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Assessment of mercury(II) bioavailability using a bioluminescent bacterial biosensor: Practical and theoretical challenges

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Abstract

Critical methodological challenges in the microbial biosensor approach to assessing Hg(II) bioavailability were evaluated from the perspective of analytical chemists. The main challenge stems from the fact that the chemical speciation of Hg(II) in natural waters exerts a major control on its bioavailability, yet its natural complexation equilibria are extensively altered during conventional bioassays. New data, obtained using a bioluminescent Hg(II)-biosensor, that illustrate these challenges are presented and potential solutions proposed.

Key words: mercury; bioavailability; biosensor; chemical speciation; bioluminescent

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Introduction

Since 1990, significant progress in understanding the biogeochemistry of Hg in freshwater ecosystems has been made due to advances in analytical methods for quantifying the concentrations of its predominant states – monomethylmercury (MeHg), divalent mercuric mercury (Hg(II)), and elemental mercury (Hg^0) – at ultra trace levels. However, our knowledge of the distribution of Hg among the diverse free, complexed and adsorbed species within each state remains limited due to the lack of analytical methods capable of distinguishing species at ultra trace levels. Largely for this reason, it is difficult to model the effects of spatiotemporal variations in chemical parameters, including pH and dissolved organic matter, on the rates of many Hg cycling processes, particularly those that are biologically-mediated.

While laboratory studies have proven that biological Hg uptake occurs via the passive diffusion of neutral Hg species such as $HgCl_2{}^0$ (aq) and $MeHgCl^0$ (aq) (Morel et al., 1998), the environmental abundance of these particular species is poorly quantified in freshwaters due to a fundamental lack of knowledge of the strength of Hg(II) (and MeHg) interactions with natural organic ligands. Thus, it remains very difficult to predict the relative bioavailability of Hg in different aquatic systems or even establish that the relative abundance of electroneutral aqueous complexes does in fact control Hg uptake *in situ*. Understanding these questions is crucial to our ability to make predictions of the interactions of Hg with major biogeochemical cycles and ultimately predict the impacts of Hg pollution at broad spatial scales.

The need to better understand Hg bioavailability in the environment has stimulated the development of a new class of Hg detection systems that use biological components as the sensory elements. In general, Hg biosensors combine both a sensitive receptor (the biological system that is responsible for the selectivity of the method) and a detector (a detection system that is capable of measuring the reaction of the biological component with the substance being monitored). While purified enzymes and antibodies can function as biological receptors, whole microorganisms are preferred in environmental work since they are more tolerant of non-ideal assay conditions and are less expensive since they can easily be cultivated and used without isolating their active biological components (Ramanathan et al., 1997).

Bioluminescent microbes make very convenient reporters since they permit researchers to quantify the *in vivo* biosensor response using widely-available optical detection systems without manipulating or disrupting the cells after exposing them to Hg-containing samples (Selifonova et al., 1993; Ramanathan et al., 1997). Since all normal processes for Hg uptake should be functioning in live biosensor cells, microbes with Hg-dependent bioluminescence can be used to directly assay the relative bioavailability of Hg in different solutions. In principle, this makes *in vivo* biosensors an ideal means of assaying Hg bioavailability in both natural and synthetic media.

However, researchers face a major challenge when developing biosensor protocols to study the bioavailability of Hg species in natural samples: that of simultaneously

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fulfilling the specific and sometimes conflicting methodological requirements of both microbiology and analytical chemistry. The crux of this challenge lies in the need to physically mix bacterial culture media and Hg-containing samples in order to conduct conventional *in vivo* bioassays. Historically, microbiologists' main concerns in culturing microorganisms have been to provide sufficient nutrients, organic compounds, vitamins and essential elements, for the metabolism and growth, and to avoid contamination by foreign organisms. In contrast, trace element chemists conducting speciation analysis of natural samples have focused on avoiding contamination by any trace elements that affect the analysis, especially the analyte of interest, and meticulously avoiding or controlling any alteration of the original speciation of the analyte. The chemists' concerns are especially challenging in the case of Hg(II). Due to its occurrence at picomolar levels in natural waters, both major and trace level components of microbiological media can potentially interfere in the analysis of its speciation.

