II)$ to obtain a final volume of 2.0 ml. The final optical density in the vial was about 0.05, which yields a cell count of about 7×10^6 cells ml^{-1}. Measurements of bioluminescence were then made. In order to examine the effect of chloride on the bioavailability of $CH_3Hg(II)$ in *E. coli* (NMZA1), a *mer-lux* assay was assembled as described above with the exception that the concentration of $CH_3Hg(II)$ was kept constant at 5 nM while the concentration of Cl^- was increased from 0.1 μM to 320 mM. Likewise, the effect of humic acids on $CH_3Hg(II)$ bioavailability was tested by keeping the concentration of $CH_3Hg(II)$ constant at 5 nM while the concentration of the humic acid was increased from 0 to 100 mg $liter^{-1}$. For assays involving humic acids, the vials were shaken for 24 h in the dark to ensure that equilibrium was attained with $CH_3Hg(II)$. A negative control was also set up with 100 mg $liter^{-1}$ of humic acid in M63 but with no $CH_3Hg(II)$ to see if the color of the medium interfered with the assay. The chloride concentration was at background level (0.1 μM). In like manner, individual assays for cysteine and glutathione were set up, with the exception that these assay mixtures were shaken gently for 2 h (60 rpm) in the dark before the addition of 1.0 ml of cells in M63 to yield a final optical density of 0.05. Experiments were done in quadruplicate, and the standard error was calculated. An *E. coli* control strain, HMS174 (3), which contains the plasmid pRB27 that constitutively expresses bioluminescence, was applied to verify that bioluminescence in our experiments resulted from induction by $CH_3Hg(II)$ only and not from other experimental variables.

RESULTS

Evaluation of the biosensor. Figure 2 shows the bioluminescence produced by bacteria that have plasmid pDES1 in response to different concentrations of $CH_3Hg(II)$. After adding a fixed quantity of cells to different aliquots of $CH_3Hg(II)$ in vials, repetitive cycles of measurements of bioluminescence were made. These measurement cycles were numbered 1 to 30 (not all are shown in Fig. 2). As there is a time lag in the production of bioluminescence due to the need for induction and protein production, the signal increases over time. Each cycle of measurement lasts approximately 10 to 12 min. The measurements of bioluminescence at concentrations from 2.5 to 10 nM $CH_3Hg(II)$ yielded a linear response. The minimum detection limit was a value between 0 and 2.5 nM $CH_3Hg(II)$ in the medium, as the sensor could not distinguish values in this range from the background luminescence.

Effects of Cl^- and NDOM on the bioavailability of $CH_3Hg(II)$. Figure 3A demonstrates the effect of Cl^- on the bioavailability of $CH_3Hg(II)$ in *E. coli*. As the concentration of Cl^- was increased, the bioavailability of $CH_3Hg(II)$ also increased, which is similar to

