Evaluation of Mercury Toxicity as a Predictor of Mercury Bioavailability

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Many studies on bioavailability of toxic metals have made the assumption that observation of toxicity is evidence that the metal was taken into the cells (i.e., was "bioavailable"). A second assumption is that results at the high concentrations necessary for toxic effect are applicable to the lower concentrations more commonly found in the environment. These assumptions were specifically tested for mercury (Hg(II)) toxicity (at concentrations of 0.25−50 nM Hg) and uptake (at lower concentrations of 0.005−0.015 nM Hg) in the aquatic bacterium, *V. anguillarum*. Toxicity was measured as reduction in levels of constitutively expressed bioluminescence in *V. anguillarum* pRB27. Hg(II) uptake was measured using the Hg(II)-inducible *mer-lux* operon in *V. anguillarum* pRB28. In experiments where the predominant Hg species was changed from $HgCl_2$ to $Hg(OH)_2$ or $Hg(NH_3)_2^{2+}$, toxicity results accurately predicted that there would be no effect of the dominant species on Hg(II) uptake at lower HgT concentrations. However, toxicity tests with these same ligands failed to predict that there would be an effect on Hg(II) uptake when conditions were changed from aerobic to anaerobic. Toxicity tests also failed to predict the effect of 5 mM histidine additions on Hg(II) uptake, as histidine addition protected cells completely from Hg toxicity under both aerobic and anaerobic conditions, at concentrations up to 50 nM Hg, but did not prevent Hg(II) uptake. Uptake occurred at low HgT concentrations (0.01 nM) at the same rate when histidine was added under aerobic conditions and was substantially increased under anaerobic conditions. Thus, toxicity assays for Hg under a variety of conditions were not always a reliable predictor of the effects of those conditions on Hg(II) uptake into the cell.

Introduction

The earliest approach to the study of cellular uptake of toxic metals in water made use of toxicity bioassays. Toxicity assays were used to infer metal uptake because there were no methods available at that time to assay directly the movement of metals into cells. Using these toxicity assays, it was demonstrated that toxicity is often decreased by the addition of ligands that form complexes with the metal (*1*). The inference is that the total concentration of a toxic metal is not as important to living cells as is the portion that is available for uptake, i.e., "bioavailable" (e.g., refs *2*−*4*).

This approach has continued to be used, including the recent use of toxicity experiments to infer mercury uptake mechanisms in *E. coli* (*5*). In the case of mercury (Hg(II)), the issue of bioavailability has been especially relevant because two of the most important biogeochemical reactions of Hg(II) in the environment are Hg(II) methylation and Hg(II) reduction to volatile Hg^0 (reviewed in ref *6*). Both of these reactions are enzymatically catalyzed within the cytoplasm of the bacterial cell, and thus will depend on uptake of Hg(II) into the cell from the bioavailable pool.

The primary assumption of the use of toxicity to study Hg(II) bioavailability is that the Hg(II) must be taken into cells to exert its toxic effect and, conversely, if no toxic effect is observed then the Hg(II) must not have been available for uptake. A second assumption required for the application of results to most natural waters, is that Hg(II) uptake will behave in the same way at low nontoxic concentrations as it does at the higher concentrations where toxicity is exhibited. Reliance on these assumptions could be problematic if uptake and toxicity were not always linked (*7*). For example, there might be sites for Hg(II) toxicity outside the cell membrane, on the cell surface or in the periplasmic space (*8*, *9*). Also, there might be circumstances where unknown detoxification mechanisms obscured the fact that Hg(II) had been taken up. As for the second assumption, it seems possible that results obtained at high Hg concentrations might not apply to lower Hg(II) concentrations, if for example different uptake processes predominate at different concentrations.

Because of the recent development of a bioreporter method to measure Hg(II) uptake by bacteria at environmentally relevant concentrations (*10*, *11*), we are now able to study both Hg toxicity and trace level Hg(II) uptake in *Vibrio anguillarum*, an aquatic species of bacteria. We carried out toxicity testing at high HgT concentrations under a certain set of ligand conditions, and used these results to make predictions about how these same ligands should affect Hg(II) uptake at lower concentrations. Then we measured Hg(II) uptake at lower (nontoxic) Hg concentrations, but with the same ligands, to determine how well the predictions applied. This type of systematic intercomparison between Hg(II) toxicity and uptake has not been undertaken previously.

Methods and Materials

Definitions of Mercury Abbreviations. Total mercury is designated as HgT. Mercury in the +2 oxidation state is designated as Hg(II). This Hg(II) can be bound to or complexed with ligands, and the compound or complex can have a neutral charge (e.g., $HgCl_2$), a positive charge (e.g., $Hg(NH3)^{2+}$, or a negative charge (e.g., $HgCl_3^-$). Some Hg(II) can be present as the free ion, which is designated as Hg^{2+}.

Bacterial Strain and Plasmids. *Vibrio anguillarum* ATCC 14181 was chosen as a representative example of a free-living natural aquatic bacterium. Hg(II) uptake was quantified by light production from the *mer-lux* bioreporter strain *V. anguillarum* (pRB28) (*10*, *11*). The (pRB28) plasmid contains a fusion of the regulatory region, *mer*Ro/p, from the Tn*21* *mer* operon to a promoterless *lux*CDABE operon from *Vibrio*

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fischeri (*12*). Transcription of the *lux* genes in this strain only occurs in response to induction by Hg(II) within the cell cytoplasm. Hg toxicity was measured using *V. anguillarum* (pRB27) (*10*, *12*). This strain contains a derivative of the (pRB28) plasmid that constitutively expresses the *lux* genes, and the effect of Hg on constitutive light production was used as the indicator of Hg toxicity. An advantage of the use of bioluminescence in determining toxicity (*14*) is that bioluminescence requires RNA and protein synthesis, ATP, $FMNH_2$, and NADPH (*15*). These are common processes or intermediates in many basic cell functions, and therefore, a toxic effect on any one of them alters a cell's ability to produce light.