Farrell et al. (1990) put forward design principles for microbiological media used in trace element speciation studies that consider both sets of concerns: (1) provide adequate nutrition for bacterial metabolism; (2) be well-enough characterized chemically that trace element speciation can be modeled; (3) be uncontaminated with respect to the trace elements studied; (4) have no medium component that masks the effects of ligands added to experimental treatments (or that occur in the natural sample in our case); and (5) maintain the above following sterilization.

To the extent that some researchers have not recognized the subtleties of these issues, dubious interpretations of bioassay results and invalid extrapolations of results to *in situ* conditions may have been reported (Hughes and Poole, 1991). In this work, we examine how the above issues can interfere with the use of current standard methods of applying biosensors to assess the bioavailability of Hg(II) species at natural levels.

1 Materials and methods

1.1 Biosensor construction

The Hg(II) biosensor employed here has a recombinant, promoter-reporter gene developed by Virta et al. (1995). The bioluminescent reporter gene, firefly luciferase (*LucFF*), was fused to the *mer* promoter of transposon Tn21 (*mer R* gene + promoter). This genetic construction was inserted in the pT0011 plasmid with a kanamycin-resistant *E. coli* (MC1061) serving as the host organism. When bioavailable Hg(II) species enter the biosensor cell, the promoter is induced. Since the reporter gene is under the control of the promoter, it is also expressed and luciferase synthesized. Upon addition of the enzyme's substrate luciferin, light is emitted in direct proportion to the amount of luciferase present, yielding a luminescence signal that is a function of the amount of Hg(II) in the cell.

To control for non-Hg(II) mediated effects of assay conditions, an isogenic strain of *E. coli* MC1061 that harbors the original plasmid pCSS810 with a luciferase gene that is not under control of the *mer* promoter is used. Since, its luciferase gene is constitutively expressed, the bioluminescence of this strain indicates the overall metabolic state of the cells (Ramanathan et al., 1997). When examining effects of medium/sample composition, parallel experiments are conducted with the Hg biosensor and the "constitutive control".

1.2 Cultivation of the biosensor and constitutive control

Long-term culture stocks of the biosensor and constitutive control strains were maintained in LB (15% glycerol) medium kept frozen at –70°C (Barrocas, 2003). To initiate an experiment, LB agar plates were streaked and isolated colonies transferred to minimal salts media and grown overnight. These media were made up either from chloride (M9Cl) or nitrate ($M9NO_3$) salts of the major ions plus glucose (2 g/L), casamino acids (1 g/L), and kanamycin (30 μg/mL) and then autoclave-sterilized (Atlas, 1997). On the morning of the bioassay, 2.0-mL aliquots of overnight cultures were transferred into nepheloflasks containing 100 mL of the appropriate M9 medium. After growing to mid-exponential phase (OD_{600} = 0.5), cultures were harvested by centrifugation (6 min at 3200 ×*g*), washed with ultrapure deionized water, and resuspended in the experimental medium. While being cultured, cells were maintained at room temperature with continuous stirring at 250 r/min using an orbital shaker (model G2, New Brunswick Scientific, USA).

1.3 Bioassay experimental conditions

The conventional, single-step bioassay consists of exposing biosensor cells to Hg(II)-containing samples while simultaneously incubating them in growth medium order to facilitate luciferase synthesis. This exposure-incubation step and subsequent measurement of light production was conducted within a luminometer using standard 96-well microplates (Luminoskan Ascent 2.4, Labsystems, Finland). Experimental conditions, cell density, incubation time, volume and pH of luciferin solution, medium composition, etc., were all optimized to achieve best biosensor response (Barrocas, 2003). Every well was added 50 μL of diluted culture slurry and appropriate volumes, typically 10–100 μL, of samples and standards. Except as noted, the concentrations of Hg(II), EDTA and other solutes discussed below refer to this stage in the experiments, i.e., the mixture of medium, Hg(II) standard, and "sample". The microplates were incubated inside the luminometer at 22°C for 1 hr. Finally, the bioluminescence measurement itself commenced when the automatic dispenser added 100 μL of 1 mmol/L D-luciferin in aqueous pH 5 sodium citrate buffer. Since with the firefly luciferase reaction, the light emission occurs almost immediately after the addition of the luciferin solution and decays rapidly (Ford and Leach, 1998), the luminometer was set to record light production for 121 sec intervals following luciferin addition. Each sample was assayed in triplicates, with the average integrated light signals reported here in relative

