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TITLE:

An Anaerobic Biosensor Assay for the Detection of Mercury and Cadmium

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SUMMARY:

Here, we present a protocol to use an anaerobic whole-cell microbial biosensor to evaluate how different environmental variables affect the bioavailability of Hg and Cd to bacteria in anoxic environments.

ABSTRACT:

Mercury (Hg) bioavailability to microbes is a key step to toxic Hg biomagnification in food webs. Cadmium (Cd) transformations and bioavailability to bacteria control the amount that will accumulate in staple food crops. The bioavailability of these metals is dependent on their speciation in solution, but more particularly under anoxic conditions where Hg is methylated to toxic monomethylmercury (MeHg) and Cd persists in the rhizosphere. Whole-cell microbial biosensors give a quantifiable signal when a metal enters the cytosol and therefore are useful tools to assess metal bioavailability. Unfortunately, most biosensing efforts have so far been constrained to oxic environments due to the limited ability of existing reporter proteins to function in the absence of oxygen. In this study, we present our effort to develop and optimize a whole-cell biosensor assay capable of functioning anaerobically that can detect metals under anoxic condition in quasi-real time and within hours. We describe how the biosensor can help assess how chemical variables relevant to the environmental cycling of metals affect their bioavailability. The following protocol includes methods to (1) prepare Hg and Cd standards under anoxic conditions, (2) prepare the biosensor in the absence of oxygen, (3) design and execute an experiment to determine how a series of variable affects Hg or Cd bioavailability, and (4) to quantify and interpret biosensor data. We show the linear ranges of the biosensors and provide examples showing the method's ability to distinguish between metal bioavailability and toxicity by utilizing both metal-inducible and constitutive strains.

INTRODUCTION:

Mercury (Hg) is a global pollutant and its bioavailability to Hg methylating microbes is the first step towards its biomagnification through food webs and its possible neurotoxic effects in human and wildlife[1]. It is currently thought that microbial Hg methylation is an intracellular process that requires: i) the species of Hg to be bioavailable[2-7] and ii) for the cell to be physiologically capable of methylating Hg [8-10]. Cadmium (Cd) bioaccumulates in organisms, but does not biomagnifies in foodwebs and is widely used in industrial and commercial processes that commonly cause acute exposures in people and the environment[11]. Although microbes play several key roles in the fate of Hg in the environment, most studies on Cd geochemistry and ecotoxicology focus on microbial eukaryotes[12]. Consumption of agricultural crops (*e.g.,* rice) is the main source of direct exposure to cadmium; in this case, the bioavailability of Cd to microbes of the rhizosphere directly influences the amount plants can accumulate[13].

Hg transport pathways are complex and possibly involve an active transport step[14]. When a transporter is involved, recent work suggested that Hg^{II} uses a Zn^{II} or Mn^{II} transporter[7,15-17]. Whereas Cd^{II} is hypothesized to be accidentally transported into the cytosol through divalent metal transport pathways (particularly Mn^{II} or Zn^{II}), mechanisms of Cd^{II} transport inside the cells remain speculative, and no Cd-specific transport pathway has been identified[13,18]. Regardless of the nature of the transporters involved, three mechanistic factors ultimately determine the ability of metals to enter a cell: i) the metal speciation in solution[2,6,15-17,19-23], ii) the biophysiochemical properties of the cell membrane[17,24-31], and iii) the ability for the metal to access a transport site[7,32]. Cd and Hg are unlikely to exists as free ions under microbial physiologically relevant conditions due to their high affinity for Dissolved Organic Matter (DOM), chelating contaminants (*e.g.,* EDTA), or reduced sulfur moieties[33-35] (Cd^{II} can exist as a free ion or form ion-pairs in the absence of these ligands). There is a lack of efficient methods in determining how these metal species are bioavailable under conditions relevant to their fate in the environment. For instance, Hg is methylated under anaerobic conditions[14], and both cadmium and Hg are soft metals (or class B cations), requiring that their speciation be investigated under conditions that maintain the integrity of reduced sulfur groups.

Microbial biosensors are bacterial cells that emit a quantifiable signal in response to the intracellular concentrations of a metal, in this case Hg or Cd. As such, they are ideal tools to understand how metals enter a cell[36], provided that exposure conditions are carefully controlled for. Hg biosensors typically contain gene fusions between the regulatory circuitry of the *mer*-operon (including genes encoding for the transcription regulator MerR as well as the operator and promoter regions of the operon), and reporting genes (*e.g., lux, gfp, lac* genes). When mercury is present in the cytoplasm, it will bind to MerR, resulting in transcription of the reporting genes and subsequent signal production[19,37]. Specific Cd biosensors are usually designed using the *cadC*, *cadAC, zntA* or *zntR* encoded transcription regulators[38], but it is worth noting that MerR has a lower, yet quantifiable affinity to Cd[5]. Due to aerobic restriction of most commonly used luminescent or fluorescent reporter proteins, until recently microbial biosensors remained unable to offer insights into the biotransformation of metals under anoxic conditions. This makes anaerobic detection of metals bioavailability very difficult over a range of conditions relevant to their environmental fate, specifically in the presence of redox sensitive ligands (*e.g.,* sulfide and thiols)[4,5,39].

To alleviate the methodological hurdle of live imaging in the absence of oxygen, Drepper *et al.* (2007) have developed a flavin-based fluorescent protein (FbFp), based on light oxygen voltage domain of SB2 protein from *P. putida*. This protein family is able to fluoresce in the absence of oxygen[40]. Building on the work of Drepper *et al.*, our lab designed an anaerobic biosensor allowing for the study of Hg bioavailability under oxic and anoxic conditions and over a wide range of salinity [17]. In the current paper, we describe how to prepare and use the biosensor to test environmental variables' influence on Hg or Cd bioavailability. Although we developed the biosensor for Hg^{II}, we chose to perform experiments with Cd^{II} as a means to draw the reader's attention to the fact that biosensors may also respond to multiple stressors that are likely to co-occur in environmental matrices; in this case Cd^{II} was investigated because it is known to bind to the transcriptional regulator MerR[5]. Here, we show representative calibration and functional linear ranges with respect to either metal. We also give an example when the biosensor's results are conclusive (Mg^{II} and Mn^{II} on Hg bioavailability) and inconclusive (Zn^{II} on Hg bioavailability).

PROTOCOL:

1. Growth Media and Exposure Media Preparation

1.1. To make 250 mL of **growth medium**:

Note: If **trace elements #1 solution** and **trace elements #2 solution** are already prepared, skip to step 1.1.5.

1.1.1. Prepare **trace elements #1 solution** in a clean volumetric flask (200 mL) to contain the final molarities of 1.5 x 10^{-3} M Na_2MoO_4, 6.5 x 10^{-4} M Na_2SeO_4, 5 x 10^{-3} M H_3BO_3, and 0.1 M NaOH.

CAUTION: Strong bases (NaOH) are corrosive. Make sure when weighing the reagents to ensure that the final molarity represents the key trace elements; Mo, Se, and B.

1.1.2. Under a sterile field, filter sterilize using a 0.22 µm polyethersulfone syringe filter in a clean/sterile plastic/Polytetrafluoroethylene (PTFE) bottle.

1.1.3. Prepare **trace elements #2 solution** in a clean volumetric flask (200 mL) to contain the final molarities of 0.01 M $MnSO_4$, 5 x 10^{-4} M $ZnSO_4$, 3.25 x 10^{-3} M $CoCl_2$, 6.25 x 10^{-3} M $NiCl_2$, and 0.1 M H_2SO_4.