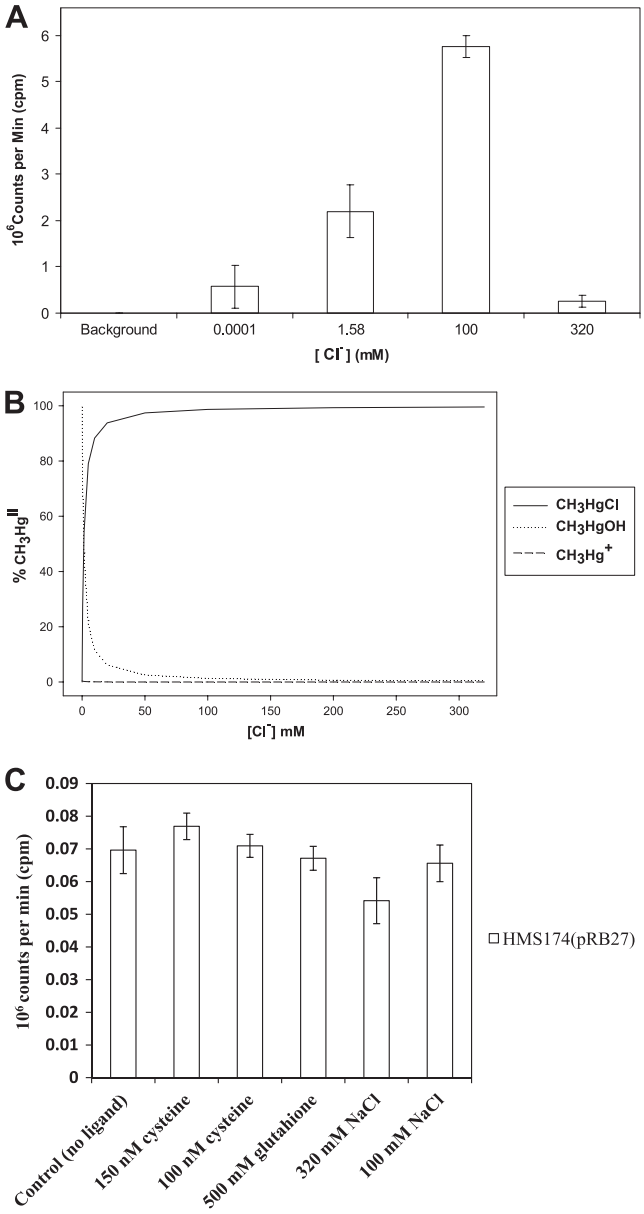

FIG 3 (A) Effect of chloride ion on $CH_3Hg(II)$ bioavailability. $CH_3Hg(II)$ was kept constant at 5 nM, while the concentration of Cl^- was increased from 0.0001 to 320 mM. The test organism was the *E. coli* strain NMZA1, while the bacterial optical density at 660 nm was 0.05. (B) Plot showing the theoretical effect of chloride ion on the speciation of $CH_3Hg(II)$ in M63. $CH_3Hg(II)$ was kept constant at 5 nM, while the concentration of Cl^- was increased from 0.0001 to 320 mM. (C) Application of the constitutive strain to various ligands used in the experiment. The test organism was the *E. coli* strain HMS174(pRB27), and the optical density at 660 nm of the medium was 0.05. The error bars indicate the standard error for replicate measurements.

what has been found by others for phytoplankton cultures (18). However, at a Cl^- concentration of 320 mM, there was a sharp decrease in bioavailability. As shown in Fig. 3B, there was no substantial change in speciation between 100 and 320 mM for $CH_3Hg(II)$, so speciation cannot explain the results observed. This decrease in bioavailability likely reflects a physiological response to the high Cl^- concentration, as discussed further below, given that *E. coli* does not grow well at high salinity. At 320 mM, it

