

Elsevier Editorial System(tm) for Chemosphere
Manuscript Draft

Manuscript Number: CHEM34688R1

Title: Immobilization of *Shewanella oneidensis* MR-1 in diffusive gradients in thin films for determining metal bioavailability

Article Type: Research Paper

Section/Category: Environmental Toxicology and Risk Assessment

Keywords: biological mobilizing DGT; *Shewanella oneidensis*; BacLight

Corresponding Author: Dr. Paul William Baker, PhD

Corresponding Author's Institution: Bangor University

First Author: Paul William Baker, PhD

Order of Authors: Paul William Baker, PhD; Christer Högstrand, BSc PhD; Jamie Lead; Roger Pickup; Hao Zhang, BSc PhD

Response to Reviewers: COMMENTS FROM EDITORS AND REVIEWERS

Reviewer #1: Immobilization of *Shewanella oneidensis* MR-1 in diffusive gradients in thin films for determining metal bioavailability

CHEM34688

Review: The integration of bacteria into the popular metal sampler DGT opens up a range of exciting new possibilities to probe and quantify complex biogeochemistries in waters and soils. Being the first study where live bacteria has been incorporated successfully into the devices, this work is original, within the remit of Chemosphere, and would appeal to the interests of the general readership.

Overall the experiments have been carefully planned and carried out. The script on the whole is well written, but in numerous places the definition of key concepts or ideas needs to be more clearly expressed. The script also still requires further editing and proof reading, as there are many minor errors or inconsistencies. No further experiments are required. The reference list is of a suitable length and variety. However, some of the measurement units presented within the text need to be changed.

Most of my comments below are relatively minor, and are intended to support and strengthen the work.

Comments:

L2. 'Assessing metal bioavailability in soil is important for assessing...' rephrase.

This sentence has been changed to "Assessing metal bioavailability in soil is important in modelling..."

L3-4. Define what is meant by current methods are limited in their availability and sensitivity. This is an important point that requires further clarification.

The sentence has been extended to include methods: “Current methods based on diffusive gradient thin films (DGTs) and Gel-Integrated Microelectrode....”

L5. 'into the thin layer of agarose...' this statement without prior knowledge of the DGT method could be a point of confusion.

The part of the sentence “we modified DGTs by incorporating *S. oneidensis*, an anaerobic iron reducing bacterium, into the thin layer of agarose to form biologically mobilizing DGT (BMDGT)”, has been replaced with “*S. oneidensis*, an anaerobic iron reducing bacterium, was incorporated into a thin layer of agarose to replace the polyacrylamide gel that is normally present in DGT to form biologically mobilizing DGT (BMDGT).”

L26. The sentence beginning 'One of these methods...' slightly awkward phrasing.

This sentence has been changed to “Diffusive gradients in thin films (DGT) are passive devices which can be easily deployed in natural environments”.

L28. In the abstract it's mentioned that the DGT used was agarose based. Yet, according to the definition presented here, it appears DGT must be based on a polyacrylamide gel matrix and employ Bio-Rad 100 resin. Is this correct? Perhaps provide a more general definition of the method.

This point has been changed in the abstract where it is stated that the agarose gel replaced the polyacrylamide gel matrix.

L37. 'In it's simplest form DGT...' this could be another point of confusion, are there 'advanced' DGT device configurations that evoke more metal cycling processes? It wasn't clear what was meant with this statement.

“In it's simplest form” has been deleted although there are other DGT devices that are in development.

L39-41. This statement could be supported with by a reference.

Reference included: Williams, P.N.; Zhang, H.; Davison, W.; Meharg, A.A.; Hossain, M.; Norton, G.J.; Brammer, H.; Islam, M.R. Organic Matter—Solid Phase Interactions Are Critical for Predicting Arsenic Release and Plant Uptake in Bangladesh Paddy Soils. *Environ. Sci. Technol.* 2011 45 (14) 6080-6087.

L41. Sentence beginning 'Some microorganisms...' a reference could be included to support this statement. Further, some description of the main mechanisms evoked that control the element solubilisation described would be welcomed.

Reference included: Gadd, G.M. Metals, minerals and microbes: geomicrobiology and bioremediation. *Microbiology* (2010), 156 (3), 609–643.

Also continued the sentence with “by siderophores, organic acids and metabolites”.

L49. Explain the shortcomings of biosensors in relation to multi-analyte detection.

Another point has been included at the end of the previous sentence “and knowledge about protein transportation of metals across the cell membrane is limited”

L50. 'should be possible to determine directly their effect on bioavailable metals.' This is a crucial aspect of the experimental rationale; this point could be elaborated on further.

To achieve this the microorganism would need to be evenly distributed throughout the DGT at a high density of viable cells and at the surface so that the microorganisms can interact with insoluble metal colloids.

L52-57. Could the processes leading to iron reduction be explained in more depth? This is important context for the study.

A sentence has been included to describe how this may occur as follows: "It is believed that this occurs by direct electron transfer between the cell membrane and the surface of the oxide when nutrient conditions are low."

L57-58. This sentence appears to have gone somewhat off message. The variable binding site affinities needs to be placed in better context with Cd tolerance. Some minor rephrasing required.

This sentence has been rephrased to "S. oneidensis MR has a high tolerance to cadmium (Mugerfield et al., 2009) and has different affinity binding sites for cadmium (Mishra et al., 2010)".

L96. Change ppb to $\mu\text{g L}^{-1}$

Both have been changed including all those throughout the document to $100 \mu\text{g L}^{-1}$

L98-102. It's not clear how the primary objective of the experiment, which is to determine the attachment of metals to the cells, is met by sub-sampling, followed by serial dilution, then plating on agar and colony counting. Pls. clarify.

A sentence has been included prior to sentence about sub-sampling as follow: "The population of bacteria were determined by culturing so that it would possible to calculate the amount of metal ions associated with each bacterial cell."

L122. The function of the diffusion layer of the DGT has not been adequately introduced prior to this sentence. This could again be a point of confusion for readers not familiar with the DGT method.

The latter part of the sentence describing the diffusion gel has been deleted because a fuller description of DGT assembly occurs in the following paragraph. Another sentence has been included within this paragraph to assist in clarifying the construction of DGT as follows: "The typical DGT is formed of Chelex-100 resin gel, overlaid with a diffusion gel layer and then a polycarbonate filter membrane that are securely held together in a plastic moulding".

L139. Comment on the necessity to keep the deployment solutions 'stationary' and un-mixed. Would there be any issue with DBL's developing?

A sentence has been included to explain the rationale behind using stationary system: "Previous experiments showed that cells were lost from the agarose layer especially during agitation and a stationary system was used in order to maintain the highest number of cells within the agarose layer."

L178. '...Ringers solution to which 1...' rephrase sentence.

"to which" has been replaced with "containing"

L179. '5x1088' is this value what was intended? Pls. check.

Has been changed to 5×10^8

L180. Explain what is meant by 'thin sections'.

"thin sections" has been replaced with "thin cross-sections in order to observe cells within the disks using."

L218-223. This sentence requires some further editing. It is difficult to follow and the text not enclosed in parenthesis doesn't flow.

Sections were deleted from the sentence that appeared to have been repeated. These are: "The deployment of" at the beginning of the sentence and "with *S. oneidensis*" in the middle of the sentence. The sentence in parenthesis has been changed to "*S. oneidensis* grown in minimal medium were immobilized DGTs".

L224. Change 'ppb' to ' $\mu\text{g L}^{-1}$ '

These units have been changed

L224. Solution concentrations are presented as averages and accompanied by a variance term. Define what this term is, i.e. either stdev or se? Furthermore, how many replicate samples were measured. What quality control procedures featured in the metals analysis? These should be described and reported.

The values in parenthesis refer to standard deviations.

The last paragraph in the methods section of 2.2 mentions that each sample was measured in triplicate. A few more words have been included at the end of this paragraph stating: "ensuring that the percentage of rhodium remained consistent throughout all the samples. They were also compared with metal standards ranging from 1-20 $\mu\text{g L}^{-1}$ ".

L225-227. The point being raised here is important, but not communicated very well. Pls. rephrase.

This is quite a complicated point to discuss and has been rewritten using Bill Davison's description as follows: "When DGTs are deployed in stationary solutions, the calculation of the hypothetical diffusible layer is apparently much thicker than it is actually which leads to lower metal concentrations associated with the binding layer. Therefore, it was not surprising that lower metal concentrations were associated with the BMDGTs compared with the concentration found in the immersion solution".

L230. The phrase '...and so measured concentrations were lower.' is not very clear. Some minor rephrasing is required.

The sentence "Lower values were obtained with the BMDGTs compared to the solution because a gradient of metal ions would have extended from the BMDGTs into the stationary solution" has been replaced with "The values in parenthesis refer to standard deviations. When DGTs are deployed in stationary solutions, the calculation of the hypothetical diffusible layer is much thicker than it is actually which leads to lower metal concentrations associated with the binding layer. Therefore, it was not surprising that lower metal concentrations were associated with the BMDGTs compared with the concentration found in the immersion solution."

This sentence has been rephrased as follows: “When BMDGTs containing cells grown in LB were deployed in metal solutions under anaerobic conditions, the concentration of Cd that was associated with BMDGTs was significantly lower compared with the Cd concentration associated with cell free DGTs containing agarose”.

L259. '...cells that the appeared green...' amend.

This sentence has been rephrased as follows: “However, the DNA in the cells that interacted mostly with SYTO-9 and could be described as live cells were green when stained beforehand with SYTO-9, and were yellow when stained afterwards with SYTO-9 and PI simultaneously”.

L263. Is there a reference that supports this conclusion? The phenomena of yellow staining must surely have been observed before.

Another sentence has been included as follows: “The phenomenon of yellow stained cells using BacLight LIVE/ DEAD staining has been observed previously (Boulos et al., 1999)”. This matter is elaborated further in the discussion section along with two references.

L268. The term 'bacterial bioavailability' is this the crux of the BMDGT method or is the measurement centred more on the labile pool that includes a bacterial mobilized fraction? Bacterial bioavailability seems to suggest uptake of metals by bacteria within the gel support, which presumably is something that is not desirable as the aim is to have the metals diffuse through to the binding layer.

Another sentence has been included to clarify that other bacteria could be incorporated into BMDGTs as follows: “Different types of microorganisms could be incorporated into BMDGT using this procedure to assess bacterial bioavailability where the mobility of metals shows an increase as a direct consequence of bacterial presence”.

L285. '...only a small proportion...' could the amount be quantitatively expressed? i.e. put a number on what is a small proportion.

Small has been changed into “3.4 femto grams”.

L287. Again, the minor effect or range of effects on the measurements described should be semi or fully quantified. Do these effects constitute less than a 10% error? Plus are they consistent?

This part of the sentence “are likely to have a minor” has been deleted and replaced with “have almost a negligible”. The point being that the presence of the cells has little impact in binding metal ions to their surfaces.

L304-307. I don't really see what the relevance of this section on biofilms is. Granted it has some importance, but it appears to me as if the discussion is deviating away from more crucial topics, especially as the deployments in this study were short term and as is stated already within the text, biofilms take a long time to develop.

This sentence was deleted.

L321. Define 'saturated soil' do you mean water saturated?

The normal deployment of DGTs in soils requires saturation but it appears that in this context it is misleading. Hence “saturated” was deleted.

L326. Appeared to or did increase?

The words “appeared to” were deleted and replaced with “showed an”.

L330-331. 'and was at in insufficient...' amend.

The words “and was at in insufficient concentration” were replaced with “although at these concentrations were unlikely”.

Figure 2. Change the units. Furthermore it would be useful to mark the solution concentrations, as measured by ICP-MS on the figures as well.

Units have been changed to 100 µg L⁻¹. I have excluded the solution concentrations in order to simplify the graph and focus on the important outcome of this experiment. This data is available within the results section.

Figure 3. There looks like there might be a trend in Fe uptake between aerobic and anaerobic treatments. What could account for the large variability in measured Fe in the anaerobic treatment? Could this have been an analytical measurement issue? Reporting the analysis QC's would help rule out this option.

If more replicates had been deployed to you think a stronger pattern would have emerged. State within the caption, whether the bars refer to stdev or se.

I had overlooked this point but it is apparent that there are large error bars associated with the anaerobic BMDGTs and possibly an increasing trend. Assuming that there is an interaction between which has not been proven, living bacterial cells of *Shewanella* sp. would be interacting with a heterogeneous suspension of hematite colloids that would result in high errors as observed. My experience has shown that increasing the number of replicates may not change the size of the error bars though strangely enough statistics will sometimes reveal a difference. It is perhaps more important to deal with the source of the error and I can only assume that this could be achieved by increasing the number of cells in the BMDGT. It is possible that the error bars could be reduced by increasing the population of bacterial cells in the BMDGT so that the interactions between bacterial cells and hematite colloids occur more frequently. I have made changes to this section as described above.

A sort sentence has been included at the bottom of the caption stating that the error bars are standard deviations.

Graphical abstract. What is diffusible gel? It seems the key message that is trying to be conveyed here is the interaction of the metal ions with the cells within the gel. However, based on the opening text, the rationale for the work is more focused the interaction of the cells within the sampler and the external medium. Pls. clarify this point and if necessary modify the graphical abstract accordingly.

The words “diffusible gel” has been changed to “Diffusive gel layer”.

The text within the arrow has been changed to “Effect of cells on mobilization of metal ions” to reflect a broader message that mobilization or immobilization of metal ions may occur.

COLEG Y GWYDDORAU NATURIOL
COLLEGE OF NATURAL SCIENCES

YSGOL YR AMGYLCHEDD, ADNODDAU NATURIOL A DAEARYDDIAETH
SCHOOL OF ENVIRONMENT, NATURAL RESOURCES AND GEOGRAPHY



21 May 2015

Dear Editor,

Please find the revised manuscript “Immobilization of *Shewanella oneidensis* MR-1 in diffusive gradients in thin films for determining metal bioavailability” and have implemented most of the suggestions provided by the reviewer. I wish to thank the reviewer for thoroughly reading the manuscript and hope that it will be published.

Yours faithfully,

Paul Baker

PRIFYSGOL BANGOR
BANGOR, GWYNEDD,
LL57 2UW, DU

FFÔN: +44 (01248) 38 2281
FFACS: +44 (01248) 354997

BANGOR UNIVERSITY
BANGOR, GWYNEDD,
LL57 2UW, UK

TEL: +44 (01248) 38 2281
FAX: +44 (01248) 354997

M.A.McDONALD, BSc, PhD
PENNAETH YR YSGOL / HEAD OF SCHOOL

RHIF UNIONGYRCHOL / DIRECT LINE: +44 (01248) 38 8076
EBOST/EMAIL: m.mcdonald@bangor.ac.uk

COMMENTS FROM EDITORS AND REVIEWERS

Reviewer #1: Immobilization of *Shewanella oneidensis* MR-1 in diffusive gradients in thin films for determining metal bioavailability

CHEM34688

Review: The integration of bacteria into the popular metal sampler DGT opens up a range of exciting new possibilities to probe and quantify complex biogeochemistries in waters and soils. Being the first study where live bacteria has been incorporated successfully into the devices, this work is original, within the remit of *Chemosphere*, and would appeal to the interests of the general readership.

Overall the experiments have been carefully planned and carried out. The script on the whole is well written, but in numerous places the definition of key concepts or ideas needs to be more clearly expressed. The script also still requires further editing and proof reading, as there are many minor errors or inconsistencies. No further experiments are required. The reference list is of a suitable length and variety. However, some of the measurement units presented within the text need to be changed.

Most of my comments below are relatively minor, and are intended to support and strengthen the work.

Comments:

L2. 'Assessing metal bioavailability in soil is important for assessing...' rephrase.

This sentence has been changed to "Assessing metal bioavailability in soil is important in modelling..."

L3-4. Define what is meant by current methods are limited in their availability and sensitivity. This is an important point that requires further clarification.

The sentence has been extended to include methods: "Current methods based on diffusive gradient thin films (DGTs) and Gel-Integrated Microelectrode...."

L5. 'into the thin layer of agarose...' this statement without prior knowledge of the DGT method could be a point of confusion.

The part of the sentence "we modified DGTs by incorporating *S. oneidensis*, an anaerobic iron reducing bacterium, into the thin layer of agarose to form biologically mobilizing DGT (BMDGT)", has been replaced with "*S. oneidensis*, an anaerobic iron reducing bacterium, was incorporated into a thin layer of agarose to replace the polyacrylamide gel that is normally present in DGT to form biologically mobilizing DGT (BMDGT)."