CAUTION: Strong acids (H_2SO_4) are corrosive. Make sure when weighing the reagents to ensure that the final molarity represents the key trace elements; Mn, Zn, Co, and Ni.

1.1.4. Under a sterile field, filter sterilize using a 0.22 µm polyethersulfone syringe filter in a clean glass bottle.

1.1.5. Under a sterile field, in a clean/sterile glass bottle (250 mL minimum); add 200 mL

ultrapure water, 42.5 mL of M9 Minimal Salts (5x; see **Table of Materials**), 2.5 mL of 2 M Glucose, 125 µL of 2 M $MgSO_4$, 1200 µL of 0.6 M Thiamine HCl from a frozen (-20 ˚C) aliquot, 1.25 mL of 4 M $NaNO_3$, 770 µL of 0.075 M L-leucine/ L-isoleucine/ valine solution, 250 µL **trace elements #1**, 250 µL **of trace element #2**, and 250 µL of 0.01 M EDTA sodium salt.

Note: All reagents in step 1.1.5 should be prepared prior and filter sterilized using a 0.22 µm polyethersulfone syringe filter. Ultrapure water may be sterilized using an autoclave.

1.1.6. Take the glass bottle now containing solution from step 1.1.5., loosely cap it and cycle it through the **anaerobic chamber** air lock.

1.1.7. In the **anaerobic chamber**, add 15 µL of 0.225 M $FeSO_4$ in 0.2 M H_2SO_4, cap bottle tightly, and shake until all white precipitates disappear.

Note: All reagents in step 1.1.7 should be prepared prior and filter sterilized using a 0.22 µm polyethersulfone syringe filter. Store the 0.225 M $FeSO_4$ in 0.2 M H_2SO_4 in the **anaerobic chamber**.

1.1.8. Remove cap from bottle, wait 1 minute for air to exchange, replace the cap from bottle, and then shake vigorously. Repeat this step once.

1.1.9. Fasten cap tightly onto the bottle, remove from **anaerobic chamber**, and store bottle in fridge (4 ˚C) until use.

1.1.10. **Growth medium** should be remade once a week by repeating steps 1.1.5 to 1.1.9.

1.2. To make 100 mL of **exposure medium** in 2 x 50 mL conical sterile polypropylene centrifuge tubes:

Note: We recommend that **exposure medium** be prepared on the day of the **exposure assay** to minimize the risk of contamination, however it can be made in advance and stored in the fridge.

1.2.1. In the **anaerobic chamber**, to each 50 mL centrifuge tube add 42 mL of anaerobic ultrapure water, 350 µL of 1 M Sodium Beta-Gylcerophosphate from a frozen (-20 ˚C) aliquot, 2 mL of 1 M MOPS free acid, 50 µL of 1 M $(NH_4)_2SO_4$, 125 µL of 2 M Glucose, 300 µL of 2.5 M KOH, and 200 µL of 2.5 M NaOH.

Note: All reagents in step 1.2.1 should be prepared prior and filter sterilized using a 0.22 µm polyethersulfone syringe filter. Ultrapure water may be heat sterilized using an autoclave. 2.5 M NaOH and 2.5 M NaOH must be stored in plastic/PTFE bottles. All reagents listed must be stored in the **anaerobic chamber** except Sodium Beta-Gylcerophosphate, which is to be stored in a (-20 ˚C) freezer. In general, it is good practice to leave all plastic or glass parts and containers for several days in the anaerobic chamber to ensure that no traces of oxygen remain.

1.2.2. Cap the 50 mL centrifuge tubes, shake well, and remove a 10 mL aliquot to measure the pH. The measured pH of this exposure media must always measure **7.00 ± 0.02 at 25 ˚C**. If the pH does not measure within this range, readjust the volume of added 2.5 M KOH to correct for this.

Note: Never titrate the solution to correct for the pH with a pH probe. pH probes represent a source of contamination for the **exposure medium**. The pH must always be a product of the added reagents in step 1.2.1.

1.3. Prepare a 5-10 mM $NaNO_3$ stock solution and leave in **anaerobic chamber** to be used during the time of the **exposure assay**.

Note: It is easier to dilute a more concentrated primary $NaNO_3$ standard as opposed to making a 5-10 mM $NaNO_3$ primary standard.

2. Preparation of Mercury and Cadmium Standards.

2.1. Preparing a 4-8 µM Mercuric (Hg^{II}) solution in 0.2 M H_2SO_4.

2.1.1. Prepare a Hg^{2+} standard by making a millimolar (1-10 mM) $HgCl_2$, $HgNO_3$ or $HgSO_4$ solution in 0.2 M H_2SO_4.

CAUTION: Mercury is highly toxic and H_2SO_4 is corrosive. Operate in fume hoods with all required personal protective equipment.

2.1.2. In a PTFE bottle, dilute standard from 2.1.1. into 0.2 M H_2SO_4 to obtain a concentration within the range of 4-8 µM Hg^{2+}.

Note: The acid used must be analytical grade H_2SO_4.

2.1.3. The Hg concentration from 2.1.2 should be validated using a mercury analyzer (see **Table of Materials**).

Note: Other methods for validating the Hg concentration may be used.

2.2. Preparing a 10 µM Cd^{II} solution in 10 mM H_2SO_4.

2.2.1. Prepare a primary standard of $CdCl_2$ (10-50 mM range for accurate weighing of powder) in 0.1 M H_2SO_4.

2.2.2. In a series of serial dilutions in clean acid rinsed 20 mL borosilicate glass vials with black phenolic screw caps with PTFE faced rubber liners, dilute the standard from 2.2.1 to 10 µM Cd^{2+}. Ensure that the concentration of H_2SO_4 in every subsequent serial dilution remains 10 mM.

3. Preparation of the Biosensor for Anaerobic Exposure Assay

3.1. Plate the mercury inducible biosensor (E. coli NEB5α harboring PUC57merR-PpFbFp) and the constitutively expressed biosensor (E. coli NEB5α harboring PUC19Balch-PpFbFp) from -80 ˚C cryostock onto Lysogeny Broth plates containing 120 ug/mL ampicillin. See our previously published work for details on the production of these biosensors[17].

3.2. At 4:30-5 PM, inoculate a culture in10 mL of LB (+ amp) and grow overnight.

Note: Starting from a plate it takes **2 days** to prepare the anaerobic culture for the **exposure assay** (*i.e.,* a culture started on Monday afternoon (4:30-5 PM) will be ready for the **exposure assay** at around noon on Wednesday). The following steps are the required microbiological techniques necessary to prepare the cultures on the day of the **Exposure assay**.

3.2.1. Take one colony from the plate culture and add to 10 mL Lysogeny Broth (LB) with **21 µL** of a 100 mg/mL stock solution of ampicillin sodium salt (final concentration is 210 ug/mL amp) in a sterile culture tube.

3.2.2. Place the culture into an incubator/shaker and grow overnight at +37 ˚C with shaking at 220 rpm.

3.3. The next morning at 9-10 AM, resuspend the culture and grow anaerobically throughout the day (20% inoculum).

3.3.1. Bring the culture from the incubator (step 3.2.1) and the **growth medium** (step 1.1.9) into the **anaerobic chamber**.