In both strains, the genes encoding Hg(II) transport in mercury resistant bacteria, *mer*TPC, were deleted from the construct so that Hg(II) bioavailability could be studied as it occurs in the majority of bacteria, which do not contain the *mer* operon, and hence, do not contain these Hg(II) specific transport systems.

Reagents and HgT Analysis. Culture flasks, Teflon centrifuge tubes, and reagent bottles were washed in 30% H_2S0_4 and rinsed thoroughly with low-Hg (<1.5 pM) Milli-Q water. Reagents were periodically analyzed for total Hg by Flett Research Ltd. using a cold vapor atomic fluorescence spectrometer (CVAFS) (Brooks Rand, Ltd. model 2) and EPA Method 1631, as adapted from Bloom and Creselius (*16*). There were trace amounts of HgT in the reagents used. Collectively, these contributed 0.0015−0.065 nM HgT in final assay vials, depending on the mixture of reagents, and especially the particular batch of phosphate buffer, which was the most variable in Hg content. However, this Hg was not bioavailable to the bioreporter, as measured by blank controls. The range of HgT concentrations in assays were obtained by serial dilution of a primary Hg standard (1 μg/mL Hg, containing 1% BrCl, provided by Flett Research Ltd.), followed by the addition of the appropriate volumes to each assay vial. These dilutions were done just prior to each set of assays.

Preparation of Anaerobic Reagents. To maintain the chemical composition and Hg(II) speciation of the anaerobic assays the same as that in the aerobic assays, no reducing agents were added to lower the redox potential. Rather, the Hungate method (equilibration with oxygen-free nitrogen gas, *17*) was used to eliminate O_2 for the reagents used in the anaerobic assays. A ChemMets O_2 Kit was used to ensure the media were O_2 free (O_2 < 1 ppb).

Growth of Cultures for Toxicity and Bioreporter Assays. For maintenance of the plasmids kanamycin (Kan) was added to a final concentration of 60 μg/mL in all the growth media. Liquid cultures were started by inoculating 5 mL of aerobic Luria−Bertani (LB) broth with a single isolated colony of either *V. anguillarum* pRB28 (uptake assays) or *V. anguillarum* pRB27 (toxicity assays) from a LB-Kan agar plate and incubated at 28 °C with shaking until late-log phase.

In preparation for anaerobic assays, the culture was brought into an anaerobic Coy glove bag, maintained under a 93% N_2:7% H_2 atmosphere. 100 μL was transferred to a serum vial containing 5.9 mL of anaerobic glucose minimal medium (GMM), described in Golding et al. (*11*). The vials were sealed with a rubber butyl stopper and crimped with an aluminum cap, removed from the glove bag and reincubated at 28 °C with shaking at 150 rpm until late/mid-log phase. The culture was brought back into the anaerobic glove bag and 20 mL of anaerobic GMM-Kan broth were added. The cultures were re-incubated at 28 °C with shaking at 150 rpm until mid-log phase.

The initial inoculum and growth of cultures for the aerobic assays was as described for the anaerobic assays with the exception that a glove bag was not used, and cultures were fully aerated throughout.

TABLE 1. Calculated Speciation of Hg(II) in the Various Defined Assay Media[a] Used in the Toxicity and *mer-lux* Bioreporter Assays Using *V. Anguillarum* as the Bacterial Host[b]

Hg(II) species	assay medium (%)	assay medium B (%)	assay medium C (%)	assay medium D (%)
$Hg(OH)_2$	1.7	48	4.4	4.3×10^{-7}
$HgOH^{1+}$	1.3×10^{-4}	3.4×10^{-3}	3.1×10^{-4}	3.3×10^{-11}
$HgNH_3^{1+}$	2.9×10^{-3}	6.9×10^{-3}	6.5×10^{-4}	6.3×10^{-10}
$Hg(NH_3)_2^{2+}$	97	24	2.3	$2.5 \times$ 10-5
$Hg(NH_3)_3^{2+}$	.08	$2.2 \times$ 10-3	$2 \times$ 10-4	$2.1 \times$ 10-8
$Hg(NH_3)_4^{2+}$	3.4×10^{-5}	9.8×10^{-8}	9×10^{-9}	8.8×10^{-12}
$HgPO_4^{1-}$	0.8	26	2.1	2.1×10^{-7}
$HgHPO_4$	.04	1.5	.12	1.1×10^{-8}
HgOHCl	2.8×10^{-3}	.08	26	7.2×10^{-10}
$HgCl_4^{2-}$	6.7×10^{-17}	1.9×10^{-15}	.02	1.7×10^{-23}
$HgCl_3^{1-}$	2.3×10^{-11}	7.5×10^{-10}	2.7	6×10^{-18}
$HgCl_2$	1.9×10^{-6}	6×10^{-5}	62	4.8×10^{-13}
$HgCl^{1+}$	4.2×10^{-7}	1.2×10^{-5}	3.7×10^{-3}	1.1×10^{-13}
Hg^{2+}	5.5×10^{-8}	1.22×10^{-6}	1.16×10^{-7}	1.42×10^{-14}
HgHis[c]	0	0	0	> 99