L26. The sentence beginning 'One of these methods...' slightly awkward phrasing.

This sentence has been changed to "Diffusive gradients in thin films (DGT) are passive devices which can be easily deployed in natural environments".

L28. In the abstract it's mentioned that the DGT used was agarose based. Yet, according to the definition presented here, it appears DGT must be based on a polyacrylamide gel matrix and employ Bio-Rad 100 resin. Is this correct? Perhaps provide a more general definition of the method.

This point has been changed in the abstract where it is stated that the agarose gel replaced the polyacrylamide gel matrix.

L37. 'In its simplest form DGT...' this could be another point of confusion, are there 'advanced' DGT device configurations that evoke more metal cycling processes? It wasn't clear what was meant with this statement.

"In its simplest form" has been deleted although there are other DGT devices that are in development.

L39-41. This statement could be supported with a reference.

Reference included: Williams, P.N.; Zhang, H.; Davison, W.; Meharg, A.A.; Hossain, M.; Norton, G.J.; Brammer, H.; Islam, M.R. Organic Matter—Solid Phase Interactions Are Critical for Predicting Arsenic Release and Plant Uptake in Bangladesh Paddy Soils. *Environ. Sci. Technol.* **2011** *45* (14) 6080-6087.

L41. Sentence beginning 'Some microorganisms...' a reference could be included to support this statement. Further, some description of the main mechanisms evoked that control the element solubilisation described would be welcomed.

Reference included: Gadd, G.M. Metals, minerals and microbes: geomicrobiology and bioremediation. *Microbiology* (2010), *156* (3), 609–643.
Also continued the sentence with "by siderophores, organic acids and metabolites".

L49. Explain the shortcomings of biosensors in relation to multi-analyte detection.

Another point has been included at the end of the previous sentence "and knowledge about protein transportation of metals across the cell membrane is limited"

L50. 'should be possible to determine directly their effect on bioavailable metals.' This is a crucial aspect of the experimental rationale; this point could be elaborated on further.

To achieve this the microorganism would need to be evenly distributed throughout the DGT at a high density of viable cells and at the surface so that the microorganisms can interact with insoluble metal colloids.

L52-57. Could the processes leading to iron reduction be explained in more depth? This is important context for the study.

A sentence has been included to describe how this may occur as follows: "It is believed that this occurs by direct electron transfer between the cell membrane and the surface of the oxide when nutrient conditions are low."

L57-58. This sentence appears to have gone somewhat off message. The variable binding site affinities needs to be placed in better context with Cd tolerance. Some minor rephrasing required.

This sentence has been rephrased to “*S. oneidensis* MR has a high tolerance to cadmium (Mugerfield et al., 2009) and has different affinity binding sites for cadmium (Mishra et al., 2010)”.

L96. Change ppb to $\mu\text{g L}^{-1}$

Both have been changed including all those throughout the document to $100 \mu\text{g L}^{-1}$

L98-102. It's not clear how the primary objective of the experiment, which is to determine the attachment of metals to the cells, is met by sub-sampling, followed by serial dilution, then plating on agar and colony counting. Pls. clarify.

A sentence has been included prior to sentence about sub-sampling as follow: “The population of bacteria were determined by culturing so that it would possible to calculate the amount of metal ions associated with each bacterial cell.”

L122. The function of the diffusion layer of the DGT has not been adequately introduced prior to this sentence. This could again be a point of confusion for readers not familiar with the DGT method.

The latter part of the sentence describing the diffusion gel has been deleted because a fuller description of DGT assembly occurs in the following paragraph. Another sentence has been included within this paragraph to assist in clarifying the construction of DGT as follows: “The typical DGT is formed of Chelex-100 resin gel, overlaid with a diffusion gel layer and then a polycarbonate filter membrane that are securely held together in a plastic moulding”.

L139. Comment on the necessity to keep the deployment solutions 'stationary' and un-mixed. Would there be any issue with DBL's developing?

A sentence has been included to explain the rationale behind using stationary system: “Previous experiments showed that cells were lost from the agarose layer especially during agitation and a stationary system was used in order to maintain the highest number of cells within the agarose layer.”

L178. '...Ringers solution to which 1...' rephrase sentence.

“to which” has been replaced with “containing”

L179. '5x1088' is this value what was intended? Pls. check.

Has been changed to 5×10^8

L180. Explain what is meant by 'thin sections'.

“thin sections” has been replaced with “thin cross-sections in order to observe cells within the disks using..”

L218-223. This sentence requires some further editing. It is difficult to follow and the text not enclosed in parenthesis doesn't flow.

Sections were deleted from the sentence that appeared to have been repeated. These are: “The deployment of” at the beginning of the sentence and “with *S. oneidensis*” in the middle of the

sentence. The sentence in parenthesis has been changed to “*S. oneidensis* grown in minimal medium were immobilized DGTs”.

L224. Change 'ppb' to '<mu>g L-1'

These units have been changed

L224. Solution concentrations are presented as averages and accompanied by a variance term. Define what this term is, i.e. either stdev or se? Furthermore, how many replicate samples were measured. What quality control procedures featured in the metals analysis? These should be described and reported.

The values in parenthesis refer to standard deviations.

The last paragraph in the methods section of 2.2 mentions that each sample was measured in triplicate. A few more words have been included at the end of this paragraph stating: “ensuring that the percentage of rhodium remained consistent throughout all the samples. They were also compared with metal standards ranging from 1-20 $\mu\text{g L}^{-1}$ ”.

L225-227. The point being raised here is important, but not communicated very well. Pls. rephrase.

This is quite a complicated point to discuss and has been rewritten using Bill Davison’s description as follows: “When DGTs are deployed in stationary solutions, the calculation of the hypothetical diffusible layer is apparently much thicker than it is actually which leads to lower metal concentrations associated with the binding layer. Therefore, it was not surprising that lower metal concentrations were associated with the BMDGTs compared with the concentration found in the immersion solution”.

L230. The phrase ‘...and so measured concentrations were lower.’ is not very clear. Some minor rephrasing is required.

The sentence “Lower values were obtained with the BMDGTs compared to the solution because a gradient of metal ions would have extended from the BMDGTs into the stationary solution” has been replaced with “The values in parenthesis refer to standard deviations. When DGTs are deployed in stationary solutions, the calculation of the hypothetical diffusible layer is much thicker than it is actually which leads to lower metal concentrations associated with the binding layer. Therefore, it was not surprising that lower metal concentrations were associated with the BMDGTs compared with the concentration found in the immersion solution.”

This sentence has been rephrased as follows: “When BMDGTs containing cells grown in LB were deployed in metal solutions under anaerobic conditions, the concentration of Cd that was associated with BMDGTs was significantly lower compared with the Cd concentration associated with cell free DGTs containing agarose”.

L259. ‘...cells that the appeared green...’ amend.

This sentence has been rephrased as follows: “However, the DNA in the cells that interacted mostly with SYTO-9 and could be described as live cells were green when stained beforehand with SYTO-9, and were yellow when stained afterwards with SYTO-9 and PI simultaneously”.

L263. Is there a reference that supports this conclusion? The phenomena of yellow staining must surely have been observed before.

Another sentence has been included as follows: “The phenomenon of yellow stained cells using *BacLight LIVE/ DEAD* staining has been observed previously (Boulos et al., 1999)”. This matter is elaborated further in the discussion section along with two references.

L268. The term 'bacterial bioavailability' is this the crux of the BMDGT method or is the measurement centred more on the labile pool that includes a bacterial mobilized fraction? Bacterial bioavailability seems to suggest uptake of metals by bacteria within the gel support, which presumably is something that is not desirable as the aim is to have the metals diffuse through to the binding layer.

Another sentence has been included to clarify that other bacteria could be incorporated into BMDGTs as follows: “Different types of microorganisms could be incorporated into BMDGT using this procedure to assess bacterial bioavailability where the mobility of metals shows an increase as a direct consequence of bacterial presence”.

L285. '...only a small proportion...' could the amount be quantitatively expressed? i.e. put a number on what is a small proportion.

Small has been changed into “3.4 femto grams”.

L287. Again, the minor effect or range of effects on the measurements described should be semi or fully quantified. Do these effects constitute less than a 10% error? Plus are they consistent?

This part of the sentence “are likely to have a minor” has been deleted and replaced with “have almost a negligible”. The point being that the presence of the cells has little impact in binding metal ions to their surfaces.

L304-307. I don't really see what the relevance of this section on biofilms is. Granted it has some importance, but it appears to me as if the discussion is deviating away from more crucial topics, especially as the deployments in this study were short term and as is stated already within the text, biofilms take a long time to develop.

This sentence was deleted.

L321. Define 'saturated soil' do you mean water saturated?

The normal deployment of DGTs in soils requires saturation but it appears that in this context it is misleading. Hence “saturated” was deleted.

L326. Appeared to or did increase?

The words “appeared to” were deleted and replaced with “showed an”.

L330-331. 'and was at in insufficient...' amend.

The words “and was at in insufficient concentration” were replaced with “although at these concentrations were unlikely”.

Figure 2. Change the units. Furthermore it would be useful to mark the solution concentrations, as measured by ICP-MS on the figures as well.

Units have been changed to $100 \mu\text{g L}^{-1}$. I have excluded the solution concentrations in order to simplify the graph and focus on the important outcome of this experiment. This data is available within the results section.

Figure 3. There looks like there might be a trend in Fe uptake between aerobic and anaerobic treatments. What could account for the large variability in measured Fe in the anaerobic treatment? Could this have been an analytical measurement issue? Reporting the analysis QC's would help rule out this option.

If more replicates had been deployed to you think a stronger pattern would have emerged. State within the caption, whether the bars refer to stdev or se.

I had overlooked this point but it is apparent that there are large error bars associated with the anaerobic BMDGTs and possibly an increasing trend. Assuming that there is an interaction between which has not been proven, living bacterial cells of *Shewanella* sp. would be interacting with a heterogeneous suspension of hematite colloids that would result in high errors as observed. My experience has shown that increasing the number of replicates may not change the size of the error bars though strangely enough statistics will sometimes reveal a difference. It is perhaps more important to deal with the source of the error and I can only assume that this could be achieved by increasing the number of cells in the BMDGT. It is possible that the error bars could be reduced by increasing the population of bacterial cells in the BMDGT so that the interactions between bacterial cells and hematite colloids occur more frequently. I have made changes to this section as described above.

A sort sentence has been included at the bottom of the caption stating that the error bars are standard deviations.

Graphical abstract. What is diffusible gel? It seems the key message that is trying to be conveyed here is the interaction of the metal ions with the cells within the gel. However, based on the opening text, the rationale for the work is more focused the interaction of the cells within the sampler and the external medium. Pls. clarify this point and if necessary modify the graphical abstract accordingly.

The words "diffusible gel" has been changed to "Diffusive gel layer".

The text within the arrow has been changed to "Effect of cells on mobilization of metal ions" to reflect a broader message that mobilization or immobilization of metal ions may occur.

Immobilization of *Shewanella oneidensis* MR-1 in diffusive gradients in thin films for determining metal bioavailability

Paul W Baker*¹, Christer Högstrand², Jamie Lead³, Roger W Pickup⁴ and Hao Zhang¹

¹ Lancaster Environmental Centre, Lancaster University, Bailrigg, Lancaster LA1 4YQ UK

² School of Biomedical Sciences, 1.14 Hodgkin Building, Guy's Campus, London LE1 1UL UK

³ School of Geography, Earth and Environmental Sciences, University of Birmingham, Edgbaston, Birmingham B15 2TT UK

⁴ Division of Biomedicine and Life Sciences, Faculty of Health and Medicine, Lancaster University, Lancaster LA1 4YQ UK

Footnote:

Present address of Paul W. Baker: SENRGY, Bangor University, Bangor, Gwynedd LL57 2UW, Wales

1 **Abstract**

2 Assessing metal bioavailability in soil is important in modelling the effects of metal toxicity on
3 the surrounding ecosystem. Current methods based on diffusive gradient thin films (DGTs) and
4 Gel-Integrated Microelectrode are limited in their availability and sensitivity. To address this, *S.*
5 *oneidensis*, an anaerobic iron reducing bacterium, was incorporated into a thin layer of agarose to
6 replace the polyacrylamide gel that is normally present in DGT to form biologically mobilizing
7 DGT (BMDGT). Viability analysis revealed that 16-35% of the cells remained viable within the
8 BMDGTs depending on the culturing conditions over a 20 h period with/without metals.

9 Deployment of BMDGTs in standardized metal solutions showed significant differences to cell-
10 free BMDGTs when cells grown in Luria Broth (LB) were incorporated into BMDGTs and
11 deployed under anaerobic conditions. Deployment of these BMDGTs in hematite revealed no
12 significant differences between BMDGTs and BMDGTs containing heat killed cells. Whether
13 heat killed cells retain the ability to affect bioavailability is uncertain. This is the first study to
14 investigate how a microorganism that was incorporated into a DGT device such as the metal
15 reducing bacteria, *S. oneidensis*, may affect the mobility of metals.

16

17 **Keywords**

18 diffusive gradient thin films (DGT)

19 biological mobilizing DGT

20 *Shewanella oneidensis*

21 *BacLight*

22

23 **1. Introduction**

24

25 Assessment of the potential toxicity of metals requires consideration of their
26 bioavailability rather than their total concentrations in an environment. Chemical speciation,
27 bioavailability and methods used in detection of bioavailable metals have been thoroughly
28 reviewed (van Leeuwen et al., 2005 and Pesavento et al., 2009). **Diffusive gradients in thin**
29 **films (DGT) are passive devices which can be easily deployed in natural environments** (Zhang,
30 2004). In DGT, labile metal diffuses through a layer of polyacrylamide gel of known thickness
31 and binds to Bio-Rad Chelex®100 resin embedded in a separate polyacrylamide gel (Zhang et
32 al., 1995). Metals binding to the Chelex-100 resin are eluted using concentrated acid and
33 measured using ICP-MS, allowing calculation of the mean flux of metals to the device during its
34 deployment. The mean metal flux can be related to the concentration of labile species in
35 solution: that is those metal species that are mobile and able to be released rapidly from
36 complexes and colloids. The labile metal measured by DGT has been shown to provide a good
37 prediction of the metal taken up by biota where mass transport by diffusion is rate limiting
38 (Degryse et al., 2009).

39 DGT perturbs a chemical environment solely by supplying a sink for metals, which
40 locally lowers the concentration of the free metal ion, in the diffusion layer of the device and its
41 adjacent immersion medium. This depleted concentration induces release of metal from
42 complexes and colloids in solution and, in the case of soils and sediments, from the solid phase

43 (Williams et al., 2011). Some microorganisms in the environment solubilise solid phases and
44 release metals by siderophores, organic acids and metabolites (Gadd, 2010). If such organisms
45 can be incorporated into DGT there is the possibility that the modified DGT will provide a
46 surrogate measurement for environmental metals that have the potential to be mobilized. Dried
47 *Saccharomyces cerevisiae* has already been successfully used in DGT as a binding agent instead
48 of Chelex-100 resin, but there have been no previous attempts to incorporate live
49 microorganisms into DGT and exploit their interactions with chemical substrates (Menegário et
50 al., 2010). Recently, a bioluminescent biosensor has been described containing *Escherichia coli*
51 reporter strains for the detection and toxicity of Cd and As (Charrier et al., 2011). However,
52 these and other biosensors cannot determine the effects of multianalyte detection and knowledge
53 about protein transportation of metals across the cell membrane is limited (Eltzov and Marks,
54 2011). By incorporating naturally occurring microorganisms into DGT it should be possible to
55 determine directly their effect on bioavailable metals. To achieve this the microorganism would
56 need to be evenly distributed throughout the DGT at a high density of viable cells and at the
57 surface so that the microorganisms can interact with insoluble metal colloids.