3.3.2. Add 8 mL of fresh **growth medium** and ampicillin (210 µg/mL) into a sterile Balch tube.

3.3.3. Collect 2 mL of the overnight grown culture and transfer into a 2 mL Microcentrifuge tube. Centrifuge at 10,000 RCF step for 90 seconds, dump supernatant and resuspend in **2 mL** of fresh **growth medium**. Add the resuspended culture to the Balch tube containing 8 mL of fresh **growth medium** and ampicillin.

3.3.4. Using sterile technique, carefully place a rubber stopper on the Balch tube. Remove from the **anaerobic chamber** and place in an incubator/shaker and grow anaerobically until 3-5 PM at +37 ˚C with shaking at 220 rpm.

3.4. Between 3 and 5 PM, perform a 1% anaerobic inoculum into fresh **growth medium** and grow overnight.

3.4.1. Bring the Balch tube (step 3.3.4) into the **anaerobic chamber** along with **growth medium**. Add **100 µL** of the culture to 10 mL of fresh **growth medium** (1% inoculum) with amp (210 ug/mL amp) in a sterile Balch tube.

3.4.2. Using sterile technique, carefully place a rubber stopper on the Balch tube. Remove from the **anaerobic chamber**, place in an incubator/shaker, and grow overnight at +37 ˚C with shaking at 220 rpm.

3.5. Between 9 and 10 AM, resuspend the culture to grow anaerobically throughout the day (20% inoculum).

3.5.1. Bring the culture from the incubator (step 3.4.1) and the **growth medium** (step 1.1.9) into the **anaerobic chamber**.

3.5.2. Add 8 mL of **growth medium** and ampicillin (210 ug/mL amp) into a sterile Balch tube.

3.5.3. Collect 2 m of the overnight grown culture and transfer into a 2 mL Microcentrifuge tube. Centrifuge at 10,000 x g for 90 seconds, dump supernatant, and resuspend in **2 mL** of fresh **growth medium**. Add the resuspended culture to the Balch tube containing 8 mL of fresh **growth medium** and ampicillin.

3.5.4. Using sterile technique, carefully place a rubber stopper on the Balch tube. Remove from chamber and place in an incubator/shaker and grow anaerobically at +37 ˚C with shaking at 220 rpm.

3.6. Monitor the growth of the culture using a spectrophotometer (step 3.5.4) until an **OD_{600} of 0.6** is reached. Be sure to vortex culture prior to each OD reading.

Note: This step takes 3-4 hours, and the **exposure medium** (step 1.2) should be prepared during this time as well as the **exposure assay** (step 4.1).

3.7. Stop the growth when the culture reaches an **OD_{600} of 0.6 (±0.1)** (3-4 hours expected growth).

3.7.1. Bring tube in the **anaerobic chamber** (step 3.6) and transfer culture into 2 x **2 mL** Microcentrifuge tubes. Centrifuge at 10,000 x g for 90 seconds, dump supernatant, and resuspend in 2 x **2 mL** of fresh **exposure medium**.

3.7.2. Repeat washing step 3.7.1. once to remove any trace of the **growth medium**.

3.8. Combine both microcentrifuge tubes of cell culture (step 3.7) into a 7 mL PTFE standard vial to obtain **Biosensor Stock** to be used in the **Exposure Assay** (step 4).

Note: Be sure to mix the **Biosensor Stock** by thoroughly yet gently pipetting back and forth prior to use. The method may be paused here for up to an hour.

4. Exposure Assay

4.1. Designing a plate layout.

Note: Be sure to have all pipetting values calculated and stocks prepared prior to starting the **exposure assay.** How to properly design an experiment and what controls to include is detailed in the text. In addition, the experiment should not be started if there is oxygen in the anaerobic chamber indicated on the anaerobic monitor.

4.1.1. Design the plate layout according to a 96 well template. To run experiments in technical replicates of 3, this will allow for 32 different treatments, which is best represented by a 4 x 8 grid to set up vials (see **Figure 1**).

[Place **Figure 1** here]

Note: When testing for the role of a variable on Hg uptake with the **mercury inducible biosensor;** two treatments are required for each variable: the **treatment** (biosensor + Hg + variable + nitrate) and its **treatment blank** (biosensor + variable + nitrate). When testing for the role of a variable on the physiology of the cell using the **constitutive biosensor,** two treatments are required for each variable: the **treatment** (biosensor + Hg + variable + nitrate) and **treatment blank** (biosensor + variable + Hg). Mercury may be replaced with Cadmium. Hg or Cd will become the variable when performing a calibration curve. The **constitutive** and **inducible** biosensors do not need to be run at the same time (in the same plate layout). A template example for the plate layout and corresponding 4 x 8 grid when testing a concentration range of magnesium (variable) is provided in **Table 1**).

[Place **Table 1** here]

4.2. Set up the 4 x 8 grid according to the assay plate layout.

4.2.1. Place 7 mL PTFE standard vials in the tray.

Note: PTFE vials should be acid washed/heat sterilized prior to use. Vials should only be handled by manipulating the outside of the vial.

4.2.2. To each vial, add the **exposure medium** volume corresponding to each **treatment**.

Note: In an exposure with a total volume of 2000 µL (2 mL), the added **exposure medium** will be: **exposure medium** µL = 2000 µL – **treatment (blank)** µL. (*e.g.,* exposure medium µL = 2000 – 100 µL (biosensor) – 40 µL (nitrate) – 100 µL (Hg) – 100 µL (variable (*e.g.,* $MgSO_4$)) = 1660 µL). Be sure that the volume added of the tested variable does not exceed 5% (100 µL) of the final volume.

4.2.3. To each vial, add the corresponding volume of the solution of the chemical variable to be tested according to the plate layout.

4.2.4. From step 1.3, add nitrate to each vial so that the final concentration is **200 µM** (40-80 µL).

Exclude this step for **constitutive biosensor treatment blanks**.

4.2.5. From step 2.1 or 2.2, add Hg (**5 nM** when testing for a variable) or Cd (**300 nM** when testing for a variable) to the vials according to the plate layout. Exclude this step for **mercury inducible biosensor treatment blanks**.

4.2.5.1. When using Hg, take the **4-8 µM** stock and shake well. Dilute the solution in **exposure medium** in a 7 mL PTFE vial to **100-250 nM** to make a working Hg solution. From this working solution add Hg to the required vials. In this case a calibration curve of Hg ranging from 0 to 12.5 nM.

Note: When testing for a variable's effect on Hg or Cd bioavailability, make sure that the [Hg] or [Cd] remains constant across all treatments. When adding Hg or Cd, be sure to use one pipette tip but never touch the exposure medium in the vials.

4.3. Shake in an orbital motion manually.

Note: The experiment may be paused now depending on the time required for Hg or Cd to speciate in solution. If left for more than an hour, place PTFE caps on the PTFE vials to prevent evaporation/contamination.

4.4. Gently pipette **Biosensor Stock** back and forth to ensure homogeneity. Add 100 µL of **Biosensor Stock** to each vial. Shake orbitally manually.

4.5. Prepare the plate reader to warm up to read with the following criteria: Temperature at **37˚C**, kinetic run for **10 hours** with reads every 2.5-5 minutes with orbital shaking in between reads, and fluorescence measurements with a **fluorescence excitation of 440 nm** and an **emission of 500 nm**.

4.6. 4.7. Pipette **200 µL** from each PTFE vials in the 4 x 8 grid into the corresponding wells of the 96 well plate (Black, 96-Well Clear-Bottom Nonbinding Surface Microplates). Pipette back and forth 5 times before transferring each 200 µL.

Note: Instead of discarding the pipette tip, leave the pipette tip in the PTFE vial to keep track of pipetting progress.

