

Microbial availability of mercury: effective detection and organic ligand effect using a whole-cell bioluminescent bioreporter

Xianghua Xu^{1,2} · Kathryn Oliff² · Tingting Xu² · Steven Ripp² · Gary Saylor² · Jie Zhuang³

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Abstract A *luxCDABE*-based genetically engineered bacterial bioreporter (*Escherichia coli* ARL1) was used to detect bioavailable ionic mercury (Hg(II)) and investigate the effects of humic acids and ethylenediaminetetraacetic acid (EDTA) on the bioavailability of mercury in *E. coli*. Results showed that the *E. coli* ARL1 bioreporter was sensitive to mercury, with a detection limit of Hg(II) of 0.5 µg/L and a linear dose/response relationship up to 2000 µg Hg(II)/L. Humic acids and EDTA decreased the Hg(II)-induced bioluminescent response of strain ARL1, suggesting that the two organic ligands reduced the bioavailability of Hg(II) via complexation with Hg(II). Compared with traditional chemical methods, the use of *E. coli* ARL1 is a cost-effective, rapid, and reliable approach for measuring aqueous mercury at very low concentrations and thus has potential for applications in field in situ monitoring.

Keywords Mercury · Bioavailability · Humic acids · EDTA · Bioreporter · *Escherichia coli* ARL1

Introduction

The release of mercury into the subsurface environment has resulted in widespread environmental contamination, particularly at United States Department of Energy (DOE) sites such as Oak Ridge National Laboratory (ORNL) where it is estimated that 108,000–212,000 kg of elemental mercury were released to the environment in the 1950s and 1960s (Brooks and Southworth 2011). Studies have revealed that ~77,000 kg of mercury are contained in the sediments and floodplain of a 15-mile length of East Fork Poplar Creek (EFPC), which has its headwaters at the Oak Ridge Y-12 plant (where mercury was used at the Oak Ridge Y-12 National Security Complex for lithium isotope separation processes) (Brooks and Southworth 2011), and that ~230 kg of mercury annually leave this watershed (Turner and Southworth 1999). These floodplain soils have been found to contain mercury in concentrations up to 3000 µg/g (Barnett and Turner 1995; Harris et al. 1996). Fly ash produced in coal-fired energy plants also contains mercury (Gibb et al. 2000). In 2008, 1.1 billion gallons (4.2 million m³) of coal fly ash slurry was released at the Tennessee Valley Authority's Kingston Fossil Plant in Roane County, Tennessee, USA (Bartov et al. 2013). The slurry traveled across the Emory River and its Swan Pond embayment, on to the opposite shore, covering up to 300 acres (1.2 km²) of the surrounding land. Ultimately, mercury released to the environment during these events can be taken up by various organisms where it is recycled and bioaccumulated in the food chain to eventually impact human health. Mercury is a priority pollutant that the U.S. Environmental Protection Agency (EPA) has a set discharge limit of 10 µg/L in wastewater and a maximum contaminant level of 2 µg/L for drinking water (USEPA 2009). The presence of mercury in drinking water has been

✉ Jie Zhuang
zhuangjie@iae.ac.cn

¹ Jiangsu Key Laboratory of Agricultural Meteorology, Nanjing University of Information Science & Technology, Nanjing 210044, China
² The Center for Environmental Biotechnology, The University of Tennessee, Knoxville, TN 37996, USA
³ Key Laboratory of Pollution Ecology and Environmental Engineering, Institute of Applied Ecology, Chinese Academy of Sciences, Shenyang 110016, China

associated with deleterious effects over a wide range of systems including the respiratory, cardiovascular, hematologic, immune, and reproductive systems (Park and Zheng 2012). Consequently, more stringent regulatory controls have been imposed for mercury in many countries (IPCS 2003).