58 Previous studies of dissimilatory metal reducing bacteria, such as *Shewanella oneidensis*
59 MR-1, showed that insoluble ferric compounds, including hematite and goethite, are reduced by
60 outer membrane bound cytochromes, but only under anaerobic conditions (Meitl et al., 2009 and
61 Ruebush et al., 2006). Other studies showed that (hydr)-oxide ferric deposits within glass beads
62 were indirectly reduced by *S. oneidensis* MR-1 cells growing outside the beads (Lies et al.,
63 2005). It is believed that this occurs by direct electron transfer between the cell membrane and
64 the surface of the oxide when nutrient conditions are low. *S. oneidensis* MR has a high tolerance
65 to cadmium (Mugerfield et al., 2009) and has different affinity binding sites for cadmium

66 (Mishra et al., 2010). Other metals such as chelated cobalt were reduced from [Co(III)-EDTA]⁻
67 to soluble, albeit toxic, [Co(II)-EDTA]²⁻ by *S. oneidensis* MR-1 (Hau et al., 2008). The above
68 evidence suggests that *S. oneidensis* could possibly reduce oxidized forms of Fe and Co at the
69 surface of a gel in which it is encapsulated. Trace metals that are released from iron oxides as
70 they reductively dissolve should be measured by the DGT device.

71 DGT accurately determines the labile metal concentrations in soils and this represents the
72 availability of metals to plants. However, in nature microorganisms may release labile metals
73 from metal colloids thereby increasing the proportion of labile metal. Therefore the aim of this
74 study was to incorporate *S. oneidensis* MR-1 into a DGT device in order to assess metal
75 bioavailability because this microorganism has the capacity for iron III reduction. Once these
76 cells were present within the DGT device, their effect on the concentrations of trace metals
77 measured by DGT in solutions, colloidal suspensions and soils would be determined relative to
78 appropriate controls.

79

80 **2. Methods**

81 *2.1. Growth of Shewanella oneidensis MR-1*

82

83 *Shewanella oneidensis* MR-1 (ATCC 700550, NCIMB 14063) was purchased from the
84 NCIMB culture collection. An aliquot of 100 µl (or 20 µl) of a previous culture in minimal
85 medium (or LB) was added to 20 ml (or 10 ml LB) minimal medium [9 mM ammonium
86 sulphate, 5.7 mM potassium dihydrogen phosphate, 2 mM sodium hydrogen carbonate, 1 mM
87 magnesium sulphate, 0.49 mM calcium chloride, 67.2 µM sodium EDTA, 56.6 µM boric acid,

88 10 μM sodium chloride, 5.4 μM iron II sulphate, 5 μM cobalt sulphate, 5 μM ammonium nickel
89 sulphate, 3.9 μM sodium molybdate, 1.5 μM sodium selenate, 1.3 μM manganese sulphate, 1
90 μM zinc sulphate, 0.2 μM copper sulphate, 20 $\mu\text{g/ml}$ L-arginine, 20 $\mu\text{g/ml}$ L-glutamate, 20
91 $\mu\text{g/ml}$ L-serine, 15 mM lactate, 2 mM fumarate, and 0.1 g/L casamino acids at pH 7.4]. LB is a
92 complex medium consisting of 10 g/L tryptone, 5 g/L yeast extract and 10 g/L sodium chloride.
93 The two different media were used in this study because minimal medium contains the basic
94 nutrients for cell growth and the low metal concentrations in the medium were unlikely to have
95 an effect on BMDGT analysis. LB was used because this medium contained plenty of nutrients
96 that enable to microorganisms to grow well and survive the embedding process during BMDGT
97 formation. However, there was a concern that the high concentration of metals could have an
98 effect on BMDGT analysis. The medium was removed once the cells had grown and were
99 unlikely to affect metal analysis using DGT. The cultures were incubated at 26°C and 110 rpm
100 for 19 h until the cultures had reached a population density of 10^9 cells per ml.

101 A growth curve, defined by culture, of *S. oneidensis* was determined by growing the
102 microorganism in 25 ml tubes containing 20 ml minimal medium or 10 ml LB. Minimal
103 medium and LB were inoculated to a density of 1.2×10^6 and 1.4×10^6 cells per ml. Both media
104 contained $100 \mu\text{g L}^{-1}$ cobalt and $100 \mu\text{g L}^{-1}$ cadmium that would be found in extremely polluted
105 environments only (Howari et al., 2004 and Yang and Rose, 2005). This experiment determined
106 the attachment of metal to the cells during the growth cycle in both types of media after they had
107 been separated from the media. **The population of bacteria were determined by culturing so that**
108 **it would possible to calculate the amount of metal ions associated with each bacterial cell.** At
109 each sampling time, triplicate tubes were removed for destructive analysis. From each tube, 1 ml
110 was removed, shaken for 1 min and a serial dilution was made in $\frac{1}{4}$ strength Ringers solution.

111 The serial dilutions (100 μ l) were plated onto agar plates containing LB and incubated at 30 °C
112 for 24 h before counting single colonies.

113

114 2.2. Preparation and deployment of Biological Mobilizing DGT (BMDGT)

115

116 *S. oneidensis* was grown until late log phase growth (19 h) in 20 ml minimal medium or
117 10 ml LB, then cells were centrifuged at 1710 x g for 10 min, the supernatant was discarded and
118 the pellet was resuspended in 1 ml sterile ¼ strength Ringers solution to remove metal ions
119 associated with the media and to provide an osmotic balance for the microorganisms. The cells
120 were centrifuged again at 674 x g for 5 min, the supernatant was discarded and the cells were
121 washed by resuspending in 1 ml ¼ strength Ringers solution. This was repeated a further three
122 times and finally the cells were resuspended in 1 ml of ¼ strength Ringers solution.

123 An agarose suspension (final volume and concentration of 20 ml and 1% (w/v),
124 respectively, of agarose) containing minimal medium (or LB) was prepared following the same
125 growth medium used to grow the *S. oneidensis* cells. This suspension was autoclaved for 15 min
126 at 121°C and 15 psi. After the agarose suspension had cooled to 40°C, 20 μ l amino acids were
127 added [final concentration of 20 μ g/L L-arginine, 20 μ g/L L-glutamate and 20 μ g/L L-serine]
128 and *S. oneidensis* cells resuspended in 1 ml of ¼ strength Ringers solution. The suspension was
129 stirred for 1 min and then dispensed between two pre-warmed glass plates that were separated by
130 a 0.05 cm spacer. All glass plates, spacers and DGT devices had been soaked in 10% HNO₃ acid
131 for several hours and then thoroughly rinsed in ultrapure water (milli Q at 18.2 M Ω -cm) until pH
132 5. Disks (2.2 cm in diameter and 0.05 cm) were cut from the gel and gently eased off the glass

133 plate into ultrapure water. The disks were briefly stirred in 0.01 M sodium nitrate. Control disks
134 were prepared as previously described except *S. oneidensis* washed cells were added to boiling
135 medium that resulted in heat killed cells or a similar volume of ¼ strength Ringers solution was
136 used instead of the cells. Similarly, 1% (w/v) agarose disks were prepared containing 1% (w/v)
137 Luria Broth (LB) that were inoculated with *S. oneidensis* grown for 19 h in LB medium. This
138 suspension was dispensed between pre-warmed plates separated by 0.1 mm spacers to determine
139 whether thickness or medium composition of the diffusive gels containing *S. oneidensis* would
140 affect diffusion of metals through the gel.

141 The agarose disks without cells, heat killed cells and live cells were incorporated into the
142 DGTs devices to form cell free DGTs, heat killed BMDGTs and BMDGTs that were used
143 throughout this study. **The typical DGT is formed of Chelex-100 resin gel, overlaid with a**
144 **diffusion gel layer and then a polycarbonate filter membrane that are securely held together in a**
145 **plastic moulding.** Polycarbonate filter membranes were placed behind the Chelex-100 resin gel
146 during the assembly of the DGTs where the thickness of the agarose disks was insufficient to fill
147 the space within the DGT device. In the classical DGT, a polycarbonate membrane is overlaid
148 onto the diffusive gel but this membrane was not used in the BMDGT to enable direct contact
149 between bacterial cells and metal colloids. Classical cell free DGTs containing agarose were
150 prepared using the standard procedure as previously described (Zhang et al., 1995).

151 Each DGT was deployed in a separate pot in triplicate in a stationary solution of 0.01 M
152 NaNO₃ containing metals at known concentrations and incubated at 30°C. **Previous experiments**
153 **showed that cells were lost from the agarose layer especially during agitation and a stationary**
154 **system was used in order to maintain the highest number of cells within the agarose layer.**
155 Simple metal solutions were prepared using CoNO₃ and CdNO₃ in 0.01 M NaNO₃. The

156 BMDGTs were disassembled after they had been deployed. The Chelex-100 resin gel was placed
157 into 1 ml of 1 M HNO₃ and shaken, while the agarose disk was placed into ¼ strength Ringers
158 solution for staining using LIVE/ DEAD *BacLight*, or culturing onto LB plates containing agar.
159 The eluates from the Chelex-100 resin gels were diluted and metals measured using ICP-MS
160 (Thermo Scientific, X7) ensuring that the percentage of rhodium remained consistent throughout
161 all the samples. They were also compared with metal standards ranging from 1-20 µg L⁻¹.

162

163 2.3. Calculation of metal associated with DGT

164

165 Previous studies have shown that there is a gradient of metal concentration within the
166 diffusive gel (1% (w/v) agarose disk in this case, an assumption is made that cells and media
167 have no effect on the diffusion coefficient) and the concentration within the gel close to the
168 Chelex-100 resin gel is negligible, as metals are constantly being absorbed by the Chelex-100
169 resin. The mass of metal, M , accumulated in the resin is given by equation 1 where C_e is the
170 concentration of metals eluted from the Chelex-100 resin into the 1 M HNO₃ that is measured by
171 ICP-MS, V_1 is the volume of 1M HNO₃ immersing the Chelex-100 resin gel, V_2 is the volume of
172 the diffusive gel and f_e is the elution factor. The values for V_2 and f_e are typically 0.15 ml and
173 0.8, respectively.

174

$$M = C_e(V_1 + V_2)/f_e \quad (\text{eqn. 1})$$

175

$$C_{DGT} = M \Delta g / (DtA) \quad (\text{eqn. 2})$$

176 The mean concentration of metals at the surface of the DGT device, C_{DGT} , can be calculated
177 using eqn. 2, where Δg is the thickness of the diffusive gel (0.05 cm for 1% (w/v) agarose gel), D
178 is the diffusion coefficient of the uncomplexed metal, t is the deployment time in seconds and A
179 is the area of the exposure window (3.14 cm² for solution DGT devices and 2.54 cm² for soil
180 DGT devices). The diffusion coefficients were derived for the appropriate temperature from
181 established values for metals diffusing through either agarose or polyacrylamide cross-linked
182 gels (Zhang and Davison, 1999).

183

184 *2.4. Determination of viability of Shewanella oneidensis MR-1 associated with agarose disks*

185

186 Viability of cells embedded in the agarose disks was assessed using two methods,
187 culturing on agar containing LB and confocal microscopy of cells within agarose disks stained
188 with LIVE/ DEAD *BacLight* kit (Invitrogen, UK). In both methods each agarose disk was
189 placed into 1 ml of ¼ strength Ringers solution and hand shaken for 1 min.

190

191 *2.4.1. Culture counts*

192

193 A serial dilution was made in ¼ strength Ringers solution and 100 µl of each serial
194 dilution was spread onto LB plates. The plates were incubated at 30°C for 24 h and then the
195 colonies were counted.

196 2.4.2. Viability assessment

197

198 *S. oneidensis* was grown in either LB or *Shewanella* minimal medium and washed three
199 times in ¼ strength Ringers solution. The determination of viability using BacLight LIVE/
200 DEAD kit requires using 3 µl of combined equal mixture of 3.34 mM Syto 9 and 20mM PI
201 added to 1 ml of cells. In one procedure the cells were stained beforehand to ensure that cells
202 could be viewed and were counterstained with PI. In this procedure, the cells were resuspended
203 in 1 ml ¼ strength Ringers solution containing 1 µl 3.34 nM SYTO-9. The agarose solution (of
204 1% (w/v) contained a final population of 5×10^8 cells in 1 nM propidium iodide (PI). Disks were
205 cut into thin cross-sections in order to observe cells within the disks using the Leica DMIRE2
206 microscope with a TCS SP2 AOBS confocal scanning system (Leica Microsystems). The argon
207 laser line Argon laser at 488 nm was selected using the AOTF and emission was collected
208 between 500 and 520 nm for SYTO 9 and between 600 and 680 nm for propidium iodide. Viable
209 cells were examined in approximately 30 cross-sections beginning at the top surface of the disk
210 and finishing at the bottom surface of the disks using the Z-stack mode. They were also
211 examined at right angles to the top and bottom surfaces. The captured images were analysed
212 using Image J software. In an alternative procedure, higher quantities of stains were used at
213 similar proportions to determine whether the initial staining procedure showed any differences
214 and to stain cells within the agarose disks. In this alternative staining procedure, the viability of
215 cells in the disks was determined after they had been deployed and to examine how the staining
216 method would affect the staining of the cells. The agarose disk containing the cells was
217 immersed for 15 min in 1 ml ¼ strength Ringers solution containing 9.98 nM SYTO-9 and 60
218 nM PI and viewed under the confocal microscope as previously described. Cells appearing

219 green were stained with Syto 9 indicating they were “live”, cells appearing red were stained with
220 PI indicating they were “dead” and cells appearing yellow were assumed to be in a transient state
221 between “live” and “dead”.

222

223 **3. Results**

224 *3.1. Absorption of metals to cells*

225

226 Growth of *S. oneidensis* in minimal medium and LB containing 100 $\mu\text{g L}^{-1}$ Co and Cd
227 showed that the microorganism was unaffected by high metal concentrations (Figure 1). As
228 expected, growth of *S. oneidensis* in minimal medium was slower than in LB, although final cells
229 numbers were similar. The association of Cd to *S. oneidensis* grown in minimal medium was
230 determined at discrete times when samples were taken for culturable counts. The association
231 with Cd was highest at 5 h, corresponding to late lag phase at 423×10^{-21} ($\pm 170 \times 10^{-21}$) g per cell,
232 and lowest at 14 h, corresponding to initial log phase at 63×10^{-21} ($\pm 24.9 \times 10^{-21}$) g per cell. The
233 association difference between late and initial log phases were significantly different using
234 Student *t*-test ($p = 0.029$), but at other times during the growth phases, there were no significant
235 differences in the association of Cd. Concentrations of Co and Cd measured in solution when *S.*
236 *oneidensis* had reached stationary phase had decreased by less than 1% showing that cells during
237 the stationary phase would be exposed to relatively equal metal concentrations to those
238 concentrations at the beginning of the experiment.

239

240 3.2. Deployment of BMDGTs in metal solutions

241

242 BMDGTs (*S. oneidensis* grown in minimal medium were immobilized DGTs) were
243 deployed in 50 ml solution containing Co and Cd in 0.01 M NaNO₃ under stationary conditions
244 showed that metal concentrations measured using BMDGTs were not significantly different to
245 those measured using cell free control DGTs (DGTs similar to BMDGT except without cells)
246 under aerobic and anaerobic conditions (data not shown). The Co and Cd concentrations in
247 solutions before deployment under aerobic conditions were 56.9 (±0.6) µg L⁻¹ and 69.6 (±0.7) µg
248 L⁻¹, respectively, while under anaerobic conditions they were 113 (±4.6) and 135 (±6.2) µg L⁻¹,
249 respectively. The values in parenthesis refer to standard deviations. When DGTs are deployed
250 in stationary solutions, the calculation of the hypothetical diffusible layer is apparently much
251 thicker than it is actually which leads to lower metal concentrations associated with the binding
252 layer. Therefore, it was not surprising that lower metal concentrations were associated with the
253 BMDGTs compared with the concentration found in the immersion solution.

254 When BMDGTs containing cells grown in LB were deployed in metal solutions under
255 anaerobic conditions, the concentration of Cd that was associated with BMDGTs was
256 significantly lower compared with the Cd concentration associated with cell free DGTs
257 containing agarose (p = 0.007 using Student's *t*-test and assuming unequal variances) (Figure 2).
258 However, there were no significant differences under aerobic conditions. The initial
259 concentrations of Co and Cd ions in the deployment solution under aerobic conditions were 113
260 (±1.0) µg L⁻¹ and 130 (±2.1) µg L⁻¹, respectively, while under anaerobic conditions they were
261 117 (±0.4) µg L⁻¹ and 129 (±1.4) µg L⁻¹. Deployment of BMDGTs caused no significant changes

262 in the metal concentrations in solution. There were no significant differences in Co and Cd
263 concentrations in the deployment solution containing the cell free DGTs compared to the
264 deployment solution containing the BMDGTs after 4 h of deployment. The aerobic and
265 anaerobic deployments of BMDGTs (Figure 2 and Figure 3) were incubated at 25°C and 30°C,
266 respectively.