4.7. Place the 96 well plate into the tray of the plate reader, then place the lid on the 96 well plate and begin the assay.

5. Quantifying the Data

5.1. The fluorescence of each **treatment** at each time point must be corrected for each individual well noise and blanked to the **treatment blank.**

5.1.1. The fluorescence for each time point (t) of each **treatment** (**T**) must be translated to account for the initial fluorescence (t0) of the first time point of each well, then averaged across the 3 **treatments** replicates (r_1-r_3)**.** This treatment average must then be blanked to the average **treatment blank** (**TB**) translated in the same manner (Equation 1).

$$Fluorescence\ (t) = average\left(\mathbf{T}_{r1(t)} - \mathbf{T}_{r1(t0)}, \mathbf{T}_{r2(t)} - \mathbf{T}_{r2(t0)}, \mathbf{T}_{r3(t)} - \mathbf{T}_{r3(t0)}\right) - average\left(\mathbf{TB}_{r1(t)} - \mathbf{TB}_{r1(t0)}, \mathbf{TB}_{r2(t)} - \mathbf{TB}_{r2(t0)}, \mathbf{TB}_{r3(t)} - \mathbf{TB}_{r3(t0)}\right) \quad (1)$$

Note: This should be made as a spreadsheet function. Proper propagation of error should also be calculated for each time point.

5.2. Graph the corrected fluorescence of each **treatment** as a function of time

[Place **Figure 2** here]

Note: There is no 0 nM Hg value on the graph, and all other Hg concentrations have been blanked to the 0 nM Hg as a **treatment blank**. Therefore, 0 nM Hg represents the x axis and any positive fluorescence represents fluorescence from Hg given any variable. It is optional to not blank the fluorescence in this manner, but the fluorescent curves will give misleading fluorescence curves if the variable tested has background fluorescence (*i.e.,* dissolved organic matter itself will fluoresce and if there is no treatment blank containing just cells and the dissolved organic matter, increasing dissolved organic matter concentration will increase the fluorescent signal).

5.3. Quantify the fluorescence peak. A quantifiable fluorescence peak will typically occur after 2.5-4 hours, representing the energy expended from the consumption of all 200 µM nitrate as a terminal electron acceptor. This peak should be quantified and represents the final fluorescence value of that specific **treatment**.

Note: In the event no fluorescence peak is observed (*i.e.,* no Hg bioavailability), the fluorescence of **treatment** at the time point of the fluorescence peak of the control (*i.e.,* no added variable or 5nM Hg) should be quantified. Alternatively, if there is fluorescence induction in the treatment, but fluorescence does not produce a well defined peak, there is likely contamination of a higher affinity electron acceptor such as O_2 and measures should be taken to remove traces of oxygen from all solutions and the experiment must be performed again.

REPRESENTATIVE RESULTS:

Once the fluorescence peaks have been quantified according to step 5.3, the result of the fluorescence peaks can be graphed according to the variable concentration illustrating how that variable affects the relative bioavailability of either Hg or Cd. For example, the calibration curve of fluorescence over [Hg^{II}] from **Figure 2** will yield the inducible data presented in **Figure 3A**. For Hg^{II} calibration, the curve will always contain 3 components for the Hg-inducible strain; a threshold response of about 1-2 nM Hg^{II} before fluorescence signal production is linearly proportional to [Hg^{II}], the linear range where **5 nM Hg^{II}** will reliably always be in the center of

that range, and a plateau where increasing [Hg^{II}] will no longer increase fluorescence signal. No change in signal production on the constitutive strain shows that toxicity from [Hg^{II}] does not affect signal production. For Cd^{II} in **Figure 3B**, there are always 2 components for the inducible strain; a linear range where **200-300 nM Cd^{II}** will reliably always be in the center of the linear range and a plateau. A decrease in fluorescence signal with increasing [Cd^{II}] shows that higher Cd concentrations are toxic to the cells and can explain a decrease in the inducible fluorescence production after the plateau at 1000 nM Cd^{II}. Therefore, when testing Hg or Cd bioavailability with respect to an environmental variable, we suggest using **5 nM for Hg^{II}** and **300 nM for Cd^{II}**.

In some instances, signal production can be properly attributed to Hg or Cd bioavailability, but in other cases, signal production can be affected by variation in the physiological state of the biosensor cell host (*e.g.,* the metal of interest or environmental conditions tested are toxic). In **Figure 4A**, 5 nM Hg bioavailability was tested over a gradient of Zn (0-10 µM). In both Hg-inducible and constitutive strains, there is a similar decrease in signal with increasing Zn concentrations. Therefore, one cannot discriminate whether the signal results from lowered bioavailability or is a result of Zn toxicity. In **Figure 4B and 4C**, 5nM Hg bioavailability was tested over a gradient of Mg^{II} (0-10 mM) and Mn^{II} (0-10 µM). Increasing Mg^{II} and Mn^{II} concentrations decreased the fluorescence signal of the inducible strain. On the other hand, the constitutive strain did not show a decrease in fluorescence with increasing Mg^{II} and Mn^{II} concentrations (Mg^{II} and Mn^{II} are beneficial for the cells in the production of the FbFp, as demonstrated by an increase in the fluorescence signal). This demonstrates that the cells are viable and the fluorescence decrease of the Hg-inducible strain results from a decrease in Hg bioavailability. **This data emphasizes how important it is for all biosensor assays to also provide constitutive measurements of overall cell fitness.**

FIGURE AND TABLE LEGENDS:

Figure 1: A 96 well plate (left) and a corresponding 4 x 8 grid containing PTFE vials (right) to be transferred to the plate.

Figure 2: Corrected fluorescence data as a function of time. Fluorescence measured as relative fluorescence units (RFU) emitted by *E. coli* NEB5α harboring the pUC57merR-Pp (Inducible strain) over time with the addition of Hg^{II} (0-12.5 nM) under anaerobic conditions. Fluorescence was the average of 3 technical replicates at 37 ˚C.

Figure 3. Linear ranges of the biosensor with Mercury and Cadmium. Maximum fluorescence measured as relative fluorescence units (RFU) ± 1 Standard Deviation emitted by *E. coli* NEB5α harboring the pUC57merR-Pp (Hg-Inducible) and pUC19Balch-Pp (Constitutive) with the addition of **A)** Hg^{II} (0-15 nM) and **B)** Cd^{II} (0-1000 nM) under anaerobic conditions. Fluorescence was the average of 3 technical replicates at 37 ˚C.

Figure 4. Example of an inconclusive result with Zinc and a conclusive result with Magnesium and Manganese. Maximum fluorescence measured as relative fluorescence units (RFU) ± 1 Standard Deviation emitted by *E. coli* NEB5α harboring the pUC57merR-Pp (Hg-Inducible) and

pUC19Balch-Pp (Constitutive) with the addition of **A)** Zn^{II} (0-10 µM), **B)** Mg^{II} (0-10 mM), and **C)** Mn^{II} (0-10 µM) under anaerobic conditions. [Hg^{II}] was set to 5 nM for all treatments and fluorescence was the average of 3 technical replicates at 37 ˚C.