Biotoxicity of mercury depends upon its speciation and bioavailability. Mercury in natural waters occurs in different forms including elemental mercury (Hg^0), ionic mercury (Hg(I) , Hg(II)), and methylated mercury [CH_3Hg^+ , $(\text{CH}_3)_2\text{Hg}$]. Mercury undergoes a variety of chemical and biological transformations in aquatic ecosystems. Microorganisms play a major role in carrying out the biological transformations, many of which affect the toxicity of mercury. For example, Hg(II) is transformed by bacteria in anaerobic sediments to monomethyl mercury (CH_3Hg^+) (Compeau and Bartha 1984), which is a more toxic form of Hg(II) and is also more effectively bioaccumulated and biomagnified up the aquatic food web than other species of mercury (Mason et al. 1996; Watras et al. 1998). For bacterial mercury methylation to occur, Hg(II) must first enter the cell, and the biotoxicity of mercury thus depends upon its concentration inside the cell. Therefore, knowledge of Hg(II) bioavailability to microorganisms is critical to understanding the factors that control the bioaccumulation and biotoxicity of Hg(II) .

Like other metals, bioavailability and mercury speciation can be altered by chelating substances from organic and inorganic ligands (Fitzgerald et al. 2007; Shi et al. 2014). Dissolved organic matter (DOM) and ethylenediaminetetraacetic acid (EDTA) have been reported to be the most important organic ligands for mercury in waters from natural and anthropogenic sources (Haitzer et al. 2002; Lamborg et al. 2004). DOM occurs in all natural sediments and waters, usually at concentrations much higher than mercury (Barkay et al. 1997; Morel et al. 1998). EDTA is extensively used as a food additive in various industrial applications and has been detected in many aquatic systems (Sillanpaa 1997). The effects of DOM and EDTA are complex and highly dependent upon their chemistry, concentration, and size. A DOM- or EDTA- rendered decrease (Klinck et al. 2005; Laporte et al. 2002; Nybroe et al. 2008) and increase (Pickhardt and Fisher 2007; Rodea-Palomares et al. 2009; Zhong and Wang 2009) of metal uptake have both been observed. Investigating how the organic ligands affect mercury uptake by microorganisms requires the measurement of mercury bioavailability. Biosensors provide an effective way to assess mercury bioavailability in the environment compared to traditional chemical methods because distinguishing bioavailable mercury from other forms of mercury in environmental samples is currently beyond our analytical capabilities.

Bioluminescent bioreporters are intact, living microorganisms genetically designed to respond to specific chemical or physical agents in their environment via the production of visible light (Xu et al. 2013). The intensity of the bioluminescent response correlates to the concentration of the specific exposure agent, reporting the target's presence and bioavailability. Bioreporters containing *mer-lux* gene fusions have been used for mercury detection and monitoring in soils (Hakkila et al. 2004; Rasmussen et al. 2000), groundwater (Barkay et al. 1998), and even arctic snows (Scott 2001; Larose et al. 2011). Among them, the whole-cell bioluminescent bioreporter strain *E. coli* ARL1, which contains a *merR-luxCDABE* gene fusion for the detection of bioavailable mercury (Dahl et al. 2011), holds promise for field applications. However, this engineered bioreporter has not been fully characterized with regard to its kinetic response to mercury, effective detection limit and effects of environmental factors (e.g., DOM, EDTA). The objectives of this research were to (1) examine the response characteristics of the *E. coli* ARL1 bioreporter to mercury in aqueous environments, and (2) apply strain ARL1 to evaluate the effect of organic ligands on the bioavailability of Hg(II) .

Materials and methods

Bioreporter strains

The *luxCDABE*-based bioluminescent bioreporter *E. coli* ARL1 was used to monitor for mercury bioavailability. Strain ARL1 contains a 505 bp region of the *mer* operon consisting of the *merR* gene and the promoter/operator region of the *mer* operon fused to the *luxCDABE* gene cassette of *Photobacterium luminescens* (Dahl et al. 2011). Its lower limit of detection for Hg(II) approaches 10 nM (2.7 $\mu\text{g/L}$). The bioluminescent bioreporter *E. coli* 652T7 consisting of a fusion of a constitutive T7 promoter with the *P. luminescens luxCDABE* gene cassette served as a control strain to monitor for general toxicity, with a reduction in bioluminescent light emission signifying whether mercury or other sample constituents were toxic to *E. coli* (Shi et al. 2014).