267

268 3.3. Deployment of BMDGTs in hematite and metal solutions

269

270 The Fe concentration of the BMDGTs appeared higher under anaerobic conditions
271 compared with aerobic conditions, although the large error bar associated with BMDGTs under
272 anaerobic conditions ensured that there was no significant difference (Figure 3). It can only be
273 assumed that hematite colloids are heterogeneously distributed and that a higher density of cells
274 within the BMDGTs may have increased the frequency of interaction between bacterial cells and
275 colloids perhaps resulting in lower error bars. However, Co and Cd concentrations associated
276 with BMDGTs under anaerobic conditions only were significantly higher than concentrations
277 associated with the BMDGTs containing heat killed cells.

278

279 3.4. Viability of cells in BMDGTs

280

281 The viability assessed using culturing indicated that the number of *S. oneidensis* cells
282 increased within the BMDGTs and remained steady for 20 h when stored at 4°C (Fig. 4). The

283 viability of *S. oneidensis* within the BMDGTs was assessed before and after deployment. LB
284 grown cells embedded in agarose that were stained with SYTO-9 and PI revealed that 35% of the
285 cells were still viable (Figure 5A). In contrast only 16% of cells that were grown in minimal
286 medium and embedded into the agarose disks were stained with SYTO-9 (Figure 5B). There
287 were a higher proportion of red (dead) stained cells with PI whether the cells were stained with
288 SYTO-9 beforehand (Figure 5A) or when the cells were stained with SYTO-9 and PI after the
289 cells had been embedded in the agarose disk (Figure 5C). However, the DNA in the cells that
290 interacted mostly with SYTO-9 and could be described as live cells were green when stained
291 beforehand with SYTO-9, and were yellow when stained afterwards with SYTO-9 and PI
292 simultaneously. The appearance of the yellow stained cells indicated that the integrity of the cell
293 membranes may have been affected and perhaps more accurately reflected the actual state of the
294 “live” cells (Figure 5C). The phenomenon of yellow stained cells using *BacLight LIVE/ DEAD*
295 staining has been observed previously (Boulos et al., 1999). After deployment of the agarose
296 disks, LIVE/ DEAD *BacLight* staining revealed that the cells stained green with SYTO-9,
297 suggesting that the integrity of the cell membranes had improved, and cells stained red with PI
298 were no longer present (Figure 5D). After 20 h incubation at 10°C, the state of the cells
299 remained unchanged (data not shown). These results indicated that a significant proportion of *S.*
300 *oneidensis* cells remained viable when grown in LB. Different types of microorganisms could be
301 incorporated into BMDGT using this procedure to assess bacterial bioavailability where the
302 mobility of metals shows an increase as a direct consequence of bacterial presence.

303

304

305 **Discussion**

306

307 This study was to determine whether *S. oneidensis* MR-1 could be used to assess
308 bioavailability when incorporated into the diffusion gradient thin films (DGTs) that are currently
309 used as a passive device for monitoring heavy metals. At the initial stage, the growth of *S.*
310 *oneidensis* MR-1 in medium containing high concentrations of metals ions showed that
311 potentially this microorganism may grow in BMDGTs during deployment in high concentrations
312 of metals. The uptake or attachment of metal ions showed only one significant difference
313 between the lag phase and at the end of the exponential phase. Only **3.4 femto grams** of metal
314 ions appeared to be associated with the cells. This demonstrated that metal ions associated with
315 cells of *S. oneidensis* MR-1 in BMDGTs will **have almost a negligible** effect on the
316 measurements of metals by DGT, even if cells responded to an increased input of nutrients.
317 Therefore, any differences caused by the presence of the microorganism could be attributed to
318 the microorganism's effect on the bioavailability of metals rather than metal absorption to cell
319 surfaces.

320 Deployment of DGTs in natural waters containing high organic loads can cause the
321 formation of algal biofilms that impede the diffusion of metal ions through DGT (Warnken et al.,
322 2008). A study using yeast as the binding agent in the DGT device showed that metals bind
323 rapidly to the yeast cells (Menegário et al., 2010). Here, despite the high surface area to volume
324 ratio of the bacterial cells and the high numbers of cells in the agarose disks containing LB, the
325 attachment of metals to bacterial cell surfaces under aerobic conditions had little effect on the
326 quantity of metals determined in the BMDGT compared to the cell free DGTs. The significant
327 proportion of dead cells is unlikely to have had a large influence on the finding, as Chubar et al.

328 (2008) found the attachment of metal ions to dead cells of *S. oneidensis* was only reduced two
329 fold compared to live cells. The concentration of Cd (0.88 nM) used in these studies was more
330 than 100 fold higher than Cd concentrations determined in a pristine river using DGTs (Warnken
331 et al., 2008). Therefore under conditions where metal concentrations are very low, bacterial
332 biofilms may have a greater impact in attachment of metal ions. However, BMDGTs containing
333 LB showed a decrease in the flux of metal ions under anaerobic conditions compared to control
334 BMDGTs without cells. This was most likely caused by Cd binding to the phosphoryl groups in
335 *S. oneidensis* when the Cd concentration is high (Bhoo et al., 2010). The BMDGT with minimal
336 medium would have low phosphate resulting in a lower proportion of phosphoryl groups. The
337 effect of metal ions attaching to bacteria may have been more pronounced in BMDGTs
338 compared to the attachment observed when disks containing cells were suspended in a solution
339 of metal ions because the flux of metal ions passing through the BMDGT may be greater than
340 metals accumulating through diffusion. However, under aerobic conditions there was no
341 decreased flux that could possibly be associated with bacterial attachment, perhaps due to
342 different protein expression profiles under aerobic and anaerobic conditions (Beliaev et al.,
343 2002). Therefore, it appears that only under unusual circumstances, such as high nutrient
344 concentrations and anaerobic conditions is it likely that *S. oneidensis* may significantly reduce
345 the flux of metal ions through BMDGTs.

346 These conditions may arise during deployments in soils with high organic contents and
347 biomass. The effect of *S. oneidensis* in the BMDGTs on hematite showed that the solubility of
348 Fe remained unchanged in contrast to a previous study that showed an increase in organic
349 soluble iron complexes, albeit at low levels (Taillefert et al., 2007). It is possible that the
350 concentrations of soluble iron were too low to be detected yet Co and Cd ion concentrations

351 showed an increase. This effect may be caused by an interaction between Co and Cd ions and
352 hematite under anaerobic conditions rather than being a biological effect. Differences may have
353 been found if the BMDGTs were immersed in a deployment suspension containing low
354 concentrations of a carbon source to enable *S. oneidensis* to become metabolically active. The
355 addition of LB to the BMDGTs most likely diffused into the deployment suspension although at
356 these concentrations were unlikely to be effective. The use of warm agarose in the preparation
357 process ensured that *S. oneidensis* MR-1 cells were evenly distributed throughout the agarose
358 disk. However, it could potentially affect the viability, metabolism and cell structure. A previous
359 study has shown that temperatures up to 42°C temporarily affected the regulation of genes,
360 especially down regulating genes involved in energy metabolism (Gao et al., 2004). Viability
361 determined using LIVE/ DEAD BacLight staining showed that many cells appeared yellow and
362 were assumed to be active because later many active cells were present embedded in the agarose
363 disks. Boulos et al. (1999) showed that LIVE/ DEAD BacLight staining of chlorinated samples
364 resulted in some yellow cells that were no longer culturable whereas another study showed the
365 presence of yellow stained cells of *Pseudomonas putida* in similar proportion to culturable
366 counts (Ivanova et al., 2010). However, culturing cells detaching from the BMDGTs appeared
367 to confirm that many cells of *S. oneidensis* were still viable within the system. After
368 deployment, LIVE/ DEAD BacLight staining revealed that the cells in the BMDGTs stained
369 green with SYTO-9 indicating that their proportion had increased. The presence of media within
370 the BMDGTs would have enabled the microorganisms to survive assuming that some of the
371 media did not diffuse from the disks. It was assumed that the remaining viable cells would be
372 sufficient to determine whether they have an effect on the bioavailability of metals and therefore
373 decrease or increase the proportion of labile metals associated with the Chelex-100 resin.

374 However, ideally a much higher proportion of viable cells would show more clearly whether
375 cells increased the bioavailability of metals. A much higher population of cells could be
376 achieved by immersing the disks containing the cells in media to stimulate growth.

377 The results of this study have shown that bacteria can be effectively incorporated into
378 modified DGTs especially if the cells are grown in LB and under certain conditions they
379 influence the metal accumulated on the Chelex-100 resin, demonstrating the effect of
380 microorganisms on the bioavailability of metals. Although this first study has demonstrated the
381 feasibility of modifying the performance of DGT using live organisms, the impact on the
382 measured accumulated metal was generally small. Future studies could be performed by
383 incorporating low concentrations of different medium into the deployment suspension to
384 encourage growth of *S. oneidensis* and using other microorganisms that may increase metal
385 mobilization. For example actinobacteria within the rhizosphere are believed to mobilize metals
386 (e.g. copper) through the production of organic acids and siderophores leading to uptake by
387 hyper-accumulator plants (Tao et al., 2003, Wang et al., 2008, Kuffner et al., 2010 and Turmel et
388 al., 2011). Microorganisms isolated from these environments could be incorporated into
389 BMDGTs to determine whether they increase mobilization of metals. This study has shown a
390 few effects of *S. oneidensis* on metal solutions, colloids and nanoparticles.

391

392 **ACKNOWLEDGEMENTS**

393

394 We wish to thank Debra Hurst with assistance in confocal microscopy.

395

396 **REFERENCES**

397
398 Beliaev A. S., Thompson, D. K., Khare, T., Lim, H., Brandt, C. C., Li, G., Murray, A. E.,
399 Heidelberg, J. F., Giometti, C. S., Yates, J. 3rd, Nealson, K. H., Tiedje, J. M., Zhoui, J.,
400 2002. Gene and protein expression profiles of *Shewanella oneidensis* during anaerobic
401 growth with different electron acceptors. OMICS. 6, 39-60.
402
403 Boulos, L., Pre´vost, M., Barbeau, B., Coallier, J., Desjardins, R., 1999. LIVE/DEAD®
404 BacLight™: application of a new rapid staining method for direct enumeration of viable
405 and total bacteria in drinking water. J. Microbiol. Meth. 37, 77–86.
406
407 Charrier, T., Chapeau, C., Bendria, L., Picart, P., Daniel, P., Thouand, G., 2011. A multi-
408 channel bioluminescent bacterial biosensor for the on-line detection of metals and
409 toxicity. Part II: technical development and proof of concept of the biosensor. Anal.
410 Bioanal. Chem. 400, 1061–1070.
411
412 Chubar, N., Behrends, T., Van Cappellen, P., 2008. Biosorption of metals (Cu^{2+} , Zn^{2+}) and
413 anions (F^- , H_2PO_4^-) by viable and autoclaved cells of the Gram-negative bacterium
414 *Shewanella putrefaciens*. Colloids Surf. B 65, 126–133.
415
416 Degryse, F., Smolders, E., Zhang, H., Davison, W., 2009. Predicting availability of mineral
417 elements to plants with the DGT technique: a review of experimental data and
418 interpretation by modeling. Environ. Chem. 6 (3), 198-218.
419

420 Eltzov, E., Marks, R. S., 2011. Whole-cell aquatic biosensors. *Anal. Bioanal. Chem.* 400, 895–
421 913.

422

423 Gadd, G.M., 2010. *Metals, minerals and microbes: geomicrobiology and bioremediation.*
424 *Microbiology* 156, 609–643.

425

426 Gao, H., Wang, G. Y., Liu, X., Yan, T., Wu, L., Alm, E., Arkin, A., Thompson, D. K., Zhou, J.,
427 2004. Global Transcriptome Analysis of the Heat Shock Response of *Shewanella*
428 *oneidensis*. *J. Bact.* 186, 7796–7803.

429

430 Hau, H. H., Gilbert, A., Coursolle, D., Gralnick, J. A., 2008. Mechanism and Consequences of
431 Anaerobic Respiration of Cobalt by *Shewanella oneidensis* Strain MR-1. *Appl Environ*
432 *Microbiol.* 74, 6880–6886.

433

434 Howari, F.M., Abu-Rukah, Y, Goodell, P.C., 2004. Heavy metal pollution of soils along North
435 Shuna-Aqaba Highway, Jordan. *Int. J. Environ. Pollut.* 22, 597-607.

436

437 Ivanova, I. A., Kambarev, S., Popova, R. A., Naumovska, E. G., Markoska, K. B., Dushkin, C.
438 D., 2010. Determination of *Pseudomonas putida* live cells with classic cultivation and
439 staining with “Live/Dead BacLight bacterial viability kit”. *Biotechnol. Biotec. Eq.* 24,
440 567-570.

441

442 Kuffner, M., De Maria, S., Puschenreiter, M., Fallmann, K., Wieshammer, G., Gorfer, M.,
443 Strauss, J., Rivelli, A. R., Sessitsch, A., 2010. Culturable bacteria from Zn- and Cd-
444 accumulating *Salix caprea* with differential effects on plant growth and heavy metal
445 availability. *J. Appl. Microbiol.* 108, 1471–1484.
446

447 Lies, D. P., Hernandez, M. E., Kappler, A., Mielke, A. E., Gralnick, J. A., Newman, D. K., 2005.
448 *Shewanella oneidensis* MR-1 Uses Overlapping Pathways for Iron Reduction at a
449 Distance and by Direct Contact under Conditions Relevant for Biofilms. *Appl. Environ.*
450 *Microbiol.* 71, 4414–4426.
451

452 Meitl, L. A., Eggleston, C. M., Colberg, P. J. S., Khare, N., Reardon, C. L., Shi, L., 2009.
453 Electrochemical interaction of *Shewanella oneidensis* MR-1 and its outer membrane
454 cytochromes OmcA and MtrC with hematite electrodes. *Geochim. Cosmochim Acta* 73,
455 5292–5307.
456

457 Menegário, A. A., Tonello, P. S., Durrant, S. F., 2010. Use of *Saccharomyces cerevisiae*
458 immobilized in agarose gel as a binding agent for diffusive gradients in thin films. *Anal.*
459 *Chim. Acta* 683 (1), 107–112.
460

461 Mishra, B., Boyanov, M., Bunker, B. A., Kelly, S. D., Kemner, K. M., Fein, J. B., 2010. High-
462 and low-affinity binding sites for Cd on the bacterial cell walls of *Bacillus subtilis* and
463 *Shewanella oneidensis*. *Geochim. Cosmochim. Acta* 74, 4219–4233.
464

465 Mugerfield, I., Law, B. A., Wickham, G. S., Thompson, D. K., 2009. A putative azoreductase
466 gene is involved in the *Shewanella oneidensis* response to heavy metal stress. *Appl.*
467 *Microbiol. Biotechnol.* 82, 1131–1141.

468

469 Pesavento, M., Alberti, G., Biesuz, R., 2009. Analytical methods for determination of free metal
470 ion concentration, labile species fraction and metal complexation capacity of
471 environmental waters: A review. *Anal. Chim. Acta* 631 (2), 129–141.

472

473 Pichette, C., Zhang, H., Davison, W., Sauve, S., 2007. Preventing biofilm development on DGT
474 devices using metals and antibiotics. *Talanta* 72, 716–722.

475

476 Ruebush, S. S., Brantley, S. L., Tien, M., 2006. Reduction of Soluble and Insoluble Iron Forms
477 by Membrane Fractions of *Shewanella oneidensis* Grown under Aerobic and Anaerobic
478 Conditions. *Appl. Environ. Microbiol.* 72, 2925–2935.

479

480 Taillefert, M., Beckler, J.S., Carey, E., Burns, J.L., Fennessey, C.M., DiChristina, T.J., 2007.
481 *Shewanella putrefaciens* produces an Fe (III)-solubilizing organic ligand during
482 anaerobic respiration on insoluble Fe (III) oxides. *J. Inorg. Biochem.* 101, 1760–1767.