[a] All media contained 5 mM glucose and a total inorganic phosphate concentration of 6.7 mM, which consisted of NaH_2PO_4 and K_2HPO_4 (pH=7); Assay medium A was supplemented with 9 mM $(NH_4)_2SO_4$; Assay medium B was supplemented with 0.9 mM $(NH_4)_2SO_4$; Assay medium C was supplemented with 0.9 mM $(NH_4)_2SO_4$ and 3.4 mM NaCl; Assay medium D was supplemented with 9 mM $(NH_4)_2SO_4$ and 1 mM histidine. [b] No reducing agents were added; anaerobic conditions were obtained by removing O_2 (see Methods and Materials). Therefore, speciation calculations were considered the same for both aerobic and anaerobic conditions using these media [c] Total of all Hg(II)-histidine complexes including HgHis, HgHHis, $Hg(His)_2$, and $Hg(HHis)_2$.

Preparation of Cell Suspension for the Toxicity and Bioreporter Assays. In preparation for anaerobic assays, the final anaerobic 25 mL mid-log culture was decanted into 50 mL nominal capacity O-ring Teflon centrifuge tubes in the glove bag. The tubes were sealed, removed from the glove bag, and centrifuged at 12 000 g for 10 min at 4 °C. The supernatant was discarded in the glove bag and the pellet was resuspended in 20 mL of anaerobic 67 mM phosphate buffer (ratio of NaH_2PO_4 and K_2HPO_4 for a pH 7). Centrifugation and resuspension in anaerobic phosphate buffer was then repeated. The supernatant was discarded in the glove bag, and the pellet was resuspended in 3−5 mL of anaerobic phosphate buffer. The resuspended pellet was aliquoted into Balch−Wolfe tubes containing 5 mL of anaerobic phosphate buffer. The tubes were sealed using a rubber butyl stopper and aluminum crimp and removed from the glove bag. The cell suspension was diluted to an optical density (OD_{600}) of 0.4 using anaerobic phosphate buffer via syringe. The cultures were brought back into the glove bag and diluted another 100-fold in anaerobic phosphate buffer to approximately 2 $\times$ 10^6 cells/mL.

Cell preparation for the aerobic assays was the same as for the anaerobic method described above, with the exception that it was carried out on the bench top with air-equilibrated reagents and cultures.

Toxicity and Bioreporter Assays. For assays where the exposure of cells to Hg took place under anaerobic conditions, the reagents (prepared anaerobically) for the Assay Medium A, B, C, or D (Table 1) were mixed into scintillation vials in the glove bag, to a total volume of 19 mL. The primary mercury standard (1 μg HgT/mL) was transferred to a Teflon vial and brought into the glove bag. The standard was diluted twice in anaerobic Milli-Q water, in Teflon vials that had been left overnight in the anaerobic glove bag, and appropriate volumes of the final dilution were added to each vial. Assays were then initiated by adding 1 mL of the cell suspension (for a final cell concentration of about 1 $\times$ 10^5 cells/mL).

TABLE 2. The Mean Generation Times of *V. Anguillarum* under Aerobic and Anaerobic Conditions in the Various Defined Assay Media

assay medium[a]	aerobic (hours/generation)	anaerobic (hours/generation)
A	4.7	5.4
B	4.2	5.6
C	4.8	5.5
D	4.7	4.8

[a] See Table 1 for media composition.

Incubation was at room temperature in the glove bag. For light measurement, the vials were removed from the glove bag, quickly opened, and shaken three times to aerate the samples, and then they were immediately placed in the scintillation counter for counting (Beckman LS 6500, set in noncoincidence mode for 30s count intervals). Aerating the anaerobic samples for this short period would not be expected to have an effect on the levels of bioluminescent enzymes that developed during the anaerobic Hg exposure since it takes 20−70 min, depending on the HgT concentration (*10*) for the bacteria to transcribe and translate the proteins required for bioluminescence.

Aerobic *mer-lux* assays are carried out as described for the anaerobic method described above, except that the scintillation vials were prepared on the bench top with aerobic components. Also, as soon as all the cells and reagents, including Hg, were mixed together, the samples were immediately placed in the scintillation counter and continuously monitored over time. Thus, both the Hg exposure and the measurement of light production were carried out at the same time, rather than sequentially as in the anaerobic assays.

Growth Measurements in Defined Assay Media. To determine how much cell growth was occurring during the 80 min assay period, growth measurements were done separately in defined assay media under aerobic and anaerobic conditions. The cells were grown and prepared in the same way as for the toxicity and bioreporter assays. Balch−Wolfe tubes containing 10 mL of the assay medium were inoculated with the bacterium for an initial cell concentration of ~1×10^5 cells/mL. To mimic the bioreporter assay conditions, the tubes were maintained at room temp without shaking and the growth was monitored over time by measuring the OD_{600} nm in a Biochrom Novaspec II spectrophotometer.

In all four media types the first measurable OD_{600} nm over background was not obtainable until after ~38 h of growth under aerobic conditions and ~50 h under anaerobic conditions. Once in exponential phase, the mean generation times of *V. anguillarum* were similar among all the media types (Table 2), but differed slightly under anaerobic and aerobic conditions. By extrapolating backward from the measurable growth data it was estimated that the bacteria were in lag phase after transferal to the assay media for ~5 and 10 h under aerobic and anaerobic conditions respectively, and so cells would be in lag phase during the Hg exposure assays, which only lasted 80 min.