Preparation of samples

Before use, all glassware was combusted at 500 °C followed by a 24 h soak in 10 % nitric acid to remove organics and mercury, respectively. Analytical grade HgCl_2 (ThermoFisher, Pittsburgh, PA) was used to prepare a 1000 mg/L stock solution of Hg(II) in dH_2O . A 1000 mg/L stock solution of humic acids (in sodium salt form; Aldrich Chemical, Milwaukee, WI) was prepared in dH_2O .

A 1000 mg/L EDTA stock solution was prepared in dH₂O with analytical grade EDTA (ThermoFisher). The humic acids and EDTA stock solutions were adjusted to a pH of 6.5, filtered through a 0.2 µm filter, and stored at 4 °C in the dark. Standard mercury solutions ranging from 0.5 to 2000 µg/L were prepared by diluting the 1000 mg/L stock solution with sterile dH₂O in volumetric flasks. The pH values of all HgCl₂ standards were adjusted to 6.5 using 0.5 M NaOH or HCl.

Before analyses, the stock solutions of humic acids and EDTA were diluted in sterile dH₂O to the various concentrations described in the Results section. The mercury stock solution was then diluted into the humic acids and EDTA solutions and shaken for 24 h at room temperature in the dark to ensure chelation of Hg(II).

Bioluminescent assay for mercury

Bioavailable Hg(II) was measured as a function of the bioluminescent intensity of the *E. coli* ARL1 bioreporter using a BioTek Synergy2 multi-mode microplate reader (Winooski, VT). To perform bioluminescent assays, the ARL1 bioreporter was grown in Luria–Bertani (LB) broth supplemented with kanamycin at 50 mg/L (K_{n50}) at 28 °C with shaking (200 rpm) to an optical density at 600 nm (OD₆₀₀) of 0.20–0.25 ($\sim 1 \times 10^8$ cfu/mL, exponential growth phase). Aliquots of 180 µL of the culture were then transferred to the wells of black 96-well microtiter plates (Corning, NY), each of which contained 20 µL of the HgCl₂/humic acids or HgCl₂/EDTA dilutions as described above. Three replicates of each 96-well plate were performed, with six replicates for each dilution in each plate. Plates were sealed with a transparent breathable membrane (Breathe-Easy, Sigma-Aldrich, St. Louis, MO) and placed in the BioTek Synergy2 plate reader for bioluminescence detection at 28 °C. Bioluminescence in relative light units (RLUs) was recorded with a one second integration time every 10 min for 24 h. Each plate contained triplicate controls consisting of *E. coli* ARL1 alone, *E. coli* ARL1 + humic acids (0 - 20 mg/L), and *E. coli* ARL1 + EDTA (0–20 mg/L). Similarly treated constitutive *E. coli* 652T7 bioreporter (180 µL/well of a 1×10^8 cfu/mL culture in LB + 20 µL sample) was included in each plate in triplicate wells to monitor for general toxicity (Shi et al. 2014).

Statistical analyses

The results are presented as the mean \pm standard deviation (SD). The differences between treatments were analyzed by one-way ANOVA followed by the Duncan test using SPSS 11.5 statistical software.