483

484 Tao, S., Chen, Y. J., Xu, F. L., Cao J., Li, B. G., 2003. Changes of copper speciation in maize
485 rhizosphere soil. *Environ. Poll.* 122, 447–454.

486

487 Turmel, M. C., Courchesne, F., Cloutier-Hurteau, B., 2011. Microbial activity and water-soluble
488 trace element species in the rhizosphere of spring wheat (*Triticum aestivum* cv. USU-
489 Perigee) J. Environ. Monit. 13, 1059-1072.

490

491 van Leeuwen, H. P., Town, R. W., Buffle, J., Cleven, R. F. M. J., Davison, W., Puy, J., van
492 Riemsdijk, W. H., Sigg, L., 2005. Dynamic Speciation Analysis and Bioavailability of
493 Metals in Aquatic Systems. Environ. Sci. Technol. 39, 8545-8556.

494

495 Wang, Y. P., Li, Q. B., Shi, J. Y., Qi Lin, Q., Chen, X. C., Wu, W., Chen, Y. X., 2008.
496 Assessment of microbial activity and bacterial community composition in the rhizosphere
497 of a copper accumulator and a non-accumulator. Soil Biol. Biochem. 40, 1167-1177.

498

499 Warnken, K. W., Davison, W., Zhang, H., 2008. Interpretation of in situ speciation
500 measurements of inorganic and organically complexed trace metals in freshwater by
501 DGT. Environ. Sci. Technol. 42, 6903–6909.

502

503 Williams, P.N., Zhang, H., Davison, W., Meharg, A.A., Hossain, M., Norton, G.J., Brammer, H.,
504 Islam, M.R., 2011. Organic matter - Solid phase interactions are critical for predicting
505 arsenic release and plant uptake in Bangladesh paddy soils. Environ. Sci. Technol. 45,
506 6080-6087.

507

508 Yang, H, Rose, N., 2005. Trace element pollution records in some UK lake sediments, their
509 history, influence factors and regional differences. Environ. Int. 31(1), 63– 75.

510

511 Zhang, H., Davison, W., 1995. Performance characteristics of diffusion gradients in thin films
512 for the in situ measurement of trace metals in aqueous solution. *Anal. Chem* 67, 3391-
513 3400.

514

515 Zhang, H., Davison, W., 1999. Diffusional characteristics of hydrogels used in DGT and DET
516 techniques. *Anal. Chim. Acta* 398, 329–340.

517

518 Zhang, H., 2004. In-situ speciation of Ni and Zn in freshwaters: Comparison between
519 DGT measurements and speciation models. *Environ. Sci. Technol.* 38, 1421-1427.

520

521 **List of figures**

522 **Fig. 1** Growth curve of *S. oneidensis* in LB □ and minimal medium specifically used for
523 growth of *Shewanella* sp. ◇. Each medium contained 100 µg L⁻¹ Co and Cd. Standard
524 deviations are shown within □ and around ◇.

525 **Fig. 2** DGT calculated metal concentrations from deployment of BMDGT without cells ■ and
526 BMDGT with *S. putrefaciens* cells □ in 50 ml metal solution under (A) aerobic conditions and
527 (B) anaerobic conditions. BMDGTs have an agarose diffusive layer that contained 1% (w/v) LB
528 and significant differences shown by * and error bars indicate standard deviations.

529 **Fig. 3** DGT calculated metal concentrations from deployment in hematite suspension
530 containing cobalt and cadmium in solution of BMDGTs with heat killed cells □ BMDGTs with
531 *S. oneidensis* cells under aerobic conditions ■ and BMDGTs with *S. oneidensis* cells under
532 anaerobic conditions ■ . Error bars indicate standard deviations.

533 **Fig. 4** Culturable counts of *S. oneidensis* associated with BMDGTs: (HK) BMDGT containing
534 heat killed cells that was deployed immediately, (4 h) BMDGT with cells that was deployed
535 immediately, (20 h) BMDGT with cells that was deployed after 20 h incubation of BMDGT at
536 4°C and (blank) un-deployed BMDGT. Significant differences shown by * and error bars
537 indicate standard deviations.

538 **Fig. 5** LIVE/ DEAD BacLight staining of *S. oneidensis* in agarose disks (A) cells stained with
539 SYTO-9 before being embedded in agarose disk containing LB (view of edge of agarose disk),
540 (B) cells stained with SYTO-9 before being embedded in agarose disk containing minimal
541 medium (C) cells in agarose disk containing LB stained after with SYTO-9 and PI, and (D) cells
542 in agarose disk containing LB stained after with SYTO-9 and PI after deployment for 4 h.

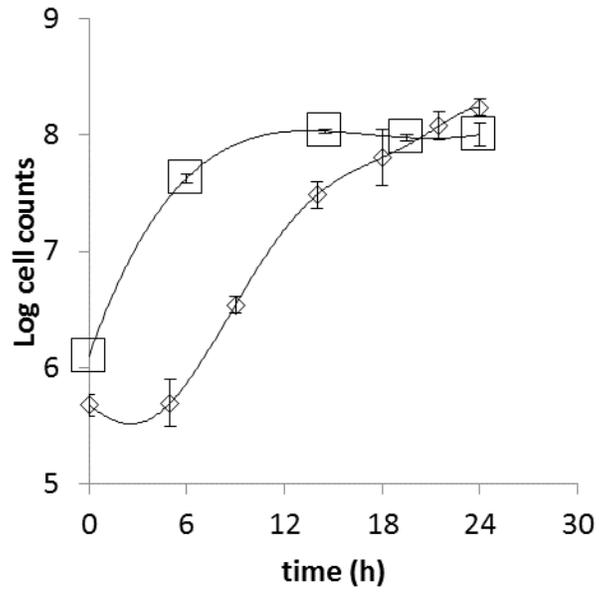


Fig. 1

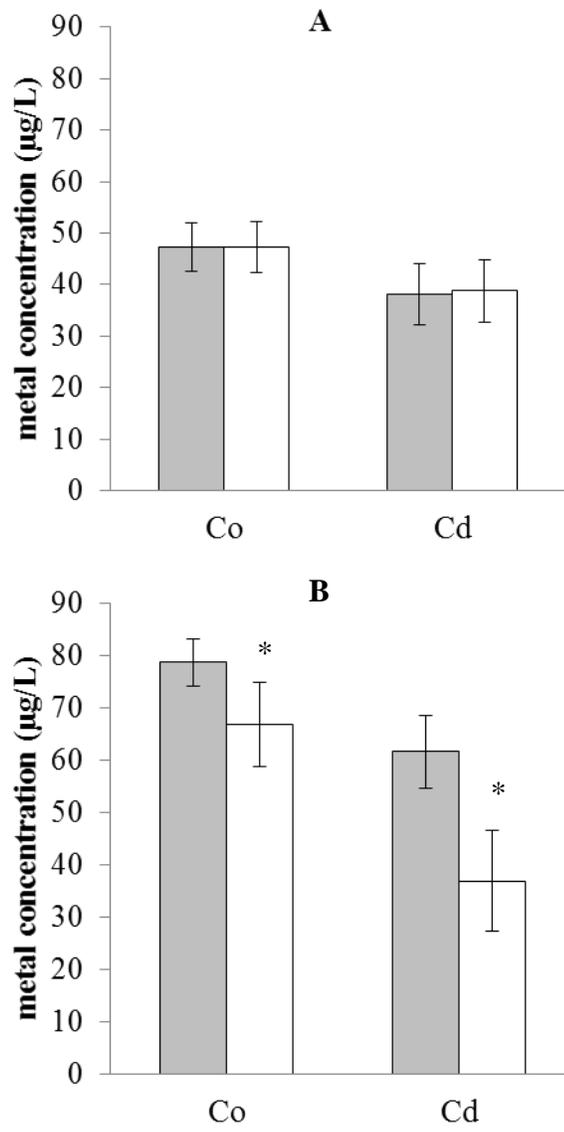


Fig. 2

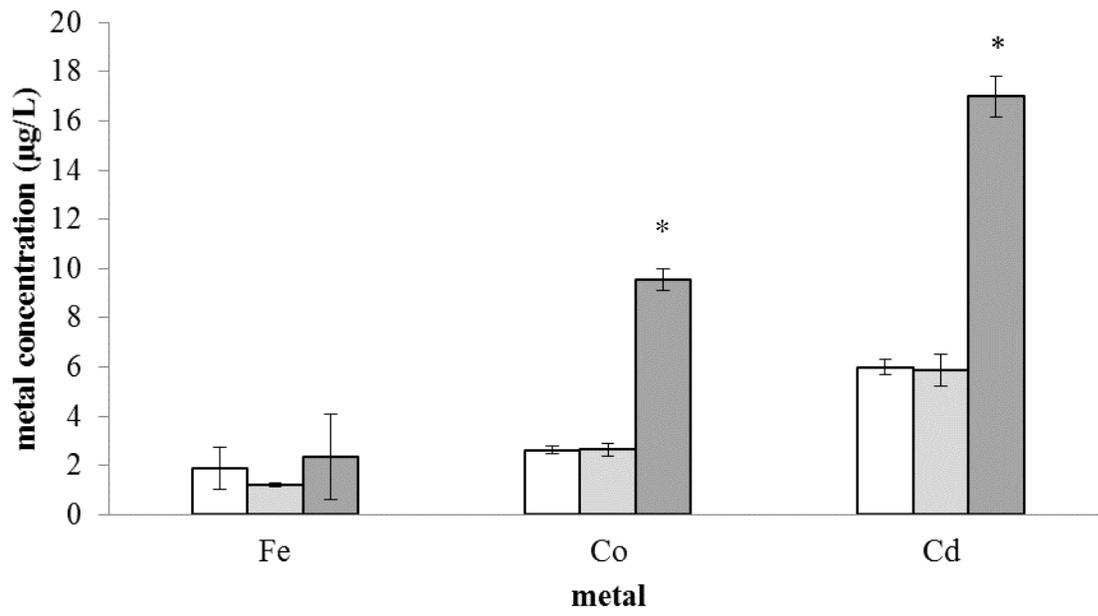


Fig. 3

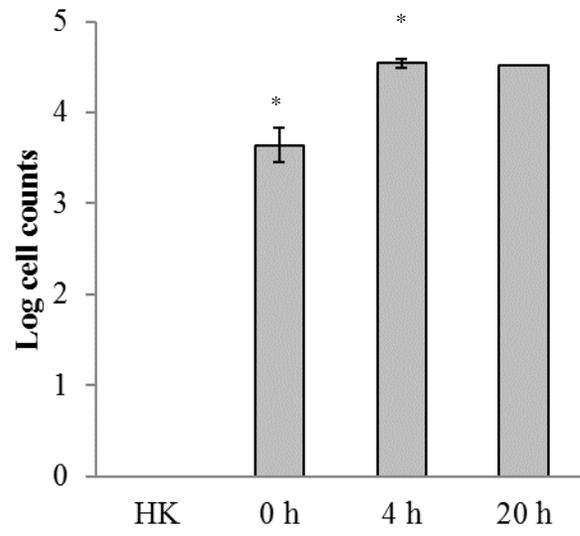


Fig. 4

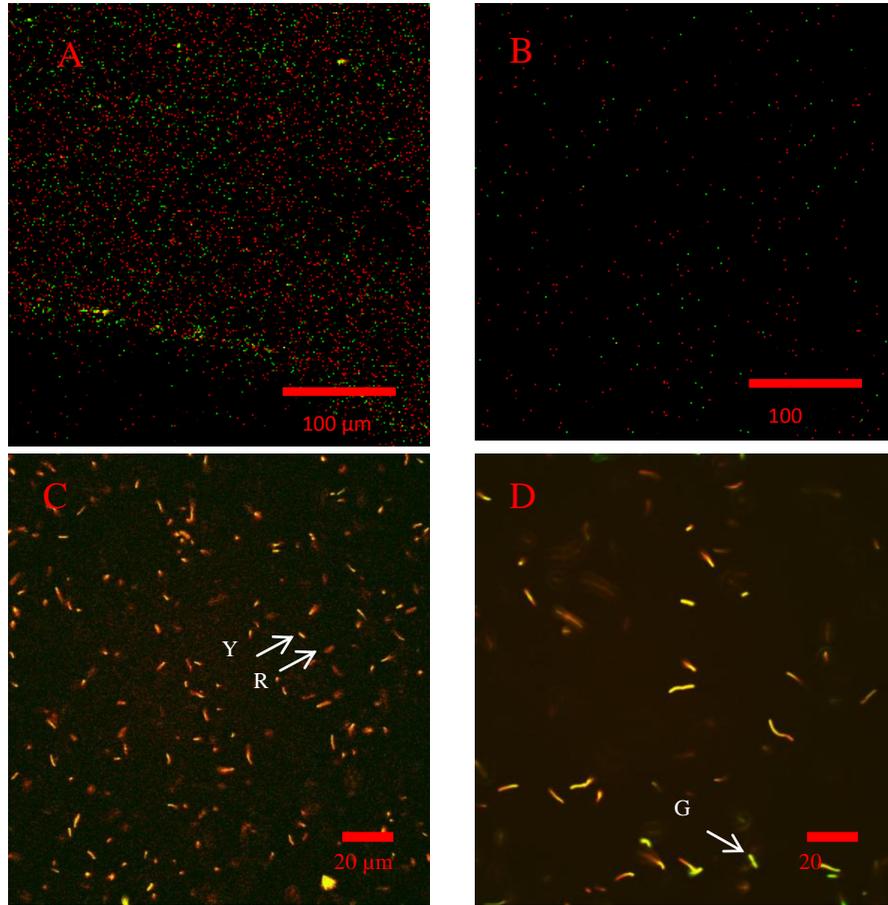


Fig. 5

Highlights

- Live bacteria were incorporated into BMDGTs
- BMDGTs showed some effects on mobilization of metals
- BMDGTs did not show increased iron mobilization by *Shewanella oneidensis*

Immobilization of *Shewanella oneidensis* MR-1 in diffusive gradients in thin films for determining metal bioavailability

Paul W Baker*¹, Christer Högstrand², Jamie Lead³, Roger W Pickup⁴ and Hao Zhang¹

¹ Lancaster Environmental Centre, Lancaster University, Bailrigg, Lancaster LA1 4YQ UK

² School of Biomedical Sciences, 1.14 Hodgkin Building, Guy's Campus, London LE1 1UL UK

³ School of Geography, Earth and Environmental Sciences, University of Birmingham, Edgbaston, Birmingham B15 2TT UK

⁴ Division of Biomedicine and Life Sciences, Faculty of Health and Medicine, Lancaster University, Lancaster LA1 4YQ UK

Footnote:

Present address of Paul W. Baker: SENRGY, Bangor University, Bangor, Gwynedd LL57 2UW, Wales

1 **Abstract**

2 Assessing metal bioavailability in soil is important in modelling the effects of metal toxicity on
3 the surrounding ecosystem. Current methods based on diffusive gradient thin films (DGTs) and
4 Gel-Integrated Microelectrode are limited in their availability and sensitivity. To address this, *S.*
5 *oneidensis*, an anaerobic iron reducing bacterium, was incorporated into a thin layer of agarose to
6 replace the polyacrylamide gel that is normally present in DGT to form biologically mobilizing
7 DGT (BMDGT). Viability analysis revealed that 16-35% of the cells remained viable within the
8 BMDGTs depending on the culturing conditions over a 20 h period with/without metals.

9 Deployment of BMDGTs in standardized metal solutions showed significant differences to cell-
10 free BMDGTs when cells grown in Luria Broth (LB) were incorporated into BMDGTs and
11 deployed under anaerobic conditions. Deployment of these BMDGTs in hematite revealed no
12 significant differences between BMDGTs and BMDGTs containing heat killed cells. Whether
13 heat killed cells retain the ability to affect bioavailability is uncertain. This is the first study to
14 investigate how a microorganism that was incorporated into a DGT device such as the metal
15 reducing bacteria, *S. oneidensis*, may affect the mobility of metals.