light units (RLU). Error bars depicted below were derived by statistical analysis (ANOVA or ANCOVA) of all data for each experiment using SAS (v. 9.2, SAS Institute) with inverse square weighting of residuals due to the wide signal ranges observed.

1.4 Chemical and biological decontamination

Materials contacting experimental solutions were cycled through at least three acid baths (3 and 0.5 mol/L HCl), which took at least one week, and were stored either inside the last acid bath or double-sealed polyethylene bags in a Class-100 tabletop laminar flow hood. All labware and solutions were either manufactured sterile or sterilized by autoclaving and carefully manipulated using strict aseptic techniques under clean-room conditions (Barrocas, 2003).

In order to control mercury contamination and/or loss, solutions or materials used during the bioassays were systematically analyzed for their total Hg content by cold vapor atomic fluorescence spectrometry (Model-2, Brooks-Rand, USA). Other trace metals were analyzed by ICP-MS (Element-1, Thermo Finnigan, Germany).

2 Results and discussion

2.1 Calibration of the biosensor response

The *sine qua non* of any quantitative analysis is the relationship between the concentration of analyte and the measured signal. Here, calibration curves relating total dissolved mercury concentration, [Hg(II)], to the integrated bioluminescence intensity (I_B) were generated by (1) mixing ultrapure water "samples" containing standard additions of Hg(II) with the cultured biosensor slurry, (2) incubating replicate samples for 1 hr to permit Hg(II) uptake and luciferase synthesis, and (3) measuring the bioluminescent flash immediately after luciferin addition. Since the experimental conditions were manipulated to achieve optimal biosensor response and unknown ligands that could sequester the Hg(II) in less bioavailable forms were excluded, these standards should exhibit the maximum biosensor response at their respective concentration of dissolved Hg(II) for the medium employed.

To clarify the conceptual model underlying the calibration curves, we define two qualitative relationships between the main variables involved. The first is between the intensity of biosensor luminescence (I_B) and the variables that (1) regulate transcription of the *LucFF* gene and luciferase production, namely Hg taken up into the cell, $(Hg)_{CELL}$, (2) are necessary for luciferase synthesis (glucose and amino acids), and (3) are substrates of luciferase (luciferin and ATP):

$$I_B = f_B\begin{pmatrix}(Hg)_{CELL,} & (\text{amino acids})_{CELL,}\\ (ATP)_{CELL,} & (\text{luciferin})_{CELL}\end{pmatrix} \quad (1)$$

The second is between cellular Hg(II) and the factors that govern Hg uptake rates, including the exposure time and the total concentration ([Hg(II)]) and speciation of Hg(II) in the medium:

$$(Hg)_{CELL} = g\begin{pmatrix}[\text{Hg(II)}], \text{aqueous Hg(II) speciation},\\ \text{exposure time}\end{pmatrix} \quad (2)$$

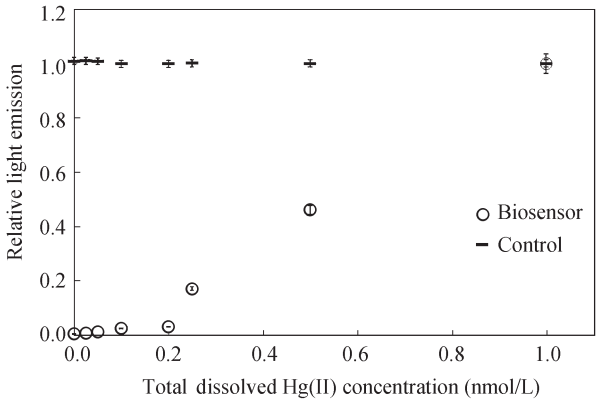

Fig. 1 Typical [Hg(II)] response by biosensor and constitutive control in M9NO$_3$ medium. *Y*-axis is ratio of I_B or I_C at each Hg standard concentration and at [Hg(II)] = 1.0 nmol/L (max I_B = 654 RLU; I_C = 1.4 RLU). Error bars are SE of ANOVA-derived means.