Results

Effect of mercury concentration on bioluminescent response kinetics

Figure 1 shows the time course of bioluminescence of the *E. coli* ARL1 bioreporter during exposure to Hg within a concentration range from 0.5 to 2,000 µg/L, with RLU values being normalized to emission measurements at time zero. Bioluminescence emission intensity increased with increasing concentration of mercury up to 1000 µg/L whereupon the *merR* regulatory system likely approached saturation and bioluminescence stabilized. Peak bioluminescence occurred within 2 h under all exposure conditions and was followed by a gradual decrease likely resulting from the consequences of reduced oxygen availability within the confined microtiter plate and reduced Hg(II) bioavailability over time. When plotting mercury exposure concentrations versus the maximum bioluminescent output by the ARL1 bioreporter, a linear relationship was indicated within the concentration ranges tested (Fig. 2). The limit of detection under these experimental conditions approached 0.5 µg Hg/L, which meets the minimum requirement (2 µg/L) of the U.S. EPA for mercury contamination in drinking water (USEPA 2009).

Effect of humic acids on the bioavailability of mercury

Humic acids were added to the mercury solutions to examine the effect of organic acids on the bioavailability of mercury. At Hg(II) concentrations ranging from 10 to 1000 µg/L, a significant decrease in light output was observed as humic acid concentrations increased from 0 to 10 mg/L (Fig. 3), suggesting that humic acids reduced the bioavailability of Hg(II) in the ARL1 bioreporter. Further increasing humic acid concentration up to 20 mg/L did not significantly change the bioavailability of mercury in terms of bioluminescence output by strain ARL1. In the absence of humic acids, bioluminescence increased with increasing Hg(II) concentrations in agreement with results represented in Fig. 1. The humic acids alone without the presence of Hg(II) was not capable of inducing significant ($p > 0.05$) light production compared to background (solid black bars, Fig. 3, inset). Additionally, light emission from the constitutive *E. coli* bioreporter 652T7 identically exposed to the HgCl₂/humic acids mixtures demonstrated no significant change in bioluminescence (data not shown), thus confirming that the chelated humic acids-mercury (HA-Hg) complexes as applied in these studies are not toxic to *E. coli*.

Fig. 1 Bioluminescent emission profiles for the *E. coli* ARL1 bioreporter after exposure to Hg(II) at concentrations ranging from 0.5 to 2000 µg/L. Error bars represent the standard deviation among three experimental replicates. RLU relative light units

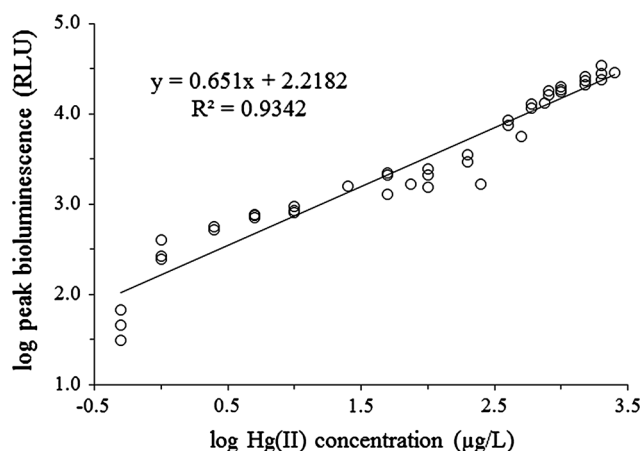
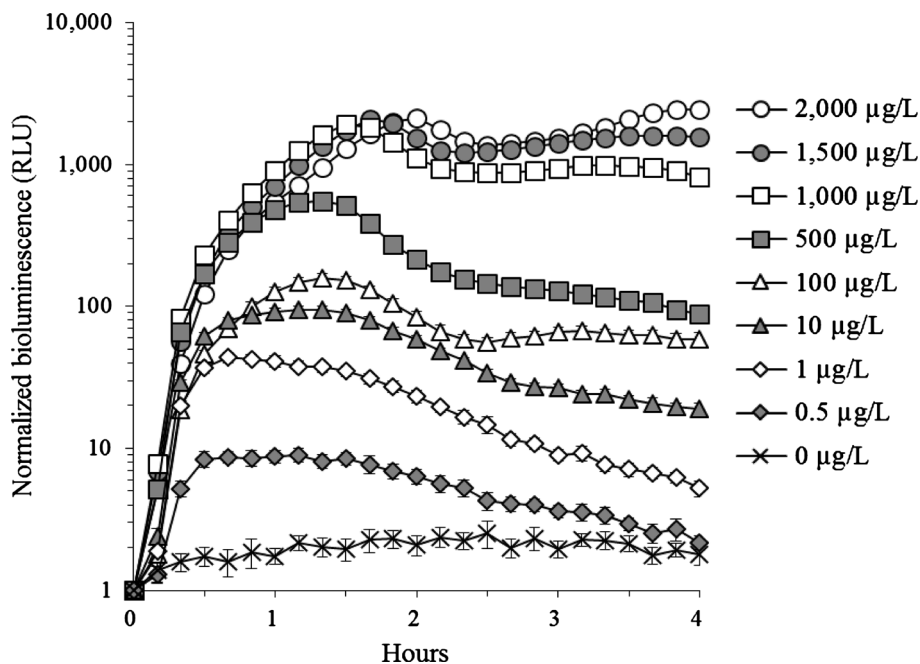