16

17 **Keywords**

18 diffusive gradient thin films (DGT)

19 biological mobilizing DGT

20 *Shewanella oneidensis*

21 *BacLight*

22

23 **1. Introduction**

24

25 Assessment of the potential toxicity of metals requires consideration of their
26 bioavailability rather than their total concentrations in an environment. Chemical speciation,
27 bioavailability and methods used in detection of bioavailable metals have been thoroughly
28 reviewed (van Leeuwen et al., 2005 and Pesavento et al., 2009). Diffusive gradients in thin
29 films (DGT) are passive devices which can be easily deployed in natural environments (Zhang,
30 2004). In DGT, labile metal diffuses through a layer of polyacrylamide gel of known thickness
31 and binds to Bio-Rad Chelex®100 resin embedded in a separate polyacrylamide gel (Zhang et
32 al., 1995). Metals binding to the Chelex-100 resin are eluted using concentrated acid and
33 measured using ICP-MS, allowing calculation of the mean flux of metals to the device during its
34 deployment. The mean metal flux can be related to the concentration of labile species in
35 solution: that is those metal species that are mobile and able to be released rapidly from
36 complexes and colloids. The labile metal measured by DGT has been shown to provide a good
37 prediction of the metal taken up by biota where mass transport by diffusion is rate limiting
38 (Degryse et al., 2009).

39 DGT perturbs a chemical environment solely by supplying a sink for metals, which
40 locally lowers the concentration of the free metal ion, in the diffusion layer of the device and its
41 adjacent immersion medium. This depleted concentration induces release of metal from
42 complexes and colloids in solution and, in the case of soils and sediments, from the solid phase

43 (Williams et al., 2011). Some microorganisms in the environment solubilise solid phases and
44 release metals by siderophores, organic acids and metabolites (Gadd, 2010). If such organisms
45 can be incorporated into DGT there is the possibility that the modified DGT will provide a
46 surrogate measurement for environmental metals that have the potential to be mobilized. Dried
47 *Saccharomyces cerevisiae* has already been successfully used in DGT as a binding agent instead
48 of Chelex-100 resin, but there have been no previous attempts to incorporate live
49 microorganisms into DGT and exploit their interactions with chemical substrates (Menegário et
50 al., 2010). Recently, a bioluminescent biosensor has been described containing *Escherichia coli*
51 reporter strains for the detection and toxicity of Cd and As (Charrier et al., 2011). However,
52 these and other biosensors cannot determine the effects of multianalyte detection and knowledge
53 about protein transportation of metals across the cell membrane is limited (Eltzov and Marks,
54 2011). By incorporating naturally occurring microorganisms into DGT it should be possible to
55 determine directly their effect on bioavailable metals. To achieve this the microorganism would
56 need to be evenly distributed throughout the DGT at a high density of viable cells and at the
57 surface so that the microorganisms can interact with insoluble metal colloids.

58 Previous studies of dissimilatory metal reducing bacteria, such as *Shewanella oneidensis*
59 MR-1, showed that insoluble ferric compounds, including hematite and goethite, are reduced by
60 outer membrane bound cytochromes, but only under anaerobic conditions (Meitl et al., 2009 and
61 Ruebush et al., 2006). Other studies showed that (hydr)-oxide ferric deposits within glass beads
62 were indirectly reduced by *S. oneidensis* MR-1 cells growing outside the beads (Lies et al.,
63 2005). It is believed that this occurs by direct electron transfer between the cell membrane and
64 the surface of the oxide when nutrient conditions are low. *S. oneidensis* MR has a high tolerance
65 to cadmium (Mugerfield et al., 2009) and has different affinity binding sites for cadmium

66 (Mishra et al., 2010). Other metals such as chelated cobalt were reduced from [Co(III)-EDTA]⁻
67 to soluble, albeit toxic, [Co(II)-EDTA]²⁻ by *S. oneidensis* MR-1 (Hau et al., 2008). The above
68 evidence suggests that *S. oneidensis* could possibly reduce oxidized forms of Fe and Co at the
69 surface of a gel in which it is encapsulated. Trace metals that are released from iron oxides as
70 they reductively dissolve should be measured by the DGT device.

71 DGT accurately determines the labile metal concentrations in soils and this represents the
72 availability of metals to plants. However, in nature microorganisms may release labile metals
73 from metal colloids thereby increasing the proportion of labile metal. Therefore the aim of this
74 study was to incorporate *S. oneidensis* MR-1 into a DGT device in order to assess metal
75 bioavailability because this microorganism has the capacity for iron III reduction. Once these
76 cells were present within the DGT device, their effect on the concentrations of trace metals
77 measured by DGT in solutions, colloidal suspensions and soils would be determined relative to
78 appropriate controls.

79

80 **2. Methods**

81 *2.1. Growth of Shewanella oneidensis MR-1*

82

83 *Shewanella oneidensis* MR-1 (ATCC 700550, NCIMB 14063) was purchased from the
84 NCIMB culture collection. An aliquot of 100 µl (or 20 µl) of a previous culture in minimal
85 medium (or LB) was added to 20 ml (or 10 ml LB) minimal medium [9 mM ammonium
86 sulphate, 5.7 mM potassium dihydrogen phosphate, 2 mM sodium hydrogen carbonate, 1 mM
87 magnesium sulphate, 0.49 mM calcium chloride, 67.2 µM sodium EDTA, 56.6 µM boric acid,

88 10 μM sodium chloride, 5.4 μM iron II sulphate, 5 μM cobalt sulphate, 5 μM ammonium nickel
89 sulphate, 3.9 μM sodium molybdate, 1.5 μM sodium selenate, 1.3 μM manganese sulphate, 1
90 μM zinc sulphate, 0.2 μM copper sulphate, 20 $\mu\text{g/ml}$ L-arginine, 20 $\mu\text{g/ml}$ L-glutamate, 20
91 $\mu\text{g/ml}$ L-serine, 15 mM lactate, 2 mM fumarate, and 0.1 g/L casamino acids at pH 7.4]. LB is a
92 complex medium consisting of 10 g/L tryptone, 5 g/L yeast extract and 10 g/L sodium chloride.
93 The two different media were used in this study because minimal medium contains the basic
94 nutrients for cell growth and the low metal concentrations in the medium were unlikely to have
95 an effect on BMDGT analysis. LB was used because this medium contained plenty of nutrients
96 that enable to microorganisms to grow well and survive the embedding process during BMDGT
97 formation. However, there was a concern that the high concentration of metals could have an
98 effect on BMDGT analysis. The medium was removed once the cells had grown and were
99 unlikely to affect metal analysis using DGT. The cultures were incubated at 26°C and 110 rpm
100 for 19 h until the cultures had reached a population density of 10^9 cells per ml.

101 A growth curve, defined by culture, of *S. oneidensis* was determined by growing the
102 microorganism in 25 ml tubes containing 20 ml minimal medium or 10 ml LB. Minimal
103 medium and LB were inoculated to a density of 1.2×10^6 and 1.4×10^6 cells per ml. Both media
104 contained $100 \mu\text{g L}^{-1}$ cobalt and $100 \mu\text{g L}^{-1}$ cadmium that would be found in extremely polluted
105 environments only (Howari et al., 2004 and Yang and Rose, 2005). This experiment determined
106 the attachment of metal to the cells during the growth cycle in both types of media after they had
107 been separated from the media. The population of bacteria were determined by culturing so that
108 it would possible to calculate the amount of metal ions associated with each bacterial cell. At
109 each sampling time, triplicate tubes were removed for destructive analysis. From each tube, 1 ml
110 was removed, shaken for 1 min and a serial dilution was made in $\frac{1}{4}$ strength Ringers solution.

111 The serial dilutions (100 μ l) were plated onto agar plates containing LB and incubated at 30 °C
112 for 24 h before counting single colonies.

113

114 2.2. Preparation and deployment of Biological Mobilizing DGT (BMDGT)

115

116 *S. oneidensis* was grown until late log phase growth (19 h) in 20 ml minimal medium or
117 10 ml LB, then cells were centrifuged at 1710 x g for 10 min, the supernatant was discarded and
118 the pellet was resuspended in 1 ml sterile ¼ strength Ringers solution to remove metal ions
119 associated with the media and to provide an osmotic balance for the microorganisms. The cells
120 were centrifuged again at 674 x g for 5 min, the supernatant was discarded and the cells were
121 washed by resuspending in 1 ml ¼ strength Ringers solution. This was repeated a further three
122 times and finally the cells were resuspended in 1 ml of ¼ strength Ringers solution.

123 An agarose suspension (final volume and concentration of 20 ml and 1% (w/v),
124 respectively, of agarose) containing minimal medium (or LB) was prepared following the same
125 growth medium used to grow the *S. oneidensis* cells. This suspension was autoclaved for 15 min
126 at 121°C and 15 psi. After the agarose suspension had cooled to 40°C, 20 μ l amino acids were
127 added [final concentration of 20 μ g/L L-arginine, 20 μ g/L L-glutamate and 20 μ g/L L-serine]
128 and *S. oneidensis* cells resuspended in 1 ml of ¼ strength Ringers solution. The suspension was
129 stirred for 1 min and then dispensed between two pre-warmed glass plates that were separated by
130 a 0.05 cm spacer. All glass plates, spacers and DGT devices had been soaked in 10% HNO₃ acid
131 for several hours and then thoroughly rinsed in ultrapure water (milli Q at 18.2 M Ω ·cm) until pH
132 5. Disks (2.2 cm in diameter and 0.05 cm) were cut from the gel and gently eased off the glass

133 plate into ultrapure water. The disks were briefly stirred in 0.01 M sodium nitrate. Control disks
134 were prepared as previously described except *S. oneidensis* washed cells were added to boiling
135 medium that resulted in heat killed cells or a similar volume of ¼ strength Ringers solution was
136 used instead of the cells. Similarly, 1% (w/v) agarose disks were prepared containing 1% (w/v)
137 Luria Broth (LB) that were inoculated with *S. oneidensis* grown for 19 h in LB medium. This
138 suspension was dispensed between pre-warmed plates separated by 0.1 mm spacers to determine
139 whether thickness or medium composition of the diffusive gels containing *S. oneidensis* would
140 affect diffusion of metals through the gel.

141 The agarose disks without cells, heat killed cells and live cells were incorporated into the
142 DGTs devices to form cell free DGTs, heat killed BMDGTs and BMDGTs that were used
143 throughout this study. The typical DGT is formed of Chelex-100 resin gel, overlaid with a
144 diffusion gel layer and then a polycarbonate filter membrane that are securely held together in a
145 plastic moulding. Polycarbonate filter membranes were placed behind the Chelex-100 resin gel
146 during the assembly of the DGTs where the thickness of the agarose disks was insufficient to fill
147 the space within the DGT device. In the classical DGT, a polycarbonate membrane is overlaid
148 onto the diffusive gel but this membrane was not used in the BMDGT to enable direct contact
149 between bacterial cells and metal colloids. Classical cell free DGTs containing agarose were
150 prepared using the standard procedure as previously described (Zhang et al., 1995).

151 Each DGT was deployed in a separate pot in triplicate in a stationary solution of 0.01 M
152 NaNO₃ containing metals at known concentrations and incubated at 30°C. Previous experiments
153 showed that cells were lost from the agarose layer especially during agitation and a stationary
154 system was used in order to maintain the highest number of cells within the agarose layer.
155 Simple metal solutions were prepared using CoNO₃ and CdNO₃ in 0.01 M NaNO₃. The

156 BMDGTs were disassembled after they had been deployed. The Chelex-100 resin gel was placed
157 into 1 ml of 1 M HNO₃ and shaken, while the agarose disk was placed into ¼ strength Ringers
158 solution for staining using LIVE/ DEAD *BacLight*, or culturing onto LB plates containing agar.
159 The eluates from the Chelex-100 resin gels were diluted and metals measured using ICP-MS
160 (Thermo Scientific, X7) ensuring that the percentage of rhodium remained consistent throughout
161 all the samples. They were also compared with metal standards ranging from 1-20 µg L⁻¹.

162

163 2.3. Calculation of metal associated with DGT

164

165 Previous studies have shown that there is a gradient of metal concentration within the
166 diffusive gel (1% (w/v) agarose disk in this case, an assumption is made that cells and media
167 have no effect on the diffusion coefficient) and the concentration within the gel close to the
168 Chelex-100 resin gel is negligible, as metals are constantly being absorbed by the Chelex-100
169 resin. The mass of metal, M , accumulated in the resin is given by equation 1 where C_e is the
170 concentration of metals eluted from the Chelex-100 resin into the 1 M HNO₃ that is measured by
171 ICP-MS, V_1 is the volume of 1M HNO₃ immersing the Chelex-100 resin gel, V_2 is the volume of
172 the diffusive gel and f_e is the elution factor. The values for V_2 and f_e are typically 0.15 ml and
173 0.8, respectively.

174

$$M = C_e(V_1 + V_2)/f_e \quad (\text{eqn. 1})$$

175

$$C_{DGT} = M \Delta g / (DtA) \quad (\text{eqn. 2})$$

176 The mean concentration of metals at the surface of the DGT device, C_{DGT} , can be calculated
177 using eqn. 2, where Δg is the thickness of the diffusive gel (0.05 cm for 1% (w/v) agarose gel), D
178 is the diffusion coefficient of the uncomplexed metal, t is the deployment time in seconds and A
179 is the area of the exposure window (3.14 cm² for solution DGT devices and 2.54 cm² for soil
180 DGT devices). The diffusion coefficients were derived for the appropriate temperature from
181 established values for metals diffusing through either agarose or polyacrylamide cross-linked
182 gels (Zhang and Davison, 1999).

183

184 2.4. Determination of viability of *Shewanella oneidensis* MR-1 associated with agarose disks

185

186 Viability of cells embedded in the agarose disks was assessed using two methods,
187 culturing on agar containing LB and confocal microscopy of cells within agarose disks stained
188 with LIVE/ DEAD *BacLight* kit (Invitrogen, UK). In both methods each agarose disk was
189 placed into 1 ml of ¼ strength Ringers solution and hand shaken for 1 min.

190

191 2.4.1. Culture counts

192

193 A serial dilution was made in ¼ strength Ringers solution and 100 µl of each serial
194 dilution was spread onto LB plates. The plates were incubated at 30°C for 24 h and then the
195 colonies were counted.

196 2.4.2. Viability assessment

197

198 *S. oneidensis* was grown in either LB or *Shewanella* minimal medium and washed three
199 times in ¼ strength Ringers solution. The determination of viability using BacLight LIVE/
200 DEAD kit requires using 3 µl of combined equal mixture of 3.34 mM Syto 9 and 20mM PI
201 added to 1 ml of cells. In one procedure the cells were stained beforehand to ensure that cells
202 could be viewed and were counterstained with PI. In this procedure, the cells were resuspended
203 in 1 ml ¼ strength Ringers solution containing 1 µl 3.34 nM SYTO-9. The agarose solution (of
204 1% (w/v) contained a final population of 5×10^8 cells in 1 nM propidium iodide (PI). Disks were
205 cut into thin cross-sections in order to observe cells within the disks using the Leica DMIRE2
206 microscope with a TCS SP2 AOBS confocal scanning system (Leica Microsystems). The argon
207 laser line Argon laser at 488 nm was selected using the AOTF and emission was collected
208 between 500 and 520 nm for SYTO 9 and between 600 and 680 nm for propidium iodide. Viable
209 cells were examined in approximately 30 cross-sections beginning at the top surface of the disk
210 and finishing at the bottom surface of the disks using the Z-stack mode. They were also
211 examined at right angles to the top and bottom surfaces. The captured images were analysed
212 using Image J software. In an alternative procedure, higher quantities of stains were used at
213 similar proportions to determine whether the initial staining procedure showed any differences
214 and to stain cells within the agarose disks. In this alternative staining procedure, the viability of
215 cells in the disks was determined after they had been deployed and to examine how the staining
216 method would affect the staining of the cells. The agarose disk containing the cells was
217 immersed for 15 min in 1 ml ¼ strength Ringers solution containing 9.98 nM SYTO-9 and 60
218 nM PI and viewed under the confocal microscope as previously described. Cells appearing

219 green were stained with Syto 9 indicating they were “live”, cells appearing red were stained with
220 PI indicating they were “dead” and cells appearing yellow were assumed to be in a transient state
221 between “live” and “dead”.