Table 1: An example plate layout for using the biosensor to test Hg bioavailability (5 nM) over a gradient of Magnesium (0-10 mM)

DISCUSSION:

Microbial biosensors are useful tools to identify mechanisms by which metals are interacting with microbes. Here, we describe a method that can anaerobically quantify Hg^{II} and Cd^{II} bioavailability to a Gram-negative host cell (*E. coli*) and give a quantifiable result within a few hours. One of the major strengths of this protocol is that it allows extensive control of metal speciation in the exposure medium by avoiding strong binding ligands or components that may lead to metal precipitation. Metal speciation has been modelled and tested in this exposure medium using PHREEQC [17], however other metal speciation software may be deployed. In the case of no added ligands, Hg speciation is expected to be present as 97% $Hg(OH)_2$ and 3% $Hg(NH_3)_2^{2+}$, while Cd speciation will be present as 59% Cd-β-Glycerophosphate, 25% Cd^{2+}, and 16% $CdSO_4$. Using a simple thermodynamic modelling software, the user can design exposure media and test for the bioavailability of the metal of interest. In addition, the biosensor host cell (*E. coli* NEB5α) is viable over a wide range of pH (5-8.5) and NaCl concentration (0-0.55 M)[17].

Hg methylation is an anaerobic process, and the protocol outlined in this study does not have a requirement for oxygen, allowing for more accurate description of anaerobic metabolism on metal bioavailability. This is important because the presence of oxygen alters gene expression profiles[48,49,52] and hence, potential transport pathways; therefore this method presents an advantage over currently exising aerobic alternatives. The biosensing construct presented here can potentially be used with other anaerobic hosts that may be more relevant for mercury methylation (*e.g., Geobacter, Desulfovibrio*), but maybe less tractable than *E. coli*. One current limitation of the approach presented here is that our limit of detection has not yet reached pM levels, contrary to existing aerobic systems[4,19,37]. It is however important to note that to achieve these low detection limits several steps need to be taken[44]: i) ligand addition is required to ensure that Hg remains in solution and does not adsorb onto the microbial cell wall (Hg will be irreversible bound to cell surface thiols preventing its bioavailability[25,27]; see the threshold response for Hg in **Figure 3A**), ii) modifications to cell density, or iii) modify the genetic construct to include transport proteins of the *mer*-operon (namely *merT* and *merP*), increasing Hg flux inside the cell[50,51]. These modifications would be beneficial in detecting low concentrations of Hg, but not necessarily ideal when assessing environmentally relevant situations. Whereas previous cadmium biosensors primarily exist as a “proof of principle”, they were designed in complex media that do not allow the investigator to assess the role of speciation on bioavailability[41-44].

The biosensor is an incredibly useful tool in determining mechanisms in which metal species are bioavailable. Because the host organism is not a Hg-methylator, it may only be used

to develop a model for how Hg may enter Gram-negative bacteria and not a definitive rationale for how Hg-methylators acquire Hg. Other methods exist for determining Hg bioavailability, such as methylation, as an outcome of uptake or the use of a mass balance approach[10,15,20,45,46]. That being said, the method presented here offers the advantage of quasi real time bioavailability data in viable cells. We do not recommend that this method be used to quantify total Hg or Cd levels in an environmental matrix. Despite the proposed use of biosensors to determine metal concentrations in the environment[36], many more readily available standard methods are available such as ICP-MS, FAAS (for Cd analysis) or Cold vapor atomic absorption spectroscopy (for Hg analysis). The biosensor can however be used to determine if a given environmental matrix has the potential to enhance or hamper bioavailability; this is achieved by performing standard additions.

The pH of the exposure media may be altered to anywhere within the range of 5 and 8.5, provided the MOPS Free acid (buffer) is exchanged with an alternate free acid of a buffer with the appropriate pKa (please see list of appropriate buffers (Ferreira *et al.* (2015)[47]) and adjusted with KOH to the appropriate pH when making the exposure media. In addition, the method is not limited to Hg and Cd, but could be extended to other metals using other transcription regulators.

The exposure assay may be modified to explore the influence of other electron acceptors (*e.g.,* fumarate) or fermentation on Hg or Cd bioavailability. In that case, important changes will need to be made to the overall method. Indeed, the anaerobic assay was optimized for cells using 200 µM of the terminal electron acceptor (NO_3^-) through dissimilatory nitrate reduction in the exposure medium. Accordingly, cells are grown on NO_3^- and have expressed the required metabolic machinery to do this. Our preliminary tests using lower energy-yielding electron acceptors such as fumarate or cells relying on fermentation, did not produce a reliable fluorescent response to Hg added and the response did not have a replicable linear range.

In summary we would like to emphasize the following points: **I)** It is imperative that the concentrations of Cd or Hg stocks are known in step 2, as these will be used to calibrate the biosensor. **II)** On the exposure day, the growth of cells must be stopped at an OD_{600} of 0.6 (± 0.1) and that care is taken when resuspending the cell cultures, as the biosensor is calibrated to this cell density. **III)** Lastly, it is important that the exposure medium is made meticulously on the exposure day. To ensure the success of the protocol, multiple cultures should be grown simultaneously (to circumvent the possibility of growth failure) and the growth medium should be remade weekly (to circumvent the metastability of the media and possible contamination). It should also be noted that biological replicates (multiple cell cultures) express variability when it comes to signal production. Although the fluorescent responses may vary from culture to culture, the fluorescent trends in response to a given variable should remain the same throughout numerous biological replicates.

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The authors have nothing to disclose.

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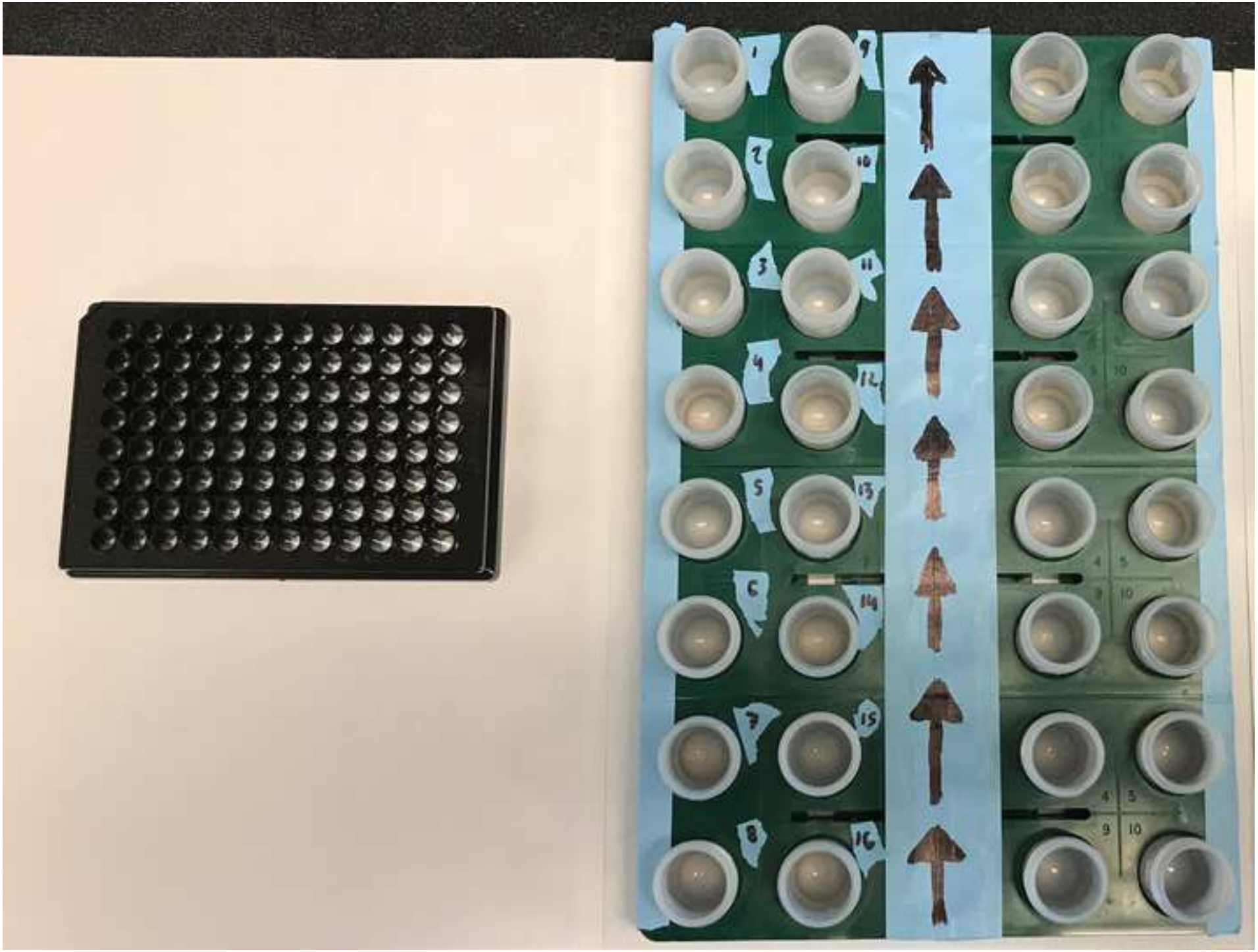