Hg(II)-Speciation Calculations. The thermodynamic speciation model program MINEQL+ version 4.5 was used to calculate the speciation of Hg(II) in the bulk solution. Constants for $Hg-PO_4$ and $Hg-HPO_4$ complexes were added from Martell and Smith (*18*). Calculations were based on the measured pH of the assay media and the calculated concentrations of the various ligands added to the sample vials. Since no reducing agents were used in the anaerobic growth media or assays, the initial redox potential and the Hg(II) speciation in assays should be the same under both aerobic and anaerobic conditions.

Chemical Speciation Under Assay Conditions. Hg(II) speciation calculations (Table 1) were done using the defined components of the assay media, and could not take into account any ligands that might have been excreted by cells during culture growth or during the assays. However, we limited the possible influence of these in two ways. First, the carry over of bacterial exudates that might have been present in the initial growth medium was limited by multiple washings and dilutions of the cells in fresh phosphate buffer prior to their use in the bioreporter and toxicity assays, as described above in the Cell Preparation section. Second, to minimize speciation changes that might occur during the assay itself, due to bacterial exudates or change in cell numbers, the nutrition content of the assay media was low, such that cells remained in lag phase for 5−10 h after transferral to these media (see the Growth Measurements section above). In this state, cells were very limited in growth, but could still sustain general metabolic functions. Since the assays were a total of only 80 min, and cells were not growing, the production of bacterial exudates should have been minimal.

Results

Toxicity and Uptake of Hg(II) in the Presence of Inorganic Ligands, with and without Oxygen. Hg toxicity was measured in three different media (Table 1), where speciation of Hg(II) was dominated by either $Hg(NH_3)_2^{2+}$(medium A), $Hg(OH)_2$ (medium B), or $HgCl_2$ (medium C). For all three assay media, there was little or no decrease in constitutive light production by *V. anguillarum* pRB27 cells for Hg concentrations up to and including 0.5 nM Hg, compared to the control containing no added Hg (Figure 1A and B). Also, for all three media, toxicity was observed at concentrations of ≥1 nM Hg, and the degree to which light production was decreased was the same (Figure 1A and B). Therefore, despite significant differences in the inorganic speciation of Hg(II), including Hg^{2+} (Table 1), toxicity was related solely to the total concentration of Hg. This was the case whether the cells were exposed to Hg under either aerobic or anaerobic conditions (Figure 1A and B).

Using the same three media (Table 1) as in the toxicity experiments, Hg(II) uptake was examined using the inducible *mer-lux* bioreporter strain, *V. anguillarum* pRB28, which only produces light when induced by Hg(II) inside the cell cytoplasm. Hg(II) uptake was observed at a much lower range of Hg concentrations (0.005−0.5 nM), compared to 0.25−4 nM used for toxicity assays. The minimum concentration for detectable Hg(II) uptake was greater when the HgT exposure took place under anaerobic conditions than under aerobic conditions. In the aerobic bioreporter assays (Figure 2A), HgT concentrations as low as 0.005 nM elicited a Hg(II)-dependent light response under aerobic conditions, whereas anaerobic conditions required Hg concentrations >0.025 nM (Figure 2B). Although the growth was slightly slower under anaerobic conditions (Table 2) the expression of bioluminescence did not appear to be limiting for the detection of Hg(II) uptake after anaerobic Hg exposure, since in the constitutive cells (*V. anguillarum* pRB27) light production was similar under anaerobic and aerobic conditions in samples containing subtoxic concentrations of Hg(II) (<1 nM, Figure 1A and B).

Under both aerobic and anaerobic conditions, Hg(II) uptake was not affected by which ligand was predominant, but rather was dependent on total Hg concentration (Figure 2A and B). This same lack of effect on Hg(II) uptake was seen previously under anaerobic conditions (*11*), and was similar to the toxicity results shown above (Figure 1A and B).

Toxicity and Uptake of Hg-Histidine Complexes, with and without Oxygen. Hg toxicity and uptake were compared in assay medium A (without histidine) and medium D (with histidine). Medium A was dominated by $Hg(NH_3)^{2+}$, and was