The fact that different transport processes take up different trace metal species at different rates is the basic tenet of experimental design in which biosensor response is measured in media of systematically varying aqueous Hg(II) speciation (Barkay et al. 1997; Golding et al., 2002).

Due to the complexity of the biosensor's response to cellular Hg(II) (Eq. (1)), the calibration curves observed here for the biosensor (Fig. 1) followed a sigmoidal rather than linear pattern, as widely documented in the literature (Ralston and O'Halloran, 1990; O'Halloran, 1993). A variety of data treatments to interpret such non-linear calibrations have been proposed, including fitting higher order polynomials to the light emission curves, calculating ratios between the sample and blank signals, or computing the rates of increase in bioluminescence signal (Barkay et al., 1998). In the data analysis employed here, we transposed the calibration curve axes, and empirically fitted polynomials in a piece-wise manner. Then defining all of the Hg(II) in calibration curve samples as bioavailable, we inferred an equivalent amount of "bioavailable Hg(II)" in other experimental and natural samples from the I_B observations (see below).

2.2 Use of a constitutive control

A fundamental requirement of *in vivo* biosensor studies is to ensure that the results only reflect the bioavailability of the chemical species assayed (Virta et al., 1995; Barkay et al., 1997, 1998; Petanen and Romantschuk, 2003). However, nearly all live biosensors can be affected by the chemical composition of samples or assay media due to biochemical effects not related to the analyte studied. Solution components known to alter biosensor response include chloride, DOC and casamino acids (Barkay et al., 1997; Golding et al., 2002). To quantify their effects on metabolism, the bioluminescence intensity of the constitutive control (I_C) was measured using the same protocols as for the biosensor. Typically, I_C was much less than I_B and little sensitivity to solution composition was observed