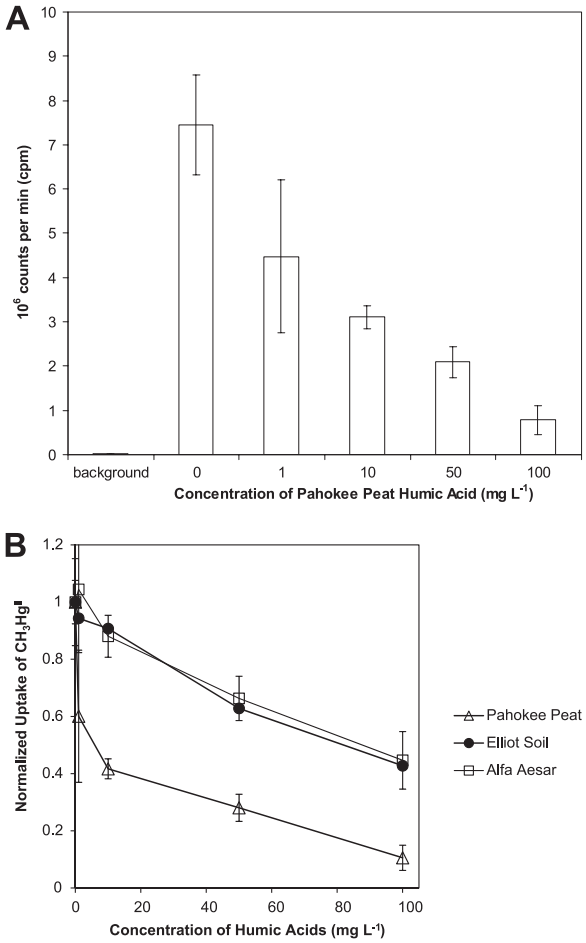

FIG 4 (A) Effect of Pahokee Peat humic acid on $CH_3Hg(II)$ bioavailability in the *E. coli* strain NMZA1. The concentration of $CH_3Hg(II)$ was kept constant at 5 nM, while the concentration of the humic acid was increased from 0 to 100 mg liter^{-1}. The optical density at 660 nm was 0.05. (B) Plot showing the impact of Pahokee Peat humic acid, Alfa Aesar humic acid, and Elliot Soil humic acid on the bioavailability of $CH_3Hg(II)$ in the *E. coli* strain NMZA1. The data shown were normalized so that the effects of different types of humic acids could be compared. The error bars indicate standard deviations.

was observed that the control strain had a reduction in bioluminescence (Fig. 3C), whereas other ligands, such as cysteine and glutathione, did not affect bioluminescence of the control strain at the concentrations tested. The principal advantage of using a minimal medium in the experiments is that the concentrations of the ions and molecules are known, and therefore, it is possible to estimate the actual speciation of Hg(II) and $CH_3Hg(II)$ in the medium, as shown in Fig. 3B.

In contrast to increased bioavailability in the presence of Cl^-, addition of humic acids to the mixture led to an overall decrease in the bioavailability of $CH_3Hg(II)$, with increasing concentrations enhancing the effect (Fig. 4A and B). Figure 4A shows the effect of Pahokee Peat humic acid on the bioavailability of $CH_3Hg(II)$ in *E. coli*. The concentration of $CH_3Hg(II)$ was kept constant at 5 nM, while the concentration of humic acid ranged from 0 to 100 mg liter^{-1}. The negative control, which consisted of a solution with 100 mg liter^{-1} of humic acid but no $CH_3Hg(II)$, indicated that the background color of the humic acid did not impact the results of the assay, i.e., the instrument could distinguish between bioluminescence and background color. In order to compare the effects of the various organic acid solutions (Pahokee Peat humic acid, Elliot Soil humic acid, and Alfa Aesar humic acid) on the bioavailability of $CH_3Hg(II)$, it is useful to normalize the data to the response found in the absence of ligands. Therefore, the data in Fig. 4A were normalized by dividing the data points by the data point corresponding to 0 mg liter^{-1} of Pahokee Peat humic acid and are displayed in Fig. 4B. In like manner, the normalized bioavailability data of assays for other forms of NDOM, amino acids and glutathione, were generated (Fig. 4B, 5, and 6).

Effects of cysteine, methionine, leucine, and glutathione. In contrast to the comparable results obtained for the various humic acid solutions, the bioavailability of $CH_3Hg(II)$ in the presence of amino acids or glutathione was mixed (Fig. 5 and 6). Figure 5 shows the normalized effects of cysteine on the bioavailability of Hg(II) and $CH_3Hg(II)$ to *E. coli* strain NMZA1 grown in M63 minimal medium. Increasing the concentration of cysteine at a constant concentration of Hg(II) enhanced the Hg(II) bioavailability rate by up to a factor of about 500. However, the relative increase in bioavailability was lower for $CH_3Hg(II)$ than for