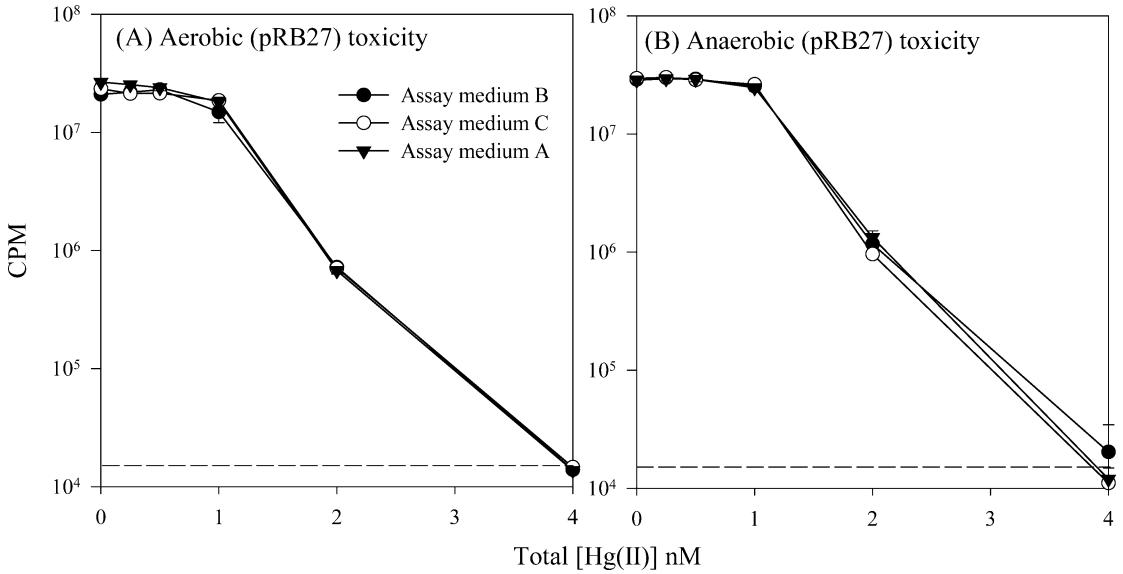

FIGURE 1. Comparison of light emission from *V. anguillarum* (pRB27), as an indicator of Hg(II) toxicity, in the presence of varying inorganic Hg(II) species. Concentrations of inorganic reagents in the assay media were manipulated so that the predominant Hg species was $Hg(NH_3)_2^{2+}$ (assay medium A), $Hg(OH)_2$ (assay medium B), or $HgCl_2$ (assay medium C) (see Table 1 for complete media composition and speciation calculations). Light production (CPM) was measured aerobically after 80 min of exposure to media containing no added Hg, or with Hg additions as indicated. The Hg exposure took place under aerobic conditions (panel A) or under anaerobic conditions (panel B). Dashed line represents background. Error bars represent the standard deviation of triplicate samples.

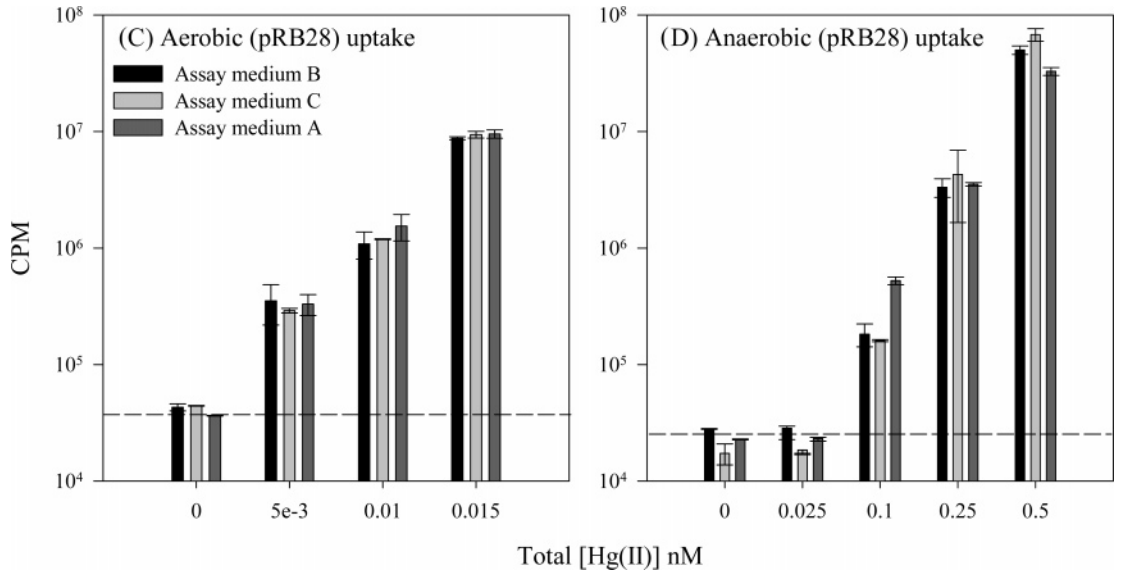

FIGURE 2. Comparison of light emission from and *V. anguillarum* (pRB28), as an indicator of Hg(II) uptake, in the presence of varying inorganic Hg(II) species. The three assay media (A, B, and C) were the same as in Figure 1 (see Table 1 for complete media composition and speciation calculations). Light production (CPM) was measured aerobically after 80 min of exposure to media containing no added Hg, or with Hg additions as indicated. The Hg exposure took place under aerobic conditions (panel A) or under anaerobic conditions (panel B). Dashed line represents background. Error bars represent the standard deviation of triplicate samples. Note the different *x* axes scales for Hg concentration, compared to Figure 1.

the same as the medium A used in the inorganic ligand experiments above. The composition of medium D was exactly the same as medium A, except that the addition of 1 mM histidine resulted in the formation of predominantly Hg(II)−histidine complexes (>99.9%) (Table 1). Under aerobic conditions, without histidine, toxicity (decreased light production) was seen in constitutive cells (*V. anguillarum* pRB27) at 5 nM Hg and above, compared to media that had no added Hg (Figure 3A). When histidine was added, however, toxicity was not observed (Figure 3A), as there was no decrease in constitutive light production even when HgT concentration was increased to 50 nM. The effect of histidine addition on toxicity was essentially the same under anaerobic conditions (Figure 3B).

Histidine itself did not affect bioluminescence, under either aerobic or anaerobic conditions. In assays where added Hg = 0, constitutive light production from *V. anguillarum* (pRB27) was the same whether histidine was included in the medium or not (Figure 3A and B).

