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 O2 and H2 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 O2 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.

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.

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.

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.

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.

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.