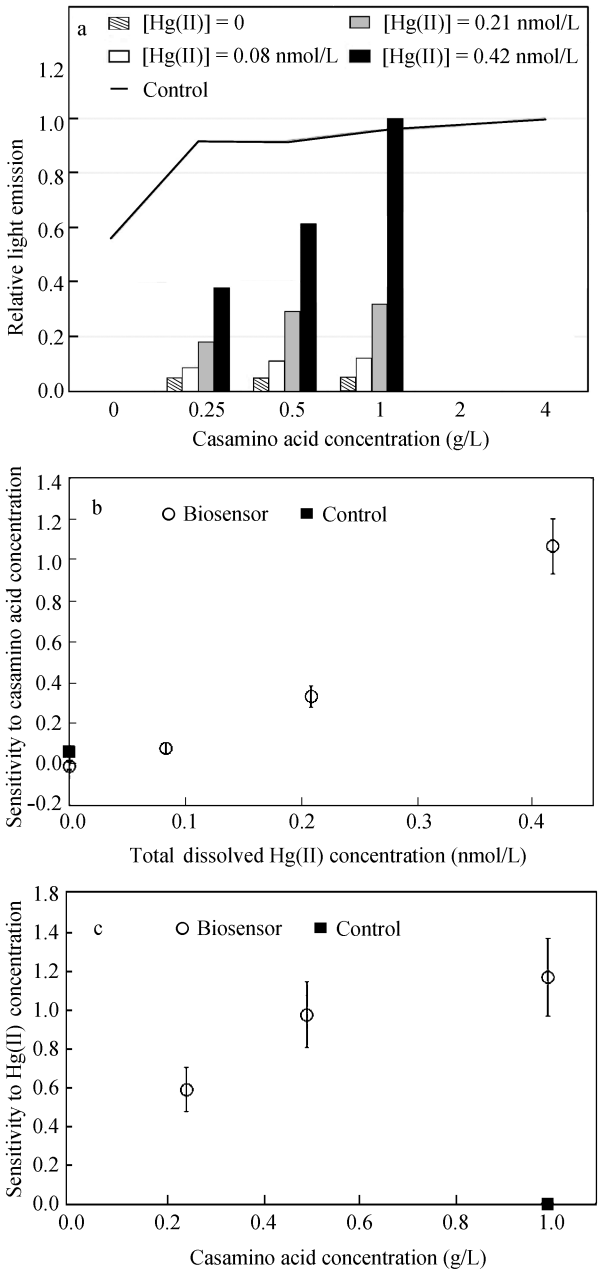

Fig. 2 Biosensor and control reponse to casamino acids (CAA). (a) observed I_B or I_C at various [Hg(II)] and [CAA] normalized by highest I_B or I_C value for each culture; (b) mean sensitivity to [CAA] at each [Hg(II)]; (c) mean sensitivity to [Hg(II)] at each [CAA]. Sensitivity ± SE in b and c derived using ANCOVA.

(Fig. 1), with the exception of a requirement that casamino acid concentrations, [CAA], exceed 0.25 g/L to attain optimal I_C (Fig. 2a).

2.3 Effects of microbiological media on biosensor response

2.3.1 Mercury contamination

The background luminescence observed when no mercury was added to the solutions could be caused either by low-level, constitutive luciferase production or by the biosensor's response to residual Hg(II) in the reagents and culture media (Tauriainen et al., 1999). In this work, much effort was devoted to identifying and eliminating sources of mercury contamination. All reagent solutions used in the bioassays were analyzed for total Hg. Occasionally, batches of common microbiological reagents contained enough Hg(II), 0.1 μg/L in LB medium, 3 μg/L in bottles of KH_2PO_4 and NH_4Cl, that they had to be discarded, since background luminescence, and hence the method detection limit, would have been elevated. In addition, since surface contamination can be a major cause of variability in blanks, all materials contacting experimental solutions were thoroughly cleaned. Cleaning procedures specific to the chemical resistance of each material and their use in the experiments were devised (see methods).

2.3.2 Mercury losses

Another factor affecting the accuracy of calibration curves was the loss of Hg(II) added to bioassay samples, especially at low levels and/or during long pre-equilibration periods. From experience working with Hg(II), we surmise that this was due to adsorption onto the surfaces of vessels used in the bioassays, such as the polystyrene microplates, polyethylene and glass tubes. Hg(II) loss during bioassays is aggravated by the fact that acidification to pH < 2, the protocol typically used by analytical chemists to keep Hg(II) in solution, could not be used due to its deleterious effects on the biosensors. Although some media guidelines recommend that metal stocks be prepared in acid solutions, they do not mention the potential instability of metals like Hg(II) added to neutral-buffered media or during autoclaving (Hughes and Poole, 1991). To minimize such problems in this work, the Hg(II) calibration standards were prepared immediately before use by diluting an acidified stock approximately 100-fold with ultrapure water only. The resulting solutions had pH above 5 and caused no harm to the biosensor as shown by the constitutive control results. To test whether losses had occurred, we measured the total mercury content of aliquots of the test solutions and corrected for any observed losses during the experiments.

2.3.3 Other trace metals

Few commercially-available growth media provide information on the levels of other trace elements, besides Fe, despite the fact that they can be present at mg/L or even higher concentrations (Hughes and Poole, 1991). Neither do Hg-biosensor research papers often address contamination by other metals, yet they must be considered since they can interfere with binding to natural or added ligands (e.g., EDTA) or even with Hg(II) uptake, to the extent uptake by facilitated mechanisms occurs. Trace element levels (in nmol/L) measured by ICP-MS were: Fe (563.7/409.7), Zn (421.6/299.4), Cu (47.72/39.78), and Pb (4.87/5.27) in M9Cl and $M9NO_3$ media.