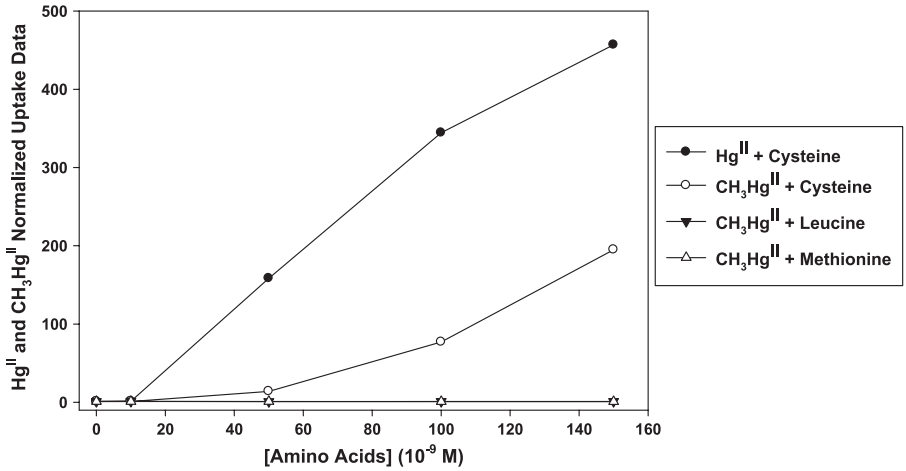

FIG 5 Effects of cysteine, leucine, and methionine on Hg(II) and $CH_3Hg(II)$ bioavailability in M63 medium. The concentrations of Hg(II) and $CH_3Hg(II)$ were kept constant at 5 nM, while the concentrations of cysteine, leucine, and methionine were increased from 0 to 150 nM. The test organism was the *E. coli* strain NMZA1. The optical density at 660 nm was 0.05.

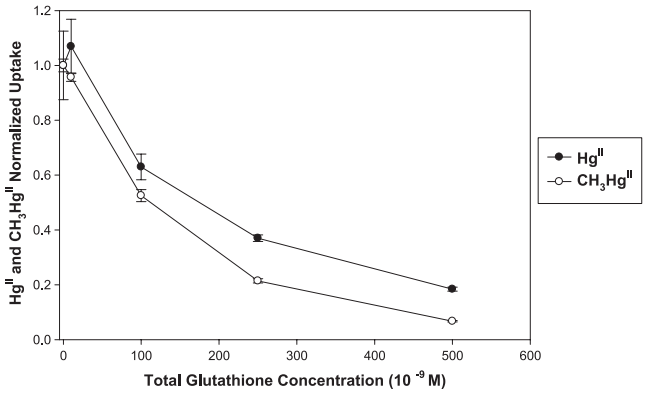

FIG 6 Effect of glutathione on Hg(II) and $CH_3Hg(II)$ bioavailability in M63 medium. The concentrations of Hg(II) and $CH_3Hg(II)$ were kept constant at 5 nM as the concentration of glutathione was increased from 0 to 500 nM. The test organism was the *E. coli* strain NMZA1, and the optical density at 660 nm of the medium was 0.05.

Hg(II) by a factor of 2 to 3. Additionally, higher concentrations of cysteine were needed to cause the initial increase in bioavailability, which is consistent with stronger binding of Hg^{2+} to cysteine compared to $CH_3Hg(II)$ (Table 2), especially for the bidentate Hg(II) complex, which dominates the speciation above 10 nM cysteine. Figure 5 also shows the normalized data on the effects of leucine and methionine on $CH_3Hg(II)$ bioavailability. Increasing the cysteine concentration resulted in enhanced bioavailability of $CH_3Hg(II)$ in the M63 medium, while equivalent concentrations of leucine and methionine did not result in increased bioavailability of $CH_3Hg(II)$. The binding constants for these two amino acids with Hg(II) and/or $CH_3Hg(II)$ are much lower, as they contain no thiol groups, and it is predicted that they have no impact on the speciation of Hg(II) or $CH_3Hg(II)$ at the concentrations added.

Figure 6 shows the normalized Hg(II) and $CH_3Hg(II)$ bioavailability data for *E. coli* strain NMZA1 with increasing concentrations of glutathione in M63 minimal medium. As before, the concentrations of Hg(II) and $CH_3Hg(II)$ were constant. In contrast to cysteine, increasing concentrations of glutathione resulted in a decrease in the bioavailability of Hg(II) and $CH_3Hg(II)$. At the highest concentration of 500 nM glutathione, the decrease in Hg(II) and $CH_3Hg(II)$ bioavailabilities were about a factor of 5 and 10, respectively. At higher concentrations of glutathione, it was likely that these complexes dominated speciation (Fig. 6), and therefore, the decrease in bioavailability suggests that there was no passive or facilitated uptake of Hg bound to glutathione.