The addition of histidine did not prevent Hg(II) uptake. In fact, Hg(II) uptake under aerobic conditions was similar whether histidine was added or not (Figure 4A). Under

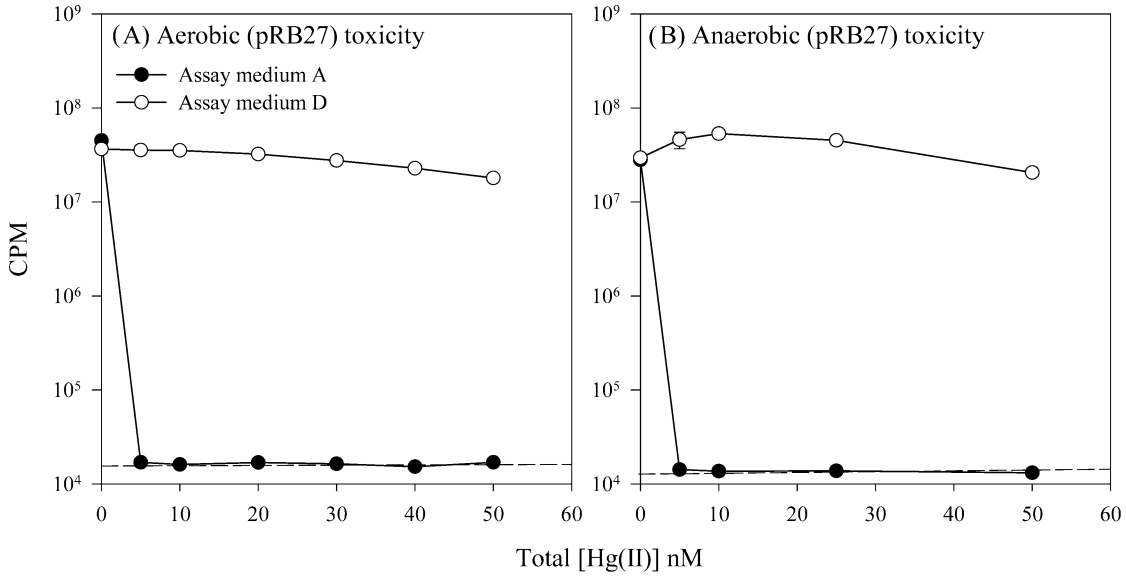

FIGURE 3. Comparison of light emission from *V. anguillarum* (pRB27), as an indicator of Hg(II) toxicity, in defined assay medium A without histidine and D with histidine (see Table 1 for complete media composition and speciation calculations). Light production (CPM) was measured aerobically after 80 min of exposure to media containing no added Hg, or with Hg additions as indicated. The Hg exposure took place under (A) aerobic conditions or under (B) anaerobic conditions. Dashed line represents background. Error bars represent the standard deviation of duplicate samples.

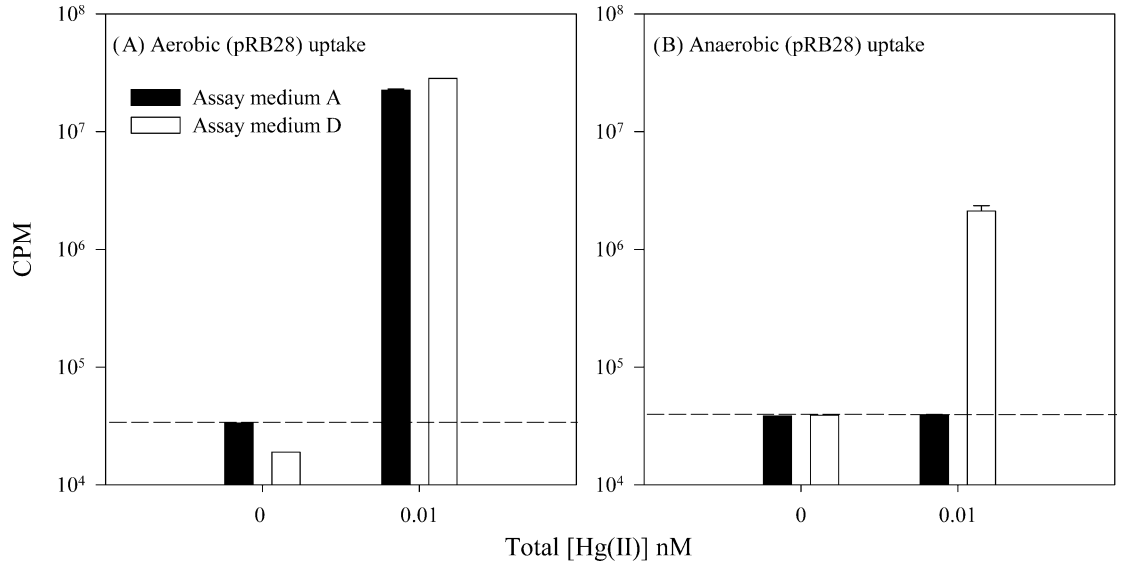

FIGURE 4. Comparison of light emission from *V. anguillarum* (pRB28), as an indicator of Hg(II) uptake, in defined assay medium A without histidine and D with histidine (see Table 1 for media composition and speciation calculations). Light production (CPM) was measured aerobically after 80 min of exposure to media containing no added Hg, or with Hg additions as indicated. The Hg exposure took place under (A) aerobic conditions or under (B) anaerobic conditions. Error bars represent the standard deviation of duplicate samples. Dashed line represents background. Note the different scale for Hg concentration on the *x* axis, compared to Figure 3.

anaerobic conditions, the addition of 1 mM histidine substantially increased Hg(II) uptake in *V. anguillarum* pRB28 (Figure 4B).