Fig. 2 The peak bioluminescent response from the *E. coli* ARL1 bioreporter increased linearly within the tested range of Hg(II) exposures. All data from three replicated experiments are plotted. RLU relative light units

Effect of EDTA on the bioavailability of mercury

The response of *E. coli* ARL1 to chelated EDTA-Hg followed the same general trend as for the humic acids exposures. The addition of EDTA decreased Hg(II) bioavailability as indicated by a decrease in the bioluminescent signal as EDTA concentrations increased from 0 to 10 mg/L (Fig. 4). Further increases in EDTA concentrations up to 20 mg/L did not significantly change the bioluminescent light output, suggesting saturation of the EDTA-Hg complexes at these higher concentrations. The effect of increasing EDTA on light production by the

constitutive strain 652T7 was the same as for the humic acids (data not shown), indicating that EDTA itself and the EDTA-Hg complexes had no significant toxic effects on *E. coli*.

Discussion

As an important characteristic, bioreporters reflect the real physiological impact of toxic compounds, and thus report on the bioavailable fraction of toxicants. Here, we demonstrated an application of strain ARL1 for monitoring Hg(II) bioavailability and explored the effects of humic acids and EDTA on Hg(II) bioavailability. The constitutively bioluminescent strain *E. coli* 652T7 was simultaneously used as a control; this enabled the monitoring of general cell fitness for better elucidation of possible deleterious effects caused by humic acids, EDTA, mercury, or other environmental factors related to cell metabolism and survival.

In this study, the bioreporter ARL1 could detect Hg(II) at concentrations as low as 0.5 µg/L, suggesting that the bioreporter is sufficiently sensitive for the detection of bioavailable Hg(II). Such higher sensitivity may be due to the inclusion of merT, an Hg(II)-binding membrane protein that allows Hg(II) to efficiently enter the cell (Hamlett et al. 1992), in the construct of the ARL1 bioreporter. Sensitivity of Hg(II) detection ability enhanced by merT has been previously reported (Pepi et al. 2006; Larose et al. 2011). The Hg(II) detection limit of 0.5 µg/L can meet the minimum requirement (2 µg/L) in drinking water by the U.S.

Fig. 3 Effect of different concentrations of humic acids on the bioluminescent response of *E. coli* ARL1 under various Hg(II) exposure conditions. Inset: Bioluminescent profile of strain ARL1 at the lower end (0 and 10 $\mu\text{g/L}$) of the Hg exposure spectrum. Error bars represent the standard deviation among three experimental replicates. Values in each column followed by different letters are significantly different at $p < 0.05$. RLU; relative light units