DISCUSSION

Evaluation of the biosensor. In Fig. 2, cycle 1 is the first set of measurements, and at this stage, the Lux proteins have not been made by the bacteria. Hence, there is no difference in the amounts of luminescence generated enzymatically by *E. coli* exposed to different concentrations of $CH_3Hg(II)$. By the 20th cycle, there is a measureable difference in the amounts of luminescence given off by bacteria exposed to larger amounts of $CH_3Hg(II)$ (5 nM, 7.5 nM, and 10 nM). These results are comparable to those found upon exposure of the bioreporter pRB28 to inorganic Hg (28). The biosensor NMZA1 contains the plasmid pDES1 and was able to detect and quantify $CH_3Hg(II)$ bioavailability when *E. coli* was exposed to 2.5 to 10 nM $CH_3Hg(II)$. The results shown in Fig. 2 demonstrate that there is an easily measured response to $CH_3Hg(II)$ and that the response appears to depend on the exposure concentration. Given that M63 minimal medium is buffered at pH 7, essentially all the $CH_3Hg(II)$ is present as CH_3HgOH; the $[CH_3HgOH]/[CH_3Hg^+]$ ratio is 316 according to the data in Table 2. It is thought that CH_3HgOH, an uncharged complex, is taken up passively by *E. coli*, as has been concluded for $Hg(OH)_2$ (18, 28) and for CH_3HgOH uptake by phytoplankton (18). Increased amounts of the neutral complex CH_3HgOH resulted in increased bioavailability. This is consistent with the mechanism of passive diffusion. The plasmid pDES1 is a slight improvement on the design of Endo et al. (8), who reported a detection limit of 50 nM for phenylmercury acetate, an organomercurial compound. A 1- to 2-order-of-magnitude improvement in the detection limit allows the examination of bioavailability at concentrations closer to those found in the environment.

Effect of Cl^-. In the Cl^- assay, the total number of $CH_3Hg(II)$ species, as shown in equation 1, was 5 nM:

$$5\ \text{nM} = [CH_3Hg^+] + [CH_3HgOH] + [CH_3HgCl] \quad (1)$$

Given that the medium was buffered at pH 7; the binding constants (log *K*) of CH_3Hg^+ with OH^- and Cl^- are 9.37 and 5.25, respectively (24); and the Cl^- concentration was increased from 0.1 μM to 320 mM, the model of the speciation of $CH_3Hg(II)$ in equilibrium can be written as equation 2:

$$5\ \text{nM} = [CH_3Hg^+]\{1 + (10^{9.37} \times\ 10^{-7}) + (10^{5.25} \times [Cl^-])\} \quad (2)$$

The percentage speciation of $CH_3Hg(II)$ in equation 2 is plotted in Fig. 3B. The figure shows that the dominant $CH_3Hg(II)$ complex at Cl^- concentrations below 1.58 mM was CH_3HgOH, while CH_3HgCl was dominant at higher Cl^- concentrations. The increased bioavailability of $CH_3Hg(II)$ observed at concentrations of Cl^- from 0.1 to 100 mM (Fig. 3A) is likely because of increased formation of the neutral chloride complex (CH_3HgCl^0). Although both CH_3HgOH and CH_3HgCl are neutrally charged complexes, they enter the cell by the mechanism of passive diffusion at different rates determined by their octanol-water partition coefficients (K_{ow}) (18). CH_3HgCl and CH_3HgOH have K_{ow} values of 1.7 and 0.07, respectively. The relationship between K_{ow} and membrane permeability is given by the Stein equation (30), which is displayed as equation 3:

$$p^* = \frac{K_{ow} D_{mem}}{I} \quad (3)$$

where P^* is the size-adjusted membrane permeability (cm s^{-1}),

TABLE 2 Binding constants used in the speciation modeling of Hg(II) and $CH_3Hg(II)$