2.3.4 Inorganic complexation

Different growth media can contain very different concentrations of inorganic Hg(II)-binding ligands, such as Cl^-, NH_3, and HPO_4^{2-}. Since these concentrations control the distribution of Hg(II) among solution complexes that have very different rates of biological transport, apparent Hg(II) "bioavailability" can vary dramatically between

media having the same concentration of added Hg(II) (Hughes and Poole, 1991), necessitating our inclusion of the "Hg speciation" variable in Eq. (2). The influence of inter-medium differences in inorganic Hg(II) speciation was investigated in an experiment where two standard addition calibration curves were generated from a single biosensor culture by resuspending cells in both M9Cl and $M9NO_3$ culture media and mixing each with the ultrapure water Hg(II) "standards". The different chloride concentrations in the two media, 32 and 5 mmol/L respectively, change the relative abundance of neutral $HgCl_2$ complexes from 60.9% to 8.8%. When biosensor (or control) cultures were split and calibrated in both M9Cl and $M9NO_3$ media, the former curve consistently exhibited higher IB values, while differences for IC were non-significant (data not shown). Such results strongly suggest that the biosensor responses differ between the two media due to higher passive $HgCl_2$ uptake in the M9Cl medium.

2.3.5 Organic complexation

Another important speciation effect was uncovered while optimizing the biosensor response to CAA (Fig. 2). CAA proved to be essential and consistent increases in biosensor sensitivity to [Hg(II)] were observed (Fig. 2) when increasing [CAA] over the range from 0.25 to 1 g/L. However, the luminescence of the constitutive control (IC) is largely insensitive to Hg(II) (Fig. 1) and exhibits only a minor increase (6%) over 0.25 to 1 g/L CAA concentration range (without Hg(II) present). Therefore, the mutually-positive interactions of [CAA] and [Hg(II)] (Fig. 2) likely were not caused by the release of nutritional limitation by CAA, but by higher Hg(II) uptake in their presence.

Golding et al. (2002) also observed strong increases in the responses of their Hg(II)-biosensors, *Vibrio anguillarum* and *E. coli*, when CAA or certain amino acids that are components of the CAA such as histidine, were added to media. They attributed the increase in sensitivity to enhanced transport, whether by passive or facilitated mechanisms, was uncertain of neutral Hg(II)-amino acid complexes. Equilibrium speciation modeling (Barrocas, 2003) based on the analysis of CAA used in this work suggests that 2:1 amino acid-Hg^{2+} complexes formed, but comprised less than 1% of [Hg(II)]. The most abundant complexes of this type, and therefore the most likely to facilitate Hg(II) uptake would have been with histidine, present at 24 μmol/L, and alanine, present at 245 μmol/L. Cystine/cysteine, which were not distinguishable analytically, was detected in the CAA. If the latter was present, its complexes would dominate Hg(II) speciation with uncertain effects on biosensor response.

2.3.6 Standardizing "unavailable" Hg(II)

EDTA reacts with many trace metals to form the paradigmatic "unavailable" aqueous metal complex. As is often done in metal speciation studies, we sought to validate the biosensor by examining Hg(II) bioavailability in EDTA-containing solutions (Fig. 3a–c). In these experiments, the Hg(II) was much more bioavailable in the presence of EDTA than expected based on equilibrium modeling and the medium's recipe, i.e., nominally no other trace metals were added. At final EDTA concentrations of 3.5, 35 and 350 nmol/L, the observed availability of Hg(II) was 100%, 60%, and 29% of that without EDTA while the bioavailable proportions predicted by equilibrium modeling (Barrocas, 2003) were 24%, 2.4%, and 0.23% respectively. This occurred despite the fact that the experiment was begun by introducing 100% of the Hg(II) in the form of EDTA complexes. A likely cause is that the trace metals in the reagents (see above) reacted with free EDTA and displaced EDTA-bound Hg^{2+}. Speciation modeling indicates that if all of the measured metals equilibrated with EDTA, only 2% of the Hg(II) should be EDTA-bound at 350 nmol/L EDTA. Another plausible explanation is that at the high inorganic ligand concentrations present in the culture media, mixed-ligand X-Hg^{2+}-Y complexes were significant enough to increase the extent of $HgEDTA^{2-}$ dissociation at equilibrium. Although few thermodynamic constants are available to create accurate models that include such complexes, this suggestion is not novel (Heitzer et al. 2002). Additional research is needed to understand the bioavailability of EDTA-bound Hg(II).