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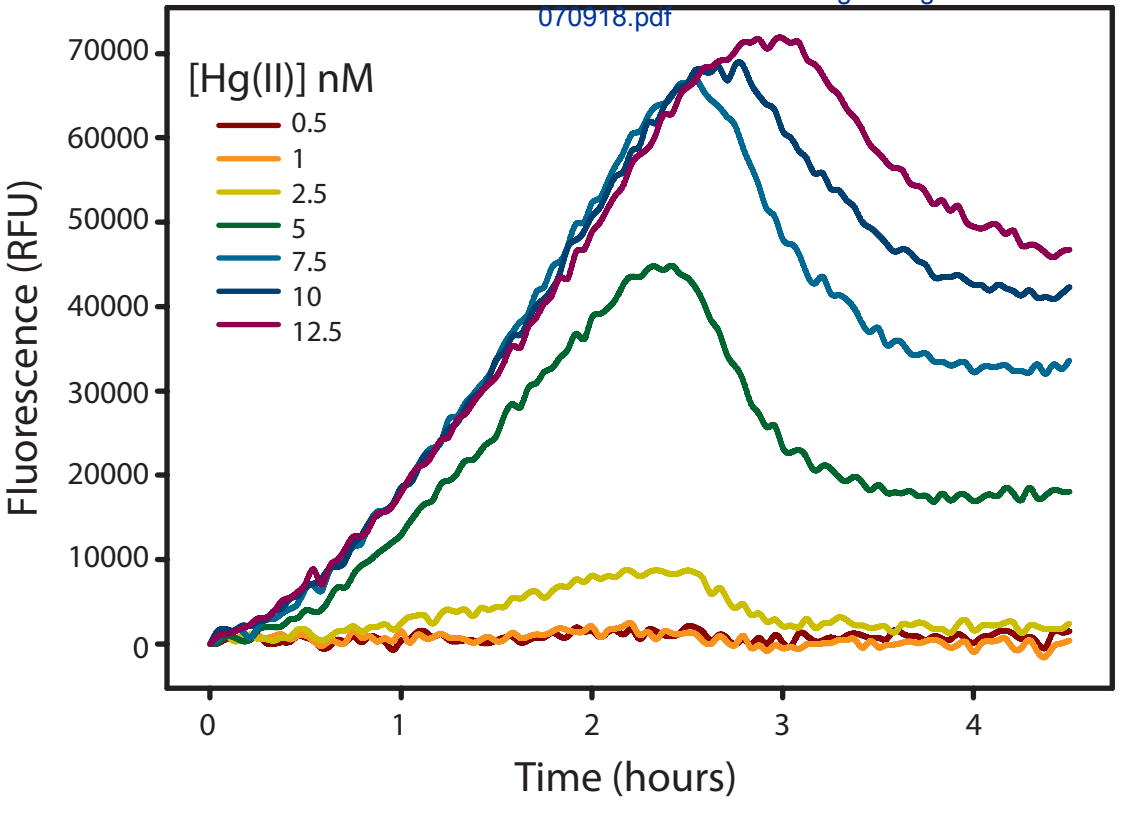

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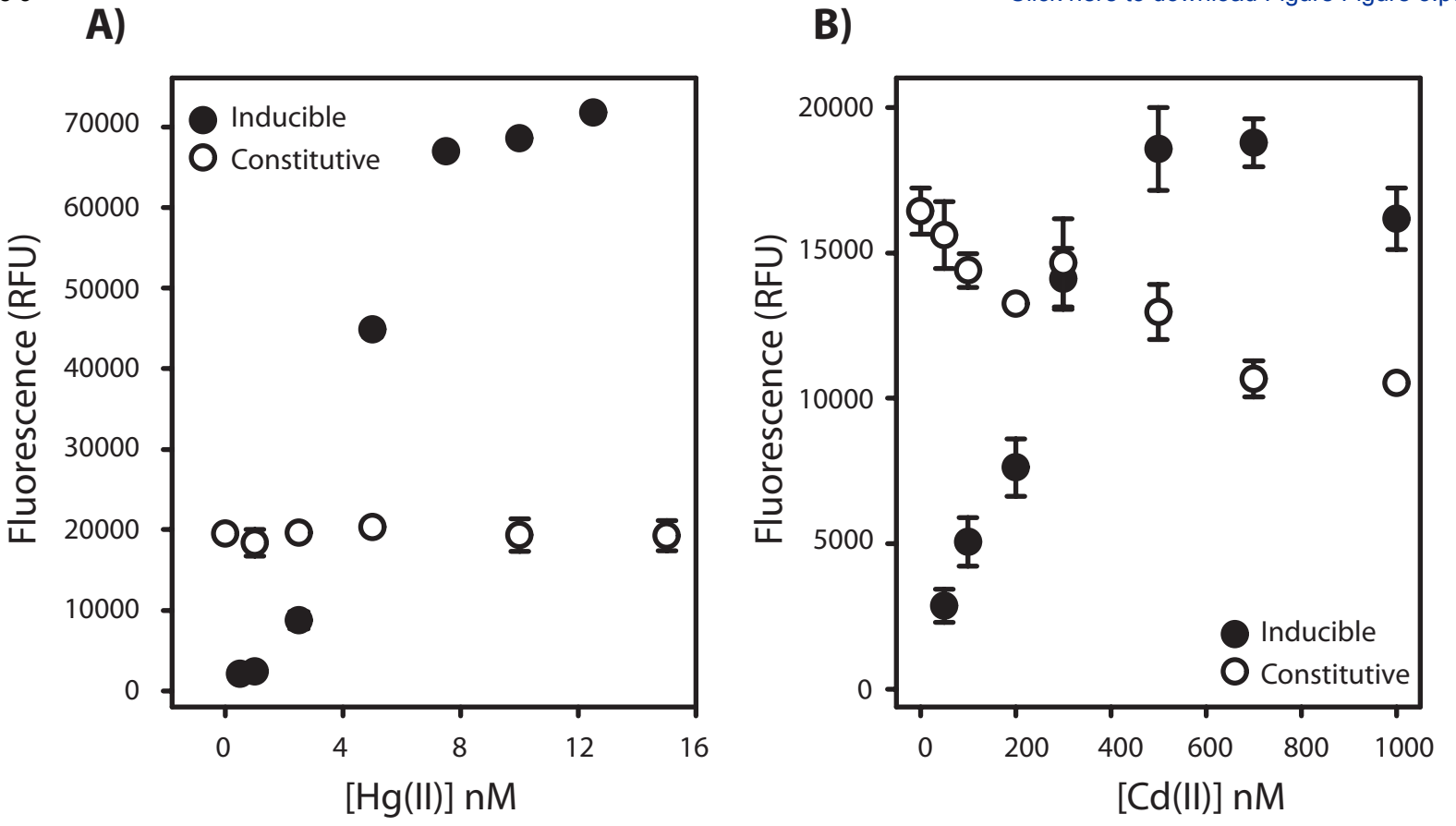