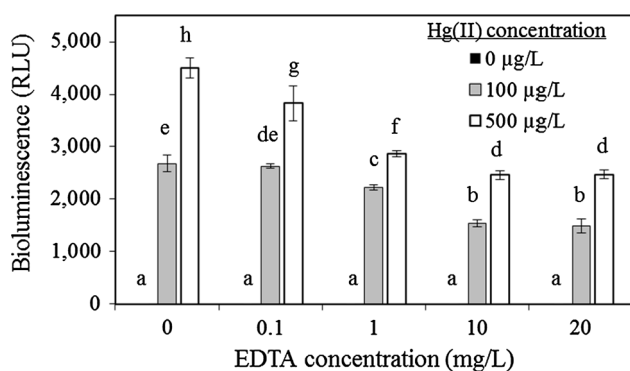
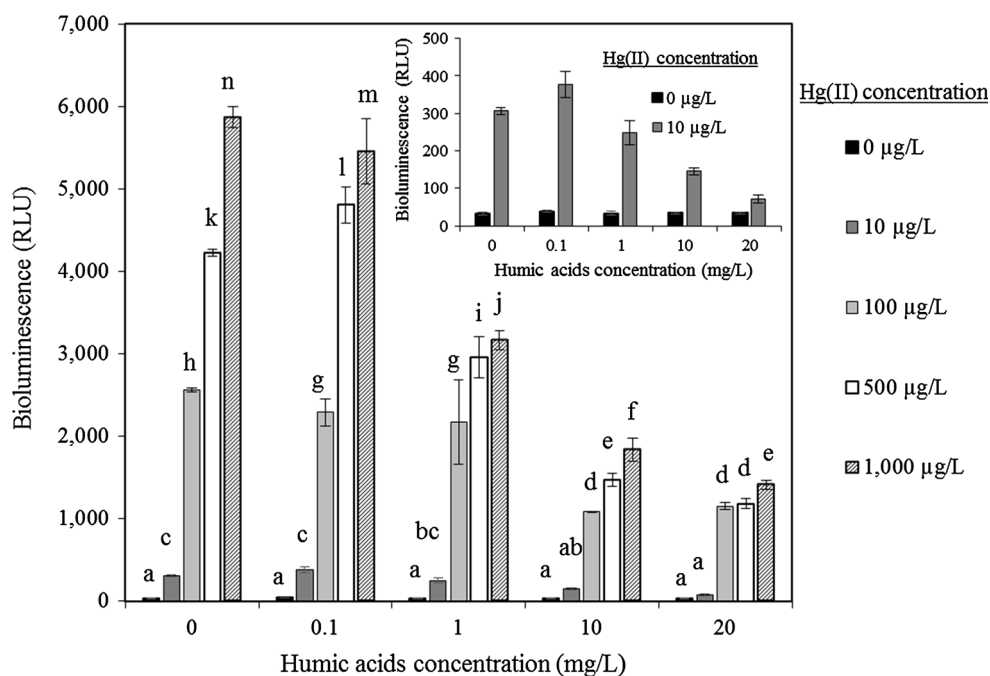


Fig. 4 Effect of differing concentrations of EDTA on the bioluminescent response of *E. coli* ARL1 under various Hg(II) exposure conditions. Error bars represent the standard deviation among three experimental replicates. Values in each column followed by different letters are significantly different at $p < 0.05$. RLU; relative light units

EPA. Therefore, such bioreporter present an effective way to assess mercury bioavailability in a water environment and provide a reference of mercury effects on human health, although mercury bioavailability can differ between tropic levels (i.e., bacterial, simple eukaryotic, human, etc.).

The complexation of heavy metals with DOM is an important process that may alter the bioavailability and toxicity of heavy metals in aquatic systems. Humic substances represent an important fraction of DOM that influences numerous biogeochemical processes (Steinberg et al. 2006). Consequently, humic acids have been extensively used as a representative natural ligand in bioavailability studies (Gu et al. 2011; Lamelas and Slaveykova 2007; Sanchez-Marin et al. 2007). Humic acids contain a