2.4 Alteration of Hg(II) speciation in natural samples by microbiological media

A concern about the use of biosensors to estimate trace-metal bioavailability under natural conditions arises from the potentially extensive alteration of the original Hg(II) complexation equilibria in natural samples after they are mixed with the biosensor slurries at the start of the conventional assay. This assay solution typically contains about 50% growth medium, which is a highly-buffered solution with ionic strengths near 0.15 mol/L and millimolar chloride, ammonium, and phosphate. In particular, given the well-known sensitivity of Hg biosensors to $[Cl^-]$ (Barkay et al., 1997), it is all but certain that the inorganic speciation of Hg(II) in both M9Cl and $M9NO_3$ incubation media differs from its speciation and thus bioavailability *in situ*. Furthermore, the apparent amino acid-mediated uptake of Hg(II) in the assay (Fig. 2) is unlikely to occur at measurable rates in the environment, since ambient amino acid concentrations are much lower than that in the bioassay solution. Thus, Hg(II) biosensor results are clearly operationally-defined.

Additional complexities arise when conducting bioassays of samples with natural Hg^{2+}-binding ligands present. The natural humic/fulvic acid fraction of Florida Everglades DOM has been shown to contain about 10 nmol/L per mg-C of strong Hg^{2+}-binding sites with association constants of approximately 1028.5 $(mol/L)^{-1}$ (Heitzer et al., 2002). There are also a variety of weaker sites, but the strongest should dominate Hg(II) speciation in freshwater samples containing similar DOM (if these sites and dissolved Hg^{2+} approach equilibrium) unless [Hg(II)] greatly exceeds ambient levels. In the lakewater titration experiments depicted here, bioavailable Hg(II) was not measurable at ambient [Hg(II)] levels, but in contrast to the model calculations, only partial complexation by DOM was observed over the entire range of Hg(II) additions in