Discussion

The primary purpose of this work was to examine the usefulness of toxicity data for predicting conditions that promote or inhibit Hg(II) uptake. The conditions examined involved two types of ligands: (1) relatively weak inorganic ligands (Cl^-, OH^-, or NH_3), and 2) a strong organic ligand, histidine. Also, both aerobic and anaerobic conditions were compared.

The sensitivity of the bioluminescence-based methods used to measure both Hg toxicity and Hg(II) uptake meant that most assays were carried out at HgT concentrations of 4 nM or lower. The sensitivity of the toxicity method used is reflected in the low Hg concentrations at which toxicity was observed. Toxicity is often reported as the concentration of toxicant that causes a 50% inhibitory response (IC_{50}). The IC_{50} in this work was slightly over 1 nM Hg in the inorganic assay media (Figure 1A and B). The IC_{50} is greatly affected by the medium used (*19*). For instance, in simple defined media, previously reported IC_{50} of Hg for bacteria were 150 nM (*20*) and 500 nM (*21*) using bioluminescence as the measure of toxicity, as compared to 600 nM using cell viability (*5*). In complex media, and using methods other than bioluminescence, the reported IC_{50} was much higher (e.g., 7400−72 700 nM) (*19*). In this work the sensitivity of toxicity

detection was maximized by using bioluminescence, simple defined assay media (Table 1), low density of cells (1×10^5 cells/mL), and ultra clean chemical techniques that provided low background levels of Hg (*11*). The IC_{50} of about 1 nM is still much higher than the concentration of Hg in most natural waters (0.01−0.1 nM), making it unlikely that aquatic bacteria would experience Hg toxicity unless there were local Hg contamination sources that caused elevated Hg concentrations in the water. Also, naturally occurring, large dissolved organic carbon (DOC) ligands, which have been shown to mitigate uptake of Hg(II) (*13*, *22*), would likely reduce toxicity compared to the simple defined media used here.

The Hg(II) uptake method was also sensitive, using bioluminescence and a *mer-lux* bioreporter (*10*−*12*). In this bioreporter, light production only occurs in response to Hg(II) uptake into the cells, and subsequent binding to the sensor protein MerR. MerR is highly sensitive and specific for Hg(II) (*23*). The increase in light production is proportional to the concentration of Hg(II) that has entered the cell (*10*). Not surprisingly, the concentrations at which Hg(II) uptake in *V. anguillarum* were observed (Figure 2) were much lower (20−400 times) than those required to produce toxicity (Figure 1). This is consistent with behavior of many toxins, which commonly have some threshold Hg(II) concentration below which there is no observable toxic effect.

Experiments with Cl^-, OH^-, or NH_3 as the Predominant Hg(II) Ligand, under Aerobic and Anaerobic Conditions. On the assumption that toxicity assays at high HgT concentrations could be used as an indicator of Hg(II) uptake into the cell at low concentrations (e.g. refs *5*, *24*, *25*), the toxicity results in the inorganic media (Figure 1A and B) would predict that similar amounts of Hg(II) would be taken up under both aerobic and anaerobic conditions for a given Hg concentration. However, this prediction was not born out by our experiments because at least 10 times higher Hg concentrations were required for Hg(II) uptake under anaerobic conditions (Figure 2B), compared to aerobic conditions (Figure 2A). The requirement for higher concentrations of Hg(II) to support detectable Hg(II) uptake under anaerobic conditions in *V. anguillarum* is consistent with previous results using a *merR-lacZ* fusion and $^{203}Hg(II)$ volatilization as indicators of Hg(II) uptake in *Pseudomonas stutzeri* (*26*). This requirement could be due to differences in gene expression. For example, in *Escherichia coli* the expression of over 200 genes is affected by the presence or absence of O_2 (*27*), which also includes metal transport systems for Mn(II) (*28*), Cu(II) (*29*), and Ni(II) (reviewed in ref *30*). The accidental uptake of Hg(II) via such differentially expressed systems could explain why there was an apparent decrease in Hg(II) uptake under anaerobic conditions.

Since differences in Hg(II) uptake occurred under aerobic and anaerobic conditions (Figure 2A and B), why was there no corresponding difference in Hg toxicity (Figure 1A and B)? One possibility is that at the approximately 10× higher concentrations used for toxicity testing (Figure 1A and B), Hg(II) uptake may have been occurring at maximal and equal rates under both conditions. We were not able to study Hg(II) uptake rates at these higher concentrations because the *mer-lux* bioreporter reached maximum inducible light production at 0.015−to 0.025 nM Hg under aerobic conditions and ∼0.5 nM under anaerobic conditions at the cell density used in this study (data not shown). Alternatively, there could be sites of toxicity outside or on the cytoplasmic membrane that were unaffected by differences in oxygen presence or absence, or by differences in transport of Hg(II) into the cells.