variety of complex organics with high affinity for metals, bind metals at their carboxylic and phenolic groups, and alter the bioavailability and toxicity of the toxicants (Benoit et al. 2001b). Results from this study demonstrated a significant decrease in light output after the addition of humic acids, suggesting that humic acids made Hg(II) less bioavailable to the ARL1 bioreporter via complexation with mercury (Fig. 3). Laporte et al. (2002) reported a similar behavior in the bioavailability of Hg to *Callinectes sapidus* in the presence of humic acids. The inhibitory effects of DOM may be attributed to the mercury species and their different uptake mechanisms. It has been shown that Hg(II) can be taken up via passive diffusion, whereas DOM-mercury complexes are unlikely to be transported across the biological membrane. The low bioavailability of DOM-mercury and possible uptake mechanism likely involves ligand exchange processes (Campbell 1995; Laporte et al. 2002). A ligand exchange is required to exchange Hg from the complex to an uptake site (Laporte et al. 2002). The effect of DOM on the metal's bioavailability is complicated and highly dependent on the molecular sizes, chemistry, and concentrations of the DOM molecules (Rui and Wen-Xiong 2010; Worms et al. 2006). Previous studies have inconsistently reported on the influence of DOM on the uptake of Hg(II), with some studies demonstrating decreased bioavailability (Klinck et al. 2005; Laporte et al. 2002; Miskimmin et al. 1992) while others indicate increased bioavailability (Gu et al. 2011; Lamelas and Slaveykova 2007; Wang et al. 2014; Zhong and Wang 2009). The humic acids-enhanced Hg(II) bioavailability may be due to the amphiphilic characteristic

of humic acids because their adsorption on the surface of living cells can affect metal interactions with the cell surface (Campbell et al. 1997; Knauer and Buffle 2001; Slaveykova and Wilkinson 2005).

EDTA is an artificially synthesized chemical substance, the property of which significantly differs from naturally occurring humic acids. Results from this study showed that EDTA significantly reduced the bioavailability of Hg(II) (Fig. 4) in *E. coli*. This result is expected and consistent with other studies (Nybroe et al. 2008; Rodea-Palomares et al. 2009). It could be inferred that the effect of the formation of the paradigmatic “unavailable” aqueous metal complex between EDTA and Hg(II) may cause the complex to be too large to cross the cell membrane via passive diffusion, which is a primary uptake process (Barrocas et al. 2010; Benoit et al. 2001a).

Like other metals, speciation of mercury is affected by complexation with organic and inorganic ligands that are present in water. The relative importance of each ligand for metal complexation may depend on the concentration of the metal and ligand, and the binding strength for the metal–ligand complex. Humic acids and EDTA have high binding constants with Hg(II) (Ravichandran 2004), suggesting that humic acids and EDTA have a strong ability to complex with Hg(II) and the complexes formed are unlikely to penetrate the plasma membrane. Hence, as the ligand concentrations (humic acids or EDTA) increase, ligand–Hg complexes (humic acids–Hg or EDTA–Hg) will be the dominant mercury species found in the solution. In this study, the humic acids and EDTA are all effective in complexing with Hg(II) and decreasing Hg(II) bioavailability at different concentrations, suggesting that the bioavailability of Hg(II) was predominately determined by the chemical speciation of mercury.

Conclusion

We report an application of the bioreporter *E. coli* ARL1 in detecting bioavailable mercury and assessing the effects of organic ligands (humic acids and EDTA) on Hg(II) bioavailability. A significant linear relationship between the bioluminescence emission and Hg(II) concentration up to 2,000 µg/L can be observed. The bioreporter ARL1 could detect Hg(II) at concentrations as low as 0.5 µg/L, which meets the minimum requirement (2 µg/L) in drinking water by the U.S. EPA. Humic acids and EDTA decreased the bioavailability of Hg(II), suggesting that mercury bioavailability mainly depends on its chemical speciation. The results have implications for predicting the long-term biological fate of mercury in the environment, assessing the biotoxicity of mercury, and developing

effective environmental quality criteria for controlling metal discharge and cleanup.

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Compliance with ethical standards

Conflict of Interest The authors declare that they have no conflict of interest.

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