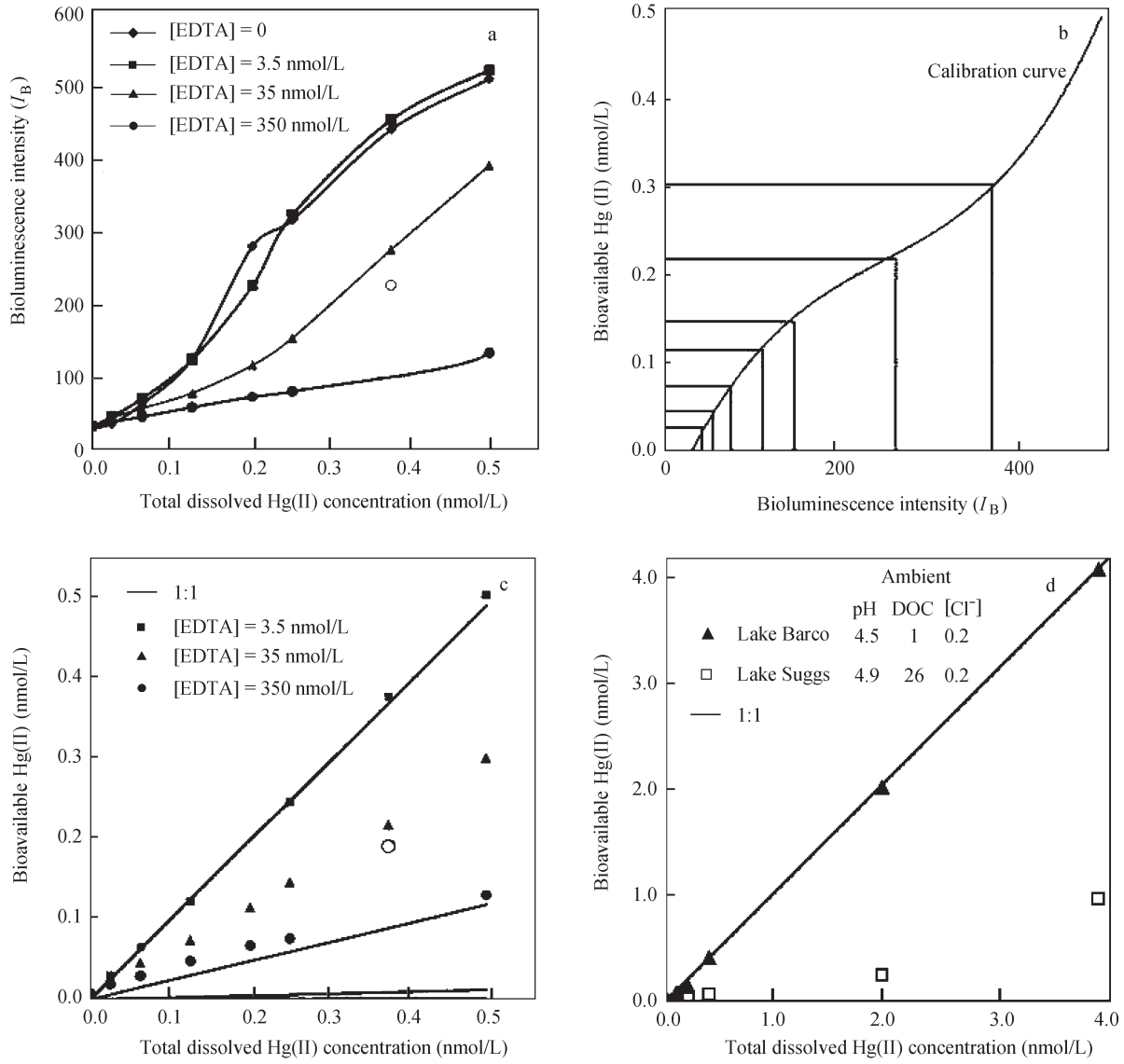

Fig. 3 Observed I_B in EDTA-containing solutions mixed with M9Cl medium (a), example use of transposed calibration curve to infer bioavailable Hg(II) for 35 nmol/L EDTA data (b), bioavailable Hg(II) inferred from data in (a) (symbols) and from MINEQL calculations with no other metals present (lines) as in Barrocas (2003) (c), inferred bioavailable Hg(II) for titrations of Lakes Barco and Suggs samples mixed with M9Cl (DOC in mg/L; [Cl^-] in mmol/L) (d). Open circles in (a) and (c) are one outlier from 350 nmol/L EDTA curve. Calibration curve for (d) not shown.

the high-DOC (26 mg/L) Lake Suggs (Fig. 3d). Unless no strong site was present in the DOM of these lakes, such results suggest that slow kinetics hindered their reaction of Hg(II). It is possible that some of the trace metals added with the culture media competed with Hg^{2+} for binding to these sites in DOM.

2.5 Addressing the challenges

These methodological challenges mandate that one carefully interpret or even reinterpret conventional Hg(II)-bioassay results. For example, if the marked dichotomy between strong and weak Hg(II)-binding sites observed by Heitzer et al. (2002) is generally important, it may be possible to interpret "bioavailable Hg(II)" in natural samples simply as Hg(II) not bound to strong sites in DOM. However, slow complexation kinetics with strong sites, as likely observed here, would make the use of biosensor titrations to quantify Hg(II)-DOM stability constants problematic. Modeling that takes kinetics into account could yield improved interpretations of extant data. It seems most certain, however, that the *in situ* effects on Hg(II) uptake of variables such as pH and [Cl^-] are very difficult, if not impossible, to quantify when the Hg(II)-exposure step involves mixtures of natural samples with bacterial growth media.

An alternative to theoretically-based data (re)interpretation is to directly address these challenges by developing novel approaches for applying the Hg(II)-biosensor technology. In fact, it has been shown (Barrocas, 2003) that the largest confounding factor can be avoided simply by separating the combined exposure/incubation step of the conventional bioassay in time. In this novel procedure, one simply rinses the biosensor cells free of growth media prior to exposing them to bioavailable Hg(II) in natural samples or laboratory solutions. After Hg(II) exposure, one incubates the microbes under optimal

nutritional conditions, i.e., in growth medium, to promote luciferase synthesis and then quantifies the response using bioluminescence measurements. Subsequent reports will describe this work.

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