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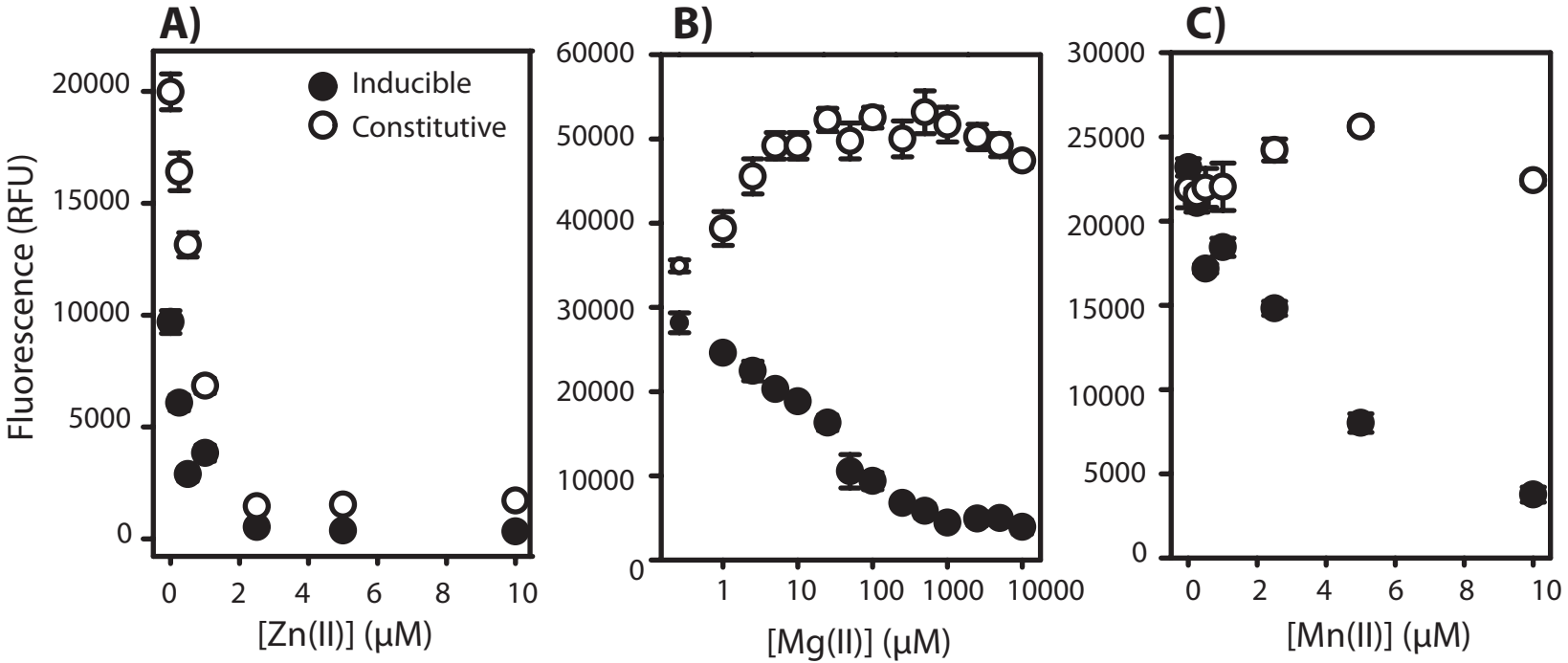

Table 1

	1	2	3	4	5	6	7	8
a	Hg induced biosensor + Hg + Nitrate + 0 mM Mg			Hg induced biosensor + Nitrate + 0 mM Mg			Constitutive bios Hg + Nitrate + 0	
b	Hg induced biosensor+ Hg + Nitrate + 0.1 mM Mg			Hg induced biosensor + Nitrate + 0.1 mM Mg			Constitutive bios Hg + Nitrate + 0.1	
c	Hg induced biosensor + Hg + Nitrate + 1 mM Mg			Hg induced biosensor + Nitrate + 1 mM Mg			Constitutive bios Hg + Nitrate + 1	
d	Hg induced biosensor + Hg + Nitrate + 10 mM Mg			Hg induced biosensor + Nitrate + 10 mM Mg			Constitutive bios Hg + Nitrate + 10	
e								

9	10	11	12
ensor + mM Mg	Constitutive biosensor + Hg + 0 mM Mg		
sensor+ mM Mg	Constitutive biosensor + Hg + 0.1 mM Mg		
ensor + mM Mg	Constitutive biosensor + Hg + 1 mM Mg		
ensor + mM Mg	Constitutive biosensor + Hg + 10 mM Mg		

Table of Materials

Click here to download Table of Materials Stenzler_JoVE_Materials_070918.xlsx

Name of Material/ Equipment	Company	Catalog Number
7 ml standard vial, rounded interior	Delta Scientific	200-007-20
Vial tray, 21 mm openings	Delta Scientific	730-2001
24 mm Closure	Delta Scientific	600-024-01
LID CORNER NOTCH BLK STR CS/50	Corning	Corning 3931
Corning 96- well clear-bottom nonbinding surface microplate	Corning	Corning 3651
Anaerobic Chamber (glovebox)		
Palladium catalyst		
Microplate reader		
450 (±10) nM filter for the microplate reader		
500 (±10) nM filter for the microplate reader		
Anaerobic culture vial (balch tubes) + rubber stoppers		
Spectrophotometer		
MA-3000 (mercury analyzer)		
pH probe		
50 mL conical sterile polypropylene centrifuge tubes.		
0.22 µm polyethersulfone syringe filter + syringe		
Sterile/clean glass bottles		
Sterile/clean plastic or PTFE bottles		

Reagents powders: Na2MoO4, Na2SeO4, H3BO3, NaOH, KOH, MnSO4, ZnSO4, CoCl2, NiCl2, ampicillin sodium salt, Difco M9 Minimal Salts, Glucose, MgSO4, Thiamine HCl, NaNO3, l-leucine, l-isoleucine valine, EDTA sodium salt, FeSO4, Sodium Beta-Gylcerophosphate, Mops free acid, (NH4)2SO4, Hg(NO3)2, CdCl2		
Sterile Milli-Q water		
Lysogeny Broth		
Analytical grade H_2SO_4		

Comments/Description
34 recommended
4 for (4 x 8 grid)
32 recommended
The air in the anaerobic chamber should be (97 % N_2 ± 2 % and 3 % H_2 ± 2 %)
Converts O_2 to H_2O in the anaerobic chamber. Not required but recommended.
Modified for anaerobic culture tubes
Any pH probe will work
For growth media and standards
For alkali solutions (NaOH/KOH)

Autoclaved or filter sterilized is fine.