The alteration of the predominant inorganic ligand to Cl^-, OH^-, or NH_3 was the only set of conditions in which toxicity results accurately predicted what would happen in the uptake experiments. Toxicity results (Figure 1A and B) predicted that changing the predominant ligand from Cl^- to OH^- to NH_3 would have no effect on Hg(II) uptake, and this was the case (Figure 2A and B). A similar lack of effect by a variety of small ligands on Hg(II) uptake at trace Hg concentrations has been observed before, in both *V. anguillarum and E. coli*. This was evidence for facilitated Hg(II) uptake in these bacteria (*10*), fitting characteristics of either kinetically controlled (*31*) or diffusion limited (*7*) facilitated uptake. However, the Hg toxicity results are different from those obtained in a previous study using *E. coli* and toxicity assays (*5*). In the work presented here, the results suggest that the same lack of effect on Hg(II) uptake of the three different inorganic ligands (Cl^-, OH^-, and NH_3) occurred at both lower Hg concentrations (0.005−0.5 nM HgT, Figure 2A and B) and higher concentrations used in the toxicity experiments (0.25−4 nM, Figure 1A and B). This was somewhat surprising, because at the higher Hg concentrations there was some expectation that passive uptake of $HgCl_2$ and $Hg(OH)_2$ might occur at a significant enough rate to observe greater toxicity in the solutions dominated by these two neutral species, compared to the solutions dominated by the positively charged $Hg(NH_3)^{2+}$. This expectation came from a study of Hg toxicity in *E. coli*, where THg concentrations were much higher (THg = 1250 nM, 3 orders of magnitude greater than used in the toxicity tests here), and where the patterns of toxicity were associated with apparent passive uptake of $HgCl_2$ that was occurring simultaneously with facilitated uptake of positively charged Hg species (*5*). Since rates of passive diffusion of $HgCl_2$ should be related to the external $HgCl_2$ concentration, this could explain why results consistent with the occurrence of passive diffusion were seen in Najera et al. (*5*), but not in this work. This also suggests that passive uptake of $HgCl_2$ by bacteria is unlikely to be important in natural waters where HgT concentrations are commonly 0.01 to 0.1 nM.

Presence or Absence of Histidine, a Strong Organic Ligand, under Aerobic and Anaerobic Conditions. Differences in Hg(II) toxicity and Hg(II) uptake results were quite obvious when examining the effect of adding histidine. Under both anaerobic and aerobic conditions the addition of 1 mM histidine greatly alleviated Hg(II) toxicity (Figure 3A and B). Therefore, on the basis of the toxicity data we would predict that histidine addition would decrease the uptake of Hg(II) into the cell. However, this was not the case, because Hg(II) was entering the cell at the same or substantially higher rate under aerobic and anaerobic conditions, respectively, than when histidine was not present (Figure 4A and B). One explanation for the observed results is that Hg(II) entered the cells in the form of its histidine complex and remained in this form after it was inside. Cotransport of Hg with amino acids has been shown in kidney cells (*32*, *33*) and is considered a means by which Hg could be "accidentally" taken into cells. This type of cotransport is also suggested by the fact that addition of histidine enhanced the rate of the Hg(II) uptake under anaerobic conditions (Figure 4B), and by previous work that showed similar stimulation with additions of a wide variety of other amino acids under anaerobic conditions (*11*). Once inside, the Hg(II) may have remained complexed to histidine, lessening the ability of the Hg(II) to exert its toxic effect, but the actual mechanism of protection is not known. Intracellular histidine has been found to mitigate the toxicity of Cu^{2+}, Co^{2+}, and Ni^{2+} in *Saccharomyces cerevisiae* (*34*) and Ni^{2+} in plants (*35*). At the same time, the *mer-lux* bioreporter genes would still be able to respond to the Hg(II) inside the cell, even in the presence of a strong ligand such as histidine, because of the even higher affinity of Hg(II) for the sensor protein MerR. This protein can bind Hg(II) even in the presence of mM concentrations of thiols (*23*).

Of particular note in evaluating the use of toxicity experiments for understanding metal uptake is the fact that if only the toxicity−histidine addition experiments (Figure 3) had been done, the results would point to the conclusion that the bioavailable form of Hg(II) was the free ion. With few exceptions (reviewed in refs *3*, *7*), metal toxicity has usually been correlated with the concentration of free metal ion, and this could also be said of the results of the Hg−histidine toxicity experiments presented here (Figure 3, Table 1). The Hg(II) uptake results give a more complex picture, however. Aerobic Hg(II) uptake was insensitive to histidine addition (Figure 4A), just as it was insensitive to the concentrations of the ligands Cl^-, OH^-, NH_3 (Figure 2), which could be explained by modifications of the free ion activity model (FIAM) (diffusion limitation (*7*) or kinetic control (*31*)). However, anaerobic Hg(II) uptake was enhanced by histidine addition (Figure 4B), which is not consistent with predictions of the FIAM (*7*). Another feature of Hg(II) uptake in these bacteria is that Hg(II) uptake increases with increasing H^+ (*22*). This is opposite to most other metals, where competition of H^+ with uptake of the positively charged free metal ion leads to a decrease in uptake (*3*). Added to these observations is the possibility that mercuric sulfide (HgS) may enter bacterial cells by passive diffusion (e.g., ref *40*). Thus, Hg(II) uptake appears to be more complicated than uptake of metals that fit the FIAM for bioavailability.

This work showed that the use of Hg toxicity as an indicator of how different conditions will affect bioavailability for Hg(II) uptake was sometimes, but not always, correct. In one case, toxicity behavior at high HgT concentrations did not predict uptake by bacteria cells behavior at low HgT concentrations. In another case, the lack of predictability appeared to be due to protection against Hg toxicity even though Hg(II) was transported into the cell. The large body of work using toxicity as an indicator of toxic metal uptake at low environmental concentrations is not necessarily incorrect, but should be interpreted with caution. Going forward, it seems prudent to use direct measurements of uptake where possible.

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