222

223 **3. Results**

224 *3.1. Absorption of metals to cells*

225

226 Growth of *S. oneidensis* in minimal medium and LB containing 100 $\mu\text{g L}^{-1}$ Co and Cd
227 showed that the microorganism was unaffected by high metal concentrations (Figure 1). As
228 expected, growth of *S. oneidensis* in minimal medium was slower than in LB, although final cells
229 numbers were similar. The association of Cd to *S. oneidensis* grown in minimal medium was
230 determined at discrete times when samples were taken for culturable counts. The association
231 with Cd was highest at 5 h, corresponding to late lag phase at 423×10^{-21} ($\pm 170 \times 10^{-21}$) g per cell,
232 and lowest at 14 h, corresponding to initial log phase at 63×10^{-21} ($\pm 24.9 \times 10^{-21}$) g per cell. The
233 association difference between late and initial log phases were significantly different using
234 Student *t*-test ($p = 0.029$), but at other times during the growth phases, there were no significant
235 differences in the association of Cd. Concentrations of Co and Cd measured in solution when *S.*
236 *oneidensis* had reached stationary phase had decreased by less than 1% showing that cells during
237 the stationary phase would be exposed to relatively equal metal concentrations to those
238 concentrations at the beginning of the experiment.

239

240 3.2. Deployment of BMDGTs in metal solutions

241

242 BMDGTs (*S. oneidensis* grown in minimal medium were immobilized DGTs) were
243 deployed in 50 ml solution containing Co and Cd in 0.01 M NaNO₃ under stationary conditions
244 showed that metal concentrations measured using BMDGTs were not significantly different to
245 those measured using cell free control DGTs (DGTs similar to BMDGT except without cells)
246 under aerobic and anaerobic conditions (data not shown). The Co and Cd concentrations in
247 solutions before deployment under aerobic conditions were 56.9 (±0.6) µg L⁻¹ and 69.6 (±0.7) µg
248 L⁻¹, respectively, while under anaerobic conditions they were 113 (±4.6) and 135 (±6.2) µg L⁻¹,
249 respectively. The values in parenthesis refer to standard deviations. When DGTs are deployed
250 in stationary solutions, the calculation of the hypothetical diffusible layer is apparently much
251 thicker than it is actually which leads to lower metal concentrations associated with the binding
252 layer. Therefore, it was not surprising that lower metal concentrations were associated with the
253 BMDGTs compared with the concentration found in the immersion solution.

254 When BMDGTs containing cells grown in LB were deployed in metal solutions under
255 anaerobic conditions, the concentration of Cd that was associated with BMDGTs was
256 significantly lower compared with the Cd concentration associated with cell free DGTs
257 containing agarose (p = 0.007 using Student's *t*-test and assuming unequal variances) (Figure 2).
258 However, there were no significant differences under aerobic conditions. The initial
259 concentrations of Co and Cd ions in the deployment solution under aerobic conditions were 113
260 (±1.0) µg L⁻¹ and 130 (±2.1) µg L⁻¹, respectively, while under anaerobic conditions they were
261 117 (±0.4) µg L⁻¹ and 129 (±1.4) µg L⁻¹. Deployment of BMDGTs caused no significant changes

262 in the metal concentrations in solution. There were no significant differences in Co and Cd
263 concentrations in the deployment solution containing the cell free DGTs compared to the
264 deployment solution containing the BMDGTs after 4 h of deployment. The aerobic and
265 anaerobic deployments of BMDGTs (Figure 2 and Figure 3) were incubated at 25°C and 30°C,
266 respectively.

267

268 *3.3. Deployment of BMDGTs in hematite and metal solutions*

269

270 The Fe concentration of the BMDGTs appeared higher under anaerobic conditions
271 compared with aerobic conditions, although the large error bar associated with BMDGTs under
272 anaerobic conditions ensured that there was no significant difference (Figure 3). It can only be
273 assumed that hematite colloids are heterogeneously distributed and that a higher density of cells
274 within the BMDGTs may have increased the frequency of interaction between bacterial cells and
275 colloids perhaps resulting in lower error bars. However, Co and Cd concentrations associated
276 with BMDGTs under anaerobic conditions only were significantly higher than concentrations
277 associated with the BMDGTs containing heat killed cells.

278

279 *3.4. Viability of cells in BMDGTs*

280

281 The viability assessed using culturing indicated that the number of *S. oneidensis* cells
282 increased within the BMDGTs and remained steady for 20 h when stored at 4°C (Fig. 4). The

283 viability of *S. oneidensis* within the BMDGTs was assessed before and after deployment. LB
284 grown cells embedded in agarose that were stained with SYTO-9 and PI revealed that 35% of the
285 cells were still viable (Figure 5A). In contrast only 16% of cells that were grown in minimal
286 medium and embedded into the agarose disks were stained with SYTO-9 (Figure 5B). There
287 were a higher proportion of red (dead) stained cells with PI whether the cells were stained with
288 SYTO-9 beforehand (Figure 5A) or when the cells were stained with SYTO-9 and PI after the
289 cells had been embedded in the agarose disk (Figure 5C). However, the DNA in the cells that
290 interacted mostly with SYTO-9 and could be described as live cells were green when stained
291 beforehand with SYTO-9, and were yellow when stained afterwards with SYTO-9 and PI
292 simultaneously. The appearance of the yellow stained cells indicated that the integrity of the cell
293 membranes may have been affected and perhaps more accurately reflected the actual state of the
294 “live” cells (Figure 5C). The phenomenon of yellow stained cells using *BacLight* LIVE/ DEAD
295 staining has been observed previously (Boulos et al., 1999). After deployment of the agarose
296 disks, LIVE/ DEAD *BacLight* staining revealed that the cells stained green with SYTO-9,
297 suggesting that the integrity of the cell membranes had improved, and cells stained red with PI
298 were no longer present (Figure 5D). After 20 h incubation at 10°C, the state of the cells
299 remained unchanged (data not shown). These results indicated that a significant proportion of *S.*
300 *oneidensis* cells remained viable when grown in LB. Different types of microorganisms could be
301 incorporated into BMDGT using this procedure to assess bacterial bioavailability where the
302 mobility of metals shows an increase as a direct consequence of bacterial presence.

303

304

305 **Discussion**

306

307 This study was to determine whether *S. oneidensis* MR-1 could be used to assess
308 bioavailability when incorporated into the diffusion gradient thin films (DGTs) that are currently
309 used as a passive device for monitoring heavy metals. At the initial stage, the growth of *S.*
310 *oneidensis* MR-1 in medium containing high concentrations of metals ions showed that
311 potentially this microorganism may grow in BMDGTs during deployment in high concentrations
312 of metals. The uptake or attachment of metal ions showed only one significant difference
313 between the lag phase and at the end of the exponential phase. Only 3.4 femto grams of metal
314 ions appeared to be associated with the cells. This demonstrated that metal ions associated with
315 cells of *S. oneidensis* MR-1 in BMDGTs will have almost a negligible effect on the
316 measurements of metals by DGT, even if cells responded to an increased input of nutrients.
317 Therefore, any differences caused by the presence of the microorganism could be attributed to
318 the microorganism's effect on the bioavailability of metals rather than metal absorption to cell
319 surfaces.

320 Deployment of DGTs in natural waters containing high organic loads can cause the
321 formation of algal biofilms that impede the diffusion of metal ions through DGT (Warnken et al.,
322 2008). A study using yeast as the binding agent in the DGT device showed that metals bind
323 rapidly to the yeast cells (Menegário et al., 2010). Here, despite the high surface area to volume
324 ratio of the bacterial cells and the high numbers of cells in the agarose disks containing LB, the
325 attachment of metals to bacterial cell surfaces under aerobic conditions had little effect on the
326 quantity of metals determined in the BMDGT compared to the cell free DGTs. The significant
327 proportion of dead cells is unlikely to have had a large influence on the finding, as Chubar et al.

328 (2008) found the attachment of metal ions to dead cells of *S. oneidensis* was only reduced two
329 fold compared to live cells. The concentration of Cd (0.88 nM) used in these studies was more
330 than 100 fold higher than Cd concentrations determined in a pristine river using DGTs (Warnken
331 et al., 2008). Therefore under conditions where metal concentrations are very low, bacterial
332 biofilms may have a greater impact in attachment of metal ions. However, BMDGTs containing
333 LB showed a decrease in the flux of metal ions under anaerobic conditions compared to control
334 BMDGTs without cells. This was most likely caused by Cd binding to the phosphoryl groups in
335 *S. oneidensis* when the Cd concentration is high (Bhoo et al., 2010). The BMDGT with minimal
336 medium would have low phosphate resulting in a lower proportion of phosphoryl groups. The
337 effect of metal ions attaching to bacteria may have been more pronounced in BMDGTs
338 compared to the attachment observed when disks containing cells were suspended in a solution
339 of metal ions because the flux of metal ions passing through the BMDGT may be greater than
340 metals accumulating through diffusion. However, under aerobic conditions there was no
341 decreased flux that could possibly be associated with bacterial attachment, perhaps due to
342 different protein expression profiles under aerobic and anaerobic conditions (Beliaev et al.,
343 2002). Therefore, it appears that only under unusual circumstances, such as high nutrient
344 concentrations and anaerobic conditions is it likely that *S. oneidensis* may significantly reduce
345 the flux of metal ions through BMDGTs.

346 These conditions may arise during deployments in soils with high organic contents and
347 biomass. The effect of *S. oneidensis* in the BMDGTs on hematite showed that the solubility of
348 Fe remained unchanged in contrast to a previous study that showed an increase in organic
349 soluble iron complexes, albeit at low levels (Taillefert et al., 2007). It is possible that the
350 concentrations of soluble iron were too low to be detected yet Co and Cd ion concentrations

351 showed an increase. This effect may be caused by an interaction between Co and Cd ions and
352 hematite under anaerobic conditions rather than being a biological effect. Differences may have
353 been found if the BMDGTs were immersed in a deployment suspension containing low
354 concentrations of a carbon source to enable *S. oneidensis* to become metabolically active. The
355 addition of LB to the BMDGTs most likely diffused into the deployment suspension although at
356 these concentrations were unlikely to be effective. The use of warm agarose in the preparation
357 process ensured that *S. oneidensis* MR-1 cells were evenly distributed throughout the agarose
358 disk. However, it could potentially affect the viability, metabolism and cell structure. A previous
359 study has shown that temperatures up to 42°C temporarily affected the regulation of genes,
360 especially down regulating genes involved in energy metabolism (Gao et al., 2004). Viability
361 determined using LIVE/ DEAD *BacLight* staining showed that many cells appeared yellow and
362 were assumed to be active because later many active cells were present embedded in the agarose
363 disks. Boulos et al. (1999) showed that LIVE/ DEAD *BacLight* staining of chlorinated samples
364 resulted in some yellow cells that were no longer culturable whereas another study showed the
365 presence of yellow stained cells of *Pseudomonas putida* in similar proportion to culturable
366 counts (Ivanova et al., 2010). However, culturing cells detaching from the BMDGTs appeared
367 to confirm that many cells of *S. oneidensis* were still viable within the system. After
368 deployment, LIVE/ DEAD *BacLight* staining revealed that the cells in the BMDGTs stained
369 green with SYTO-9 indicating that their proportion had increased. The presence of media within
370 the BMDGTs would have enabled the microorganisms to survive assuming that some of the
371 media did not diffuse from the disks. It was assumed that the remaining viable cells would be
372 sufficient to determine whether they have an effect on the bioavailability of metals and therefore
373 decrease or increase the proportion of labile metals associated with the Chelex-100 resin.

374 However, ideally a much higher proportion of viable cells would show more clearly whether
375 cells increased the bioavailability of metals. A much higher population of cells could be
376 achieved by immersing the disks containing the cells in media to stimulate growth.

377 The results of this study have shown that bacteria can be effectively incorporated into
378 modified DGTs especially if the cells are grown in LB and under certain conditions they
379 influence the metal accumulated on the Chelex-100 resin, demonstrating the effect of
380 microorganisms on the bioavailability of metals. Although this first study has demonstrated the
381 feasibility of modifying the performance of DGT using live organisms, the impact on the
382 measured accumulated metal was generally small. Future studies could be performed by
383 incorporating low concentrations of different medium into the deployment suspension to
384 encourage growth of *S. oneidensis* and using other microorganisms that may increase metal
385 mobilization. For example actinobacteria within the rhizosphere are believed to mobilize metals
386 (e.g. copper) through the production of organic acids and siderophores leading to uptake by
387 hyper-accumulator plants (Tao et al., 2003, Wang et al., 2008, Kuffner et al., 2010 and Turmel et
388 al., 2011). Microorganisms isolated from these environments could be incorporated into
389 BMDGTs to determine whether they increase mobilization of metals. This study has shown a
390 few effects of *S. oneidensis* on metal solutions, colloids and nanoparticles.

391

392 **ACKNOWLEDGEMENTS**

393

394 We wish to thank Debra Hurst with assistance in confocal microscopy.

395

396 **REFERENCES**

397
398 Beliaev A. S., Thompson, D. K., Khare, T., Lim, H., Brandt, C. C., Li, G., Murray, A. E.,
399 Heidelberg, J. F., Giometti, C. S., Yates, J. 3rd, Nealson, K. H., Tiedje, J. M., Zhoui, J.,
400 2002. Gene and protein expression profiles of *Shewanella oneidensis* during anaerobic
401 growth with different electron acceptors. OMICS. 6, 39-60.
402
403 Boulos, L., Pre´vost, M., Barbeau, B., Coallier, J., Desjardins, R., 1999. LIVE/DEAD®
404 BacLight™: application of a new rapid staining method for direct enumeration of viable
405 and total bacteria in drinking water. J. Microbiol. Meth. 37, 77–86.
406
407 Charrier, T., Chapeau, C., Bendria, L., Picart, P., Daniel, P., Thouand, G., 2011. A multi-
408 channel bioluminescent bacterial biosensor for the on-line detection of metals and
409 toxicity. Part II: technical development and proof of concept of the biosensor. Anal.
410 Bioanal. Chem. 400, 1061–1070.
411
412 Chubar, N., Behrends, T., Van Cappellen, P., 2008. Biosorption of metals (Cu^{2+} , Zn^{2+}) and
413 anions (F^- , H_2PO_4^-) by viable and autoclaved cells of the Gram-negative bacterium
414 *Shewanella putrefaciens*. Colloids Surf. B 65, 126–133.
415
416 Degryse, F., Smolders, E., Zhang, H., Davison, W., 2009. Predicting availability of mineral
417 elements to plants with the DGT technique: a review of experimental data and
418 interpretation by modeling. Environ. Chem. 6 (3), 198-218.
419

420 Eltzov, E., Marks, R. S., 2011. Whole-cell aquatic biosensors. *Anal. Bioanal. Chem.* 400, 895–
421 913.

422

423 Gadd, G.M., 2010. Metals, minerals and microbes: geomicrobiology and bioremediation.
424 *Microbiology* 156, 609–643.

425

426 Gao, H., Wang, G. Y., Liu, X., Yan, T., Wu, L., Alm, E., Arkin, A., Thompson, D. K., Zhou, J.,
427 2004. Global Transcriptome Analysis of the Heat Shock Response of *Shewanella*
428 *oneidensis*. *J. Bact.* 186, 7796–7803.

429

430 Hau, H. H., Gilbert, A., Coursolle, D., Gralnick, J. A., 2008. Mechanism and Consequences of
431 Anaerobic Respiration of Cobalt by *Shewanella oneidensis* Strain MR-1. *Appl Environ*
432 *Microbiol.* 74, 6880–6886.

433

434 Howari, F.M., Abu-Rukah, Y, Goodell, P.C., 2004. Heavy metal pollution of soils along North
435 Shuna-Aqaba Highway, Jordan. *Int. J. Environ. Pollut.* 22, 597-607.

436

437 Ivanova, I. A., Kambarev, S., Popova, R. A., Naumovska, E. G., Markoska, K. B., Dushkin, C.
438 D., 2010. Determination of *Pseudomonas putida* live cells with classic cultivation and
439 staining with “Live/Dead *BacLight* bacterial viability kit”. *Biotechnol. Biotec. Eq.* 24,
440 567-570.