Complex	Formation constant (log *K*)	Reference
$RSH + H_2O \leftrightarrows RS^- + H^+$	−10.77	20
$Hg^{2+} + 2RS^- \leftrightarrows Hg(SR)_2$	41.6	7
$Hg^{2+} + RS^- \leftrightarrows HgSR^+$	22.1	7
$CH_3Hg^+ + RS^- \leftrightarrows CH_3HgSR$	16.3	24
$CH_3Hg^+ + OH^- \leftrightarrows CH_3HgOH$	9.37	24
$CH_3Hg^+ + Cl^- \leftrightarrows CH_3HgCl$	5.25	24
$Mg^{2+} + RS^- \leftrightarrows MgSR^+$	4	5
$Fe^{2+} + RS^- \leftrightarrows FeSR^+$	6.2	5
$Fe^{2+} + 2RS^- \leftrightarrows Fe(SR)^{2-}$	11.7	5

D_{mem} is the molecular diffusion coefficient in the lipid bilayer ($cm^2\ s^{-1}$), and I is the membrane thickness (cm). It has been demonstrated that CH_3HgCl has a higher membrane permeability than CH_3HgOH, and as there is a strong correlation between the log P^* and log K_{ow} of the neutral species, this confirms the Stein equation (18). Therefore, as CH_3HgCl has a much higher K_{ow} value, conditions that favor its increased formation will most likely result in higher levels of bioavailability, as shown in Fig. 3A. Thus, the results in the presence of Cl^- fit with the expectation that uptake of either of the neutral complexes is one pathway for CH_3Hg(II) accumulation. The sharp reduction of bioavailability when the concentration of Cl^- was 320 mM was probably due to the low salt tolerance of *E. coli* (10, 11). Although the reduction of bioavailability at 320 mM could be attributed to a change in speciation, there is no evidence in the literature that no higher Cl^- complexes, such as $CH_3HgCl_2^-$, are formed, as CH_3Hg^+ forms only a 1:1 association with both inorganic and organic ligands (31).

Effect of NDOM on the bioavailability of CH_3Hg(II). Humic acid solutions are an ill-defined mixture of dissolved organic compounds varying in molecular weight with several functional groups but few that can bind CH_3Hg^+ effectively compared to inorganic ligands, such as Cl^-. The strength of binding is likely a function of the relative thiol content of the NDOM. Humic acids have high binding constants with Hg(II) (25) and CH_3Hg(II) (14, 15), but it has been observed that humic acids bind significantly less to CH_3Hg^+ than they do to Hg^{2+} (27). The reduced binding of CH_3Hg^+ to humic acids and other forms of NDOM is a result of the Hg in CH_3Hg^+ being weakened in acidity by the methyl group (24, 35). Humic acids are bulky and tend to have several charged chemical groups, such as the carboxylic acid groups. They also have high and varying molecular weights. Thus, when these substances bind to CH_3Hg^+, it is unlikely that the complexes formed will penetrate the plasma membrane. Hence, as the concentration of humic acid increases, the concentration of neutral species will decrease and the speciation of CH_3Hg(II) will be dominated by the CH_3Hg-R complex, where R represents the humic acid. Peat humic acid (100 mg $liter^{-1}$) reduced the bioavailability of CH_3Hg(II) by 80%, while the same concentration of soil humic acid reduced bioavailability by 50%, with the two different solutions having similar impacts. This difference likely resulted from the differences in the amounts of the Hg binding groups, mostly likely thiols, which are present in both NDOM mixtures. Peat is a much more degraded material and forms in the absence of oxygen, and therefore, it is entirely reasonable that the material would have a higher reduced sulfur content than the soil humic acid.

Effects of cysteine, glutathione, methionine, and leucine on the bioavailability of CH_3Hg(II). Hg-thiol complexes are bulky and polar and have a tendency to be charged, depending on the pH of the solution, as the thiol groups and carboxylic acid groups have different pK_as. Thus, uptake of these complexes is not likely to occur rapidly by passive diffusion due to repulsion by the phospholipid cell membrane, and facilitated uptake is likely to dominate, especially for compounds that may be biochemically useful. For example, cysteine and its conjugates have been shown to be imported into *E. coli* cells with the aid of transport proteins (4). Accordingly, we propose that the cysteine-Hg-cysteine and CH_3Hg-cysteine complexes, being simple conjugates of cysteine, are taken up with the assistance of cysteine transport proteins, and such transport is the most likely mechanism of uptake of these complexes in environmental solutions where concentrations of thiols are sufficient to control complexation. This facilitated transport mechanism explains the observed trend of increasing bioavailability of Hg(II) and CH_3Hg(II) when cysteine concentrations are increased. The absence of increased bioavailability in the assays of leucine and methionine suggests that CH_3Hg^+ binding with these amino acids at pH 7 is minimal, as the amino acids lack thiol groups. This is not surprising but is consistent with the fact that the log K of the binding of CH_3Hg^+ with cysteine, which is 16 (5), is large relative to the other amino acids, e.g., it is several orders of magnitude higher than that of methionine, which is 7.4 (5).