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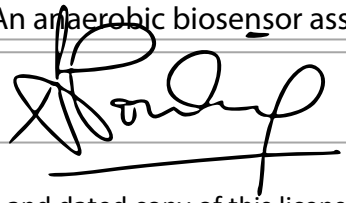
Signature:	
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Dear Dr. Steindel,

Thank you for providing two evaluations of our manuscript, JoVE58324 "An Anaerobic Biosensor Assay for the Detection of Mercury and Cadmium". We have addressed both the editorial comments as well as those of the two anonymous reviewers. We added Jessica Gaudet as an author on this paper because Jessica was able, following the protocol described in this paper, to produce novel knowledge on the bioavailability of Hg and Cd. Her contribution was helpful in testing that the protocol was understandable by an undergraduate student. You will find the manuscript with track changes attached to this submission. We provide answers to the reviewers' comments below.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Done
2. Figure legends: Please define SD. Done
3. Please provide an email address for each author. Was already present in manuscript.
4. Please rephrase the Short Abstract to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …" Done
5. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc. Done
6. Please include a space between all numbers and their corresponding units: 15 mL, 0.2 M, 37 °C, 60 s; etc. Done
7. 1.1.5: Is Disco a typo? Yes, the correct spelling is "Difco" and it's a trademark brand (which we included) for the reagent we used.
8. 3.6: Please define OD600 and describe how is it measured. OD600 is now defined, however further explaining how to use a spectrophotometer to the reader is needlessly superfluous.
9. 4.1.2/4.1.3/5.3: Please revise these steps to contain only action items that direct the reader to do something. The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Done.
10. There is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader. We reduced the highlighted text to 2.75 pages and organized into a concise narrative.
11. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Done.
12. Please revise to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the

outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included. The representative results describe what would occur should the technique be performed properly.
13. References: Please do not abbreviate journal titles. Fixed.

Reviewer #1:
Manuscript Summary:
This manuscript presented an anaerobic microbial biosensor to assess the affects of limited environmental factors on the bioavailability of Hg and Cd. The idea is novel and the biosensor will be useful in future application. In addition, the whole process of protocol is reasonable and was introduced in detail.

Major Concerns:
1. In the anaerobic exposure assay, there should be some methods to test oxygen content and ensure there is no interfered oxygen in the medium. We used an Anaerobic monitor (CAM-12) which detects real time O_2 and H_2 concentrations within the anaerobic chamber. We've included this in the protocol as a test to know whether the chamber is ideal to start the exposure assay. As all reagents being used require equilibration within the chamber air for several days, there is unlikely to be any oxygen present. Typically, a conclusive test to determine the presence of O_2 within solution would be to use redox dyes (e.g., resazurin). However, adding a redox sensitive dye to a fluorescence assay that isn't testing for redox conditions is not the most prudent of ideas. Coincidentally, the best test for the presence of oxygen is intrinsic within the protocol itself. Because *E. coli* is a facultative anaerobe, any trace oxygen that is present within the medium will be used up quickly and is unlikely to impact the experiment; should enough oxygen be present to have a physiological impact on *E. coli*, there will not be a fluorescent peak from nitrate metabolism. We've added this explanation to interpreting the results. We've also included several recommendations for redox indicators for the chamber in the 'materials' in the event the user does have an anaerobic monitor.

2. A standard curve is necessary to show the response range of the biosensor. This is already included for both Hg and Cd.
2. What should be explained more clearly is why there was increasing signal for constitutive strain/decreasing signal for the inducible biosensor with the adding of viable magnesium in the Figure 4-A. Theoretically, the results should be the stable or getting down for the constitutive strain . When the constitutive biosensor is producing more signal it's because whatever variable being added is conducive to the production of the flavin based fluorescent protein. Magnesium is an essential macronutrient for *E. coli* and adding this to a medium that has no Magnesium at all will predictable be beneficial to pretty much any aspect of metabolism. We've succinctly explained this in the results.
3. Test the response of biosensor to zinc which will give more explanations for the inconclusive result (Figure 4-B). We've tested zinc concentrations with the constitutive and mercury inducible

reporter numerous times and it always has a negative affect on fluorescent signal. The rational for including zinc was because it gives an inconclusive result. There are many potential explanations as to why zinc reduces fluorescence signal, beyond being toxic, but that falls outside the scope of the paper and will detract meaning away from the key message of this method study.

Minor Concerns:
1. Correct the reference format:the name of bacteria should be in italic; uniform the abbreviation of journal. Updated all the references to ensure they are all properly formatted.
2. Check and correct the space between number and units in the whole manuscript. Fixed.
3. E.coli in Line225 should not be abbreviated. Fixed
4. There is a sorting error from 4.5 to 4.7 (Line 363-379). Fixed.

In this paper, the authors determined developed a biosensor assay capable of functioning anaerobically that can detect metals under anoxic condition in quasi-real time. In addition, the authors described how the biosensor can help assess how chemical variables relevant to the environmental cycling of metals affect their bioavailability. Last but not the least, this paper provided an example to distinguish between metal bioavailability and toxicity by utilizing together metal-inducible and constitutive strains. After a careful reading of the manuscript and consideration of the current state of this papers, I have come to the conclusion this manuscript can offer enough novelty to stand out as a research manuscript. Therefore, I would like to recommend this manuscript for publication in the Journal of Visualized Experiments after all corrections have been done.

1. General comments

I have prepared some comments on the manuscript, which should be considered by the authors in case the review process continues. The detailed information please refer to the track changes in the manuscript.

1) The manuscript is very well written. However, there are still some missing words, connectors and in some cases it is difficult to understand what is written.

2) This manuscript doesn't have a clear objective. The authors need to add 1-2 sentences to elaborate what the purpose of this research and why this research is important. In the introduction the authors described "we describe how to prepare and use the biosensor to test environmental variables' influence on Hg or Cd bioavailability." This is not an objective of a research paper. This appears to be outside the guidelines given by the

Journal detailing how to write the manuscript.

3) Please delete the content of the protocol. A laboratory protocol is not appropriate to include in a research paper. This appears to be outside the guidelines given by the Journal detailing how to write the manuscript.

4) Please reorganize the methodology part as the following:

2. Methodology
2.1. Preparation of Mercury and Cadmium standards
2.1.1. XXXX
2.1.2. XXXX
2.2. Preparation of the biosensor for anaerobic exposure assay
2.3. The exposure assay
2.4. Quantifying the data

This appears to be outside the guidelines given by the Journal detailing how to write the manuscript.

5) Figure 1 is not appropriate to put into the manuscript. Please put it into the supporting data.

Supporting information is not part of the format of the journal.

6) There is no error bar of any data in Figure 2.

We have made the modification to the figure caption to exclude error bars in the figure. Each point is the average of 3 technical replicates so putting error on the line graph is possible, but this would distract from the overall message of the figure. In addition the error is not large enough to actually change any trend or way to depict the graph and the decision to exclude error is more of a design choice.

7) Line 401-405: Regarding the projection models, the authors need to briefly introduce why Hg concentrations have been blanked to the 0 nM Hg as a treatment blank. In addition, it is also necessary to explain why the fluorescent curves will give misleading fluorescence curves if the variable tested has background fluorescence.

An explanation for blanking is explicitly described for what a misleading fluorescent signal could mean if not blanked this way.