441

442 Kuffner, M., De Maria, S., Puschenreiter, M., Fallmann, K., Wieshammer, G., Gorfer, M.,
443 Strauss, J., Rivelli, A. R., Sessitsch, A., 2010. Culturable bacteria from Zn- and Cd-
444 accumulating *Salix caprea* with differential effects on plant growth and heavy metal
445 availability. *J. Appl. Microbiol.* 108, 1471–1484.
446

447 Lies, D. P., Hernandez, M. E., Kappler, A., Mielke, A. E., Gralnick, J. A., Newman, D. K., 2005.
448 *Shewanella oneidensis* MR-1 Uses Overlapping Pathways for Iron Reduction at a
449 Distance and by Direct Contact under Conditions Relevant for Biofilms. *Appl. Environ.*
450 *Microbiol.* 71, 4414–4426.
451

452 Meitl, L. A., Eggleston, C. M., Colberg, P. J. S., Khare, N., Reardon, C. L., Shi, L., 2009.
453 Electrochemical interaction of *Shewanella oneidensis* MR-1 and its outer membrane
454 cytochromes OmcA and MtrC with hematite electrodes. *Geochim. Cosmochim. Acta* 73,
455 5292–5307.
456

457 Menegário, A. A., Tonello, P. S., Durrant, S. F., 2010. Use of *Saccharomyces cerevisiae*
458 immobilized in agarose gel as a binding agent for diffusive gradients in thin films. *Anal.*
459 *Chim. Acta* 683 (1), 107–112.
460

461 Mishra, B., Boyanov, M., Bunker, B. A., Kelly, S. D., Kemner, K. M., Fein, J. B., 2010. High-
462 and low-affinity binding sites for Cd on the bacterial cell walls of *Bacillus subtilis* and
463 *Shewanella oneidensis*. *Geochim. Cosmochim. Acta* 74, 4219–4233.
464

465 Mugerfield, I., Law, B. A., Wickham, G. S., Thompson, D. K., 2009. A putative azoreductase
466 gene is involved in the *Shewanella oneidensis* response to heavy metal stress. *Appl.*
467 *Microbiol. Biotechnol.* 82, 1131–1141.

468

469 Pesavento, M., Alberti, G., Biesuz, R., 2009. Analytical methods for determination of free metal
470 ion concentration, labile species fraction and metal complexation capacity of
471 environmental waters: A review. *Anal. Chim. Acta* 631 (2), 129–141.

472

473 Pichette, C., Zhang, H., Davison, W., Sauve, S., 2007. Preventing biofilm development on DGT
474 devices using metals and antibiotics. *Talanta* 72, 716–722.

475

476 Ruebush, S. S., Brantley, S. L., Tien, M., 2006. Reduction of Soluble and Insoluble Iron Forms
477 by Membrane Fractions of *Shewanella oneidensis* Grown under Aerobic and Anaerobic
478 Conditions. *Appl. Environ. Microbiol.* 72, 2925–2935.

479

480 Taillefert, M., Beckler, J.S., Carey, E., Burns, J.L., Fennessey, C.M., DiChristina, T.J., 2007.
481 *Shewanella putrefaciens* produces an Fe (III)-solubilizing organic ligand during
482 anaerobic respiration on insoluble Fe (III) oxides. *J. Inorg. Biochem.* 101, 1760–1767.

483

484 Tao, S., Chen, Y. J., Xu, F. L., Cao J., Li, B. G., 2003. Changes of copper speciation in maize
485 rhizosphere soil. *Environ. Poll.* 122, 447–454.

486

487 Turmel, M. C., Courchesne, F., Cloutier-Hurteau, B., 2011. Microbial activity and water-soluble
488 trace element species in the rhizosphere of spring wheat (*Triticum aestivum* cv. USU-
489 Perigee) J. Environ. Monit. 13, 1059-1072.

490

491 van Leeuwen, H. P., Town, R. W., Buffle, J., Cleven, R. F. M. J., Davison, W., Puy, J., van
492 Riemsdijk, W. H., Sigg, L., 2005. Dynamic Speciation Analysis and Bioavailability of
493 Metals in Aquatic Systems. Environ. Sci. Technol. 39, 8545-8556.

494

495 Wang, Y. P., Li, Q. B., Shi, J. Y., Qi Lin, Q., Chen, X. C., Wu, W., Chen, Y. X., 2008.
496 Assessment of microbial activity and bacterial community composition in the rhizosphere
497 of a copper accumulator and a non-accumulator. Soil Biol. Biochem. 40, 1167-1177.

498

499 Warnken, K. W., Davison, W., Zhang, H., 2008. Interpretation of in situ speciation
500 measurements of inorganic and organically complexed trace metals in freshwater by
501 DGT. Environ. Sci. Technol. 42, 6903–6909.

502

503 Williams, P.N., Zhang, H., Davison, W., Meharg, A.A., Hossain, M., Norton, G.J., Brammer, H.,
504 Islam, M.R., 2011. Organic matter - Solid phase interactions are critical for predicting
505 arsenic release and plant uptake in Bangladesh paddy soils. Environ. Sci. Technol. 45,
506 6080-6087.

507

508 Yang, H, Rose, N., 2005. Trace element pollution records in some UK lake sediments, their
509 history, influence factors and regional differences. Environ. Int. 31(1), 63– 75.

510

511 Zhang, H., Davison, W., 1995. Performance characteristics of diffusion gradients in thin films
512 for the in situ measurement of trace metals in aqueous solution. *Anal. Chem* 67, 3391-
513 3400.

514

515 Zhang, H., Davison, W., 1999. Diffusional characteristics of hydrogels used in DGT and DET
516 techniques. *Anal. Chim. Acta* 398, 329–340.

517

518 Zhang, H., 2004. In-situ speciation of Ni and Zn in freshwaters: Comparison between
519 DGT measurements and speciation models. *Environ. Sci. Technol.* 38, 1421-1427.

520

521 **List of figures**

522 **Fig. 1** Growth curve of *S. oneidensis* in LB □ and minimal medium specifically used for
523 growth of *Shewanella* sp. ◇. Each medium contained 100 µg L⁻¹ Co and Cd. Standard
524 deviations are shown within □ and around ◇.

525 **Fig. 2** DGT calculated metal concentrations from deployment of BMDGT without cells ■ and
526 BMDGT with *S. putrefaciens* cells □ in 50 ml metal solution under (A) aerobic conditions and
527 (B) anaerobic conditions. BMDGTs have an agarose diffusive layer that contained 1% (w/v) LB
528 and significant differences shown by * and error bars indicate standard deviations.

529 **Fig. 3** DGT calculated metal concentrations from deployment in hematite suspension
530 containing cobalt and cadmium in solution of BMDGTs with heat killed cells □ BMDGTs with
531 *S. oneidensis* cells under aerobic conditions ■ and BMDGTs with *S. oneidensis* cells under
532 anaerobic conditions ■ . Error bars indicate standard deviations.

533 **Fig. 4** Culturable counts of *S. oneidensis* associated with BMDGTs: (HK) BMDGT containing
534 heat killed cells that was deployed immediately, (4 h) BMDGT with cells that was deployed
535 immediately, (20 h) BMDGT with cells that was deployed after 20 h incubation of BMDGT at
536 4°C and (blank) un-deployed BMDGT. Significant differences shown by * and error bars
537 indicate standard deviations.

538 **Fig. 5** LIVE/ DEAD BacLight staining of *S. oneidensis* in agarose disks (A) cells stained with
539 SYTO-9 before being embedded in agarose disk containing LB (view of edge of agarose disk),
540 (B) cells stained with SYTO-9 before being embedded in agarose disk containing minimal
541 medium (C) cells in agarose disk containing LB stained after with SYTO-9 and PI, and (D) cells
542 in agarose disk containing LB stained after with SYTO-9 and PI after deployment for 4 h.

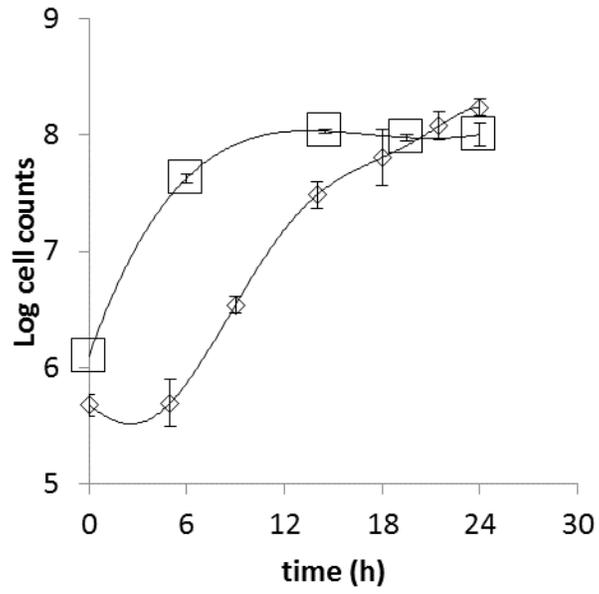


Fig. 1

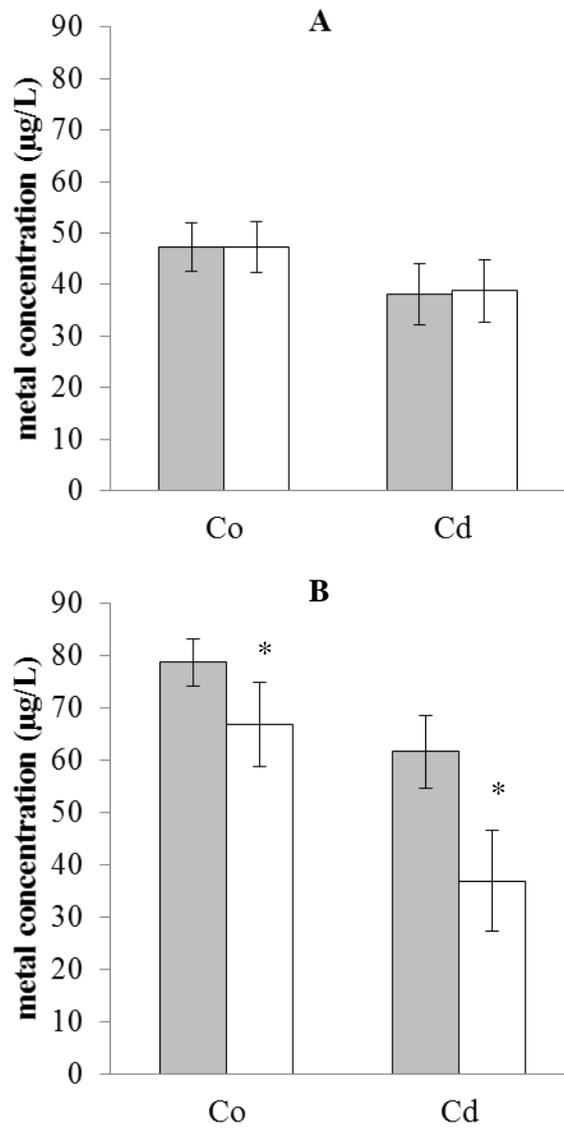


Fig. 2

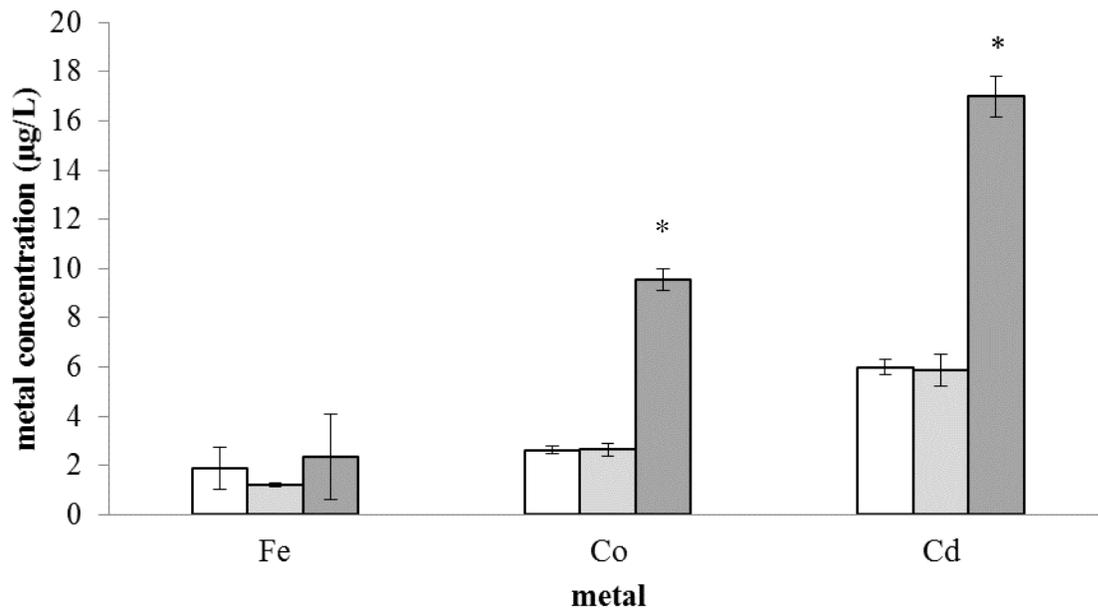


Fig. 3

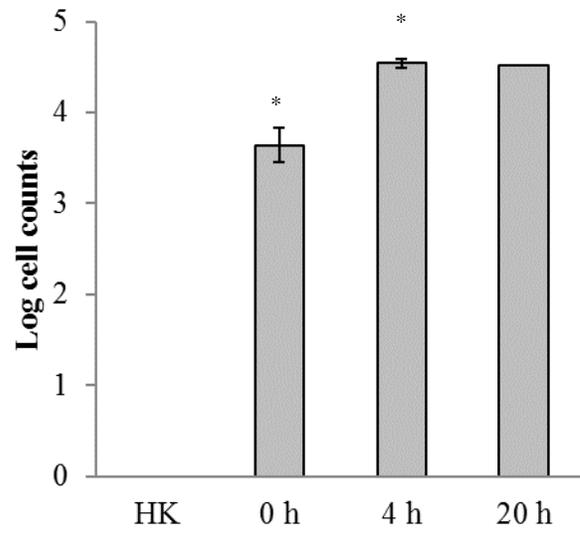


Fig. 4

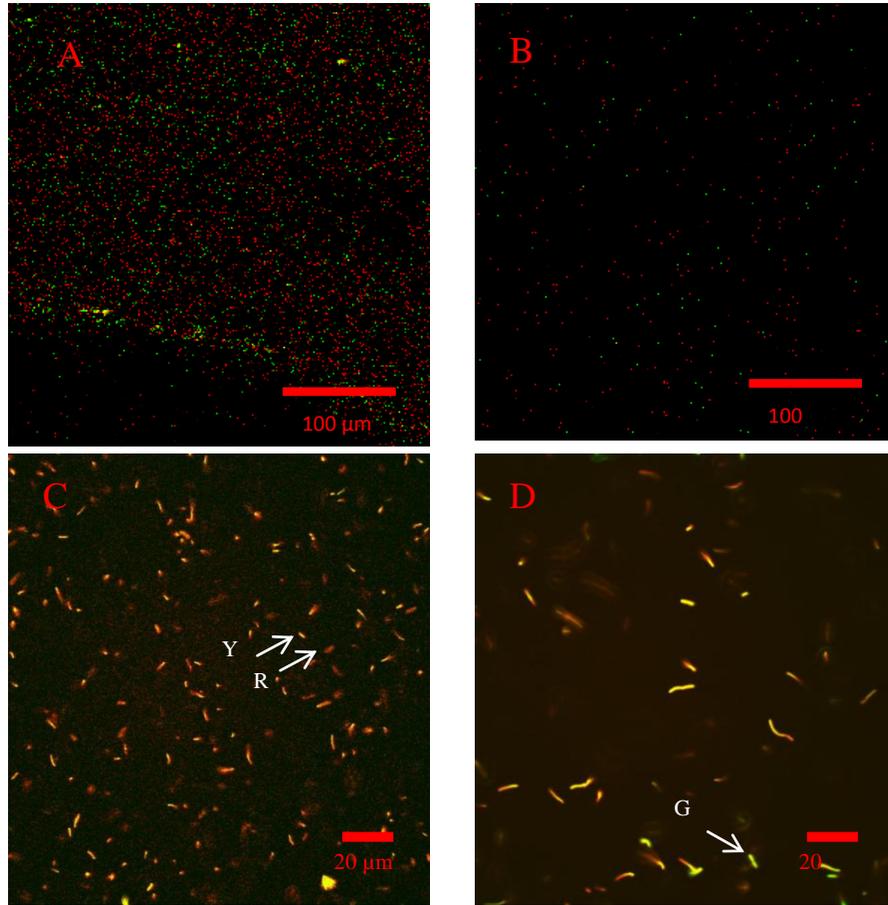


Fig. 5