Berger and Heppel (4) identified two kinetically distinct systems for cystine (the oxidized form of cysteine) uptake, which they called the cystine general transport system ($K_m = 3 \times 10^{-7}$ M) and cystine specific transport system ($K_m = 2 \times 10^{-8}$ M). The general system was reported to also assist in the uptake of diaminopimelic acid and was inhibited by several analogues of cystine, whereas the specific system was inhibited by only a few compounds. Of the 2 transport systems, the cystine specific transport system was the one that functioned in minimal medium while the cystine general transport system was inactivated in minimal medium. The gene that encodes the cystine binding protein for the general system was identified as *fliY* (21). However, the genes responsible for the specific transport system have not been identified to date. We suspect that this cystine specific transport system is the means of uptake because it functions well in minimal medium and therefore is likely functional under the conditions of our experiments.

Finally, it was observed that in the M63 medium, increasing the concentration of glutathione did not result in increased bioavailability of Hg(II) and CH_3Hg(II) in the *E. coli* strain NMZA1 (Fig. 6). Instead, increasing concentrations of glutathione bound more of the Hg(II) and CH_3Hg(II) as the complex and resulted in decreased bioavailability of both mercury species. This reduction of bioavailability could be explainable if the increased size and charge of the complex hindered uptake by passive diffusion and this was the primary uptake mechanism. The lack of uptake suggests either that this pathway does not exist or that it was not activated under the experimental conditions. We suspected the latter reason and wondered if there was a lack of a counterion for cotransport, which is often necessary for facilitated uptake. One idea was that there was a lack of a cotransporter in the medium, but addition of sodium to the medium did not enhance the bioavailability of Hg(II) and CH_3Hg(II) (results not shown), so the idea of lack of a cotransporter does not appear to be valid. There are other reasons why the facilitated uptake was not activated in the medium used in this study, and we hope to further study these potential factors in our future work. However, as the minimal medium conditions used in these studies are relatively close to those of the environment, we suggest that the results observed here are likely environmentally relevant and that the presence of glutathione in the aquatic medium hinders rather than enhances the uptake of Hg(II) and CH_3Hg(II) into microbes.

Conclusion. A limitation of the bioreporter method in terms of *in situ* detection is the high limit of detection relative to environmental concentrations. The measured concentrations of CH_3Hg(II) in the water column of coastal waters, such as Long Island Sound, tend to range from <0.5 to 3 pM (9). However, we used a CH_3Hg(II) concentration of 5 nM in this study. This is

because the sensor used in this work cannot distinguish subnanomolar values of $CH_3Hg(II)$ (Fig. 2). However, the 5 nM concentration used in these studies is much lower than the concentrations of NDOM used in the experiments and the $CH_3Hg(II)$/NDOM ratios used are comparable to those in the environment, and therefore, the results are applicable to the natural environment, as there is excess binding capacity in the NDOM.

The speciation of $CH_3Hg(II)$ in the medium determines its bioavailability in *E. coli*. Binding of CH_3Hg^+ with cysteine and inorganic ions (OH^- and Cl^-) to form a CH_3Hg-cysteine complex and neutral complexes (CH_3HgOH and CH_3HgCl) resulted in increased bioavailability. Binding to NDOM and glutathione reduced the bioavailability rate. Thus, to estimate the bioavailable portion of the total dissolved $CH_3Hg(II)$, one must take into account the sum of all the neutral complexes, both inorganic and organic, and the concentration of low-molecular-weight thiol complexes, such as CH_3Hg-cysteine.

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