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Keywords: biological mobilizing DGT; Shewanella oneidensis; BacLight

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Response to Reviewers: COMMENTS FROM EDITORS AND REVIEWERS

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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.

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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 provider 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

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***Revision Notes**
Click here to download Revision Notes: COMMENTS.doc

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Paul W Baker*[1], Christer Högstrand[2], Jamie Lead[3], Roger W Pickup[4] and Hao Zhang[1]

[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

Abstract

Assessing metal bioavailability in soil is important in modelling the effects of metal toxicity on the surrounding ecosystem. Current methods based on diffusive gradient thin films (DGTs) and Gel-Integrated Microelectrode are limited in their availability and sensitivity. To address this, *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). Viability analysis revealed that 16-35% of the cells remained viable within the BMDGTs depending on the culturing conditions over a 20 h period with/without metals. Deployment of BMDGTs in standardized metal solutions showed significant differences to cell-free BMDGTs when cells grown in Luria Broth (LB) were incorporated into BMDGTs and deployed under anaerobic conditions. Deployment of these BMDGTs in hematite revealed no significant differences between BMDGTs and BMDGTs containing heat killed cells. Whether heat killed cells retain the ability to affect bioavailability is uncertain. This is the first study to investigate how a microorganism that was incorporated into a DGT device such as the metal reducing bacteria, *S. oneidensis*, may affect the mobility of metals.

Keywords

diffusive gradient thin films (DGT)

biological mobilizing DGT

Shewanella oneidensis

*Bac*Light

1. Introduction

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

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

(Williams et al., 2011). Some microorganisms in the environment solubilise solid phases and release metals by siderophores, organic acids and metabolites (Gadd, 2010). If such organisms can be incorporated into DGT there is the possibility that the modified DGT will provide a surrogate measurement for environmental metals that have the potential to be mobilized. Dried *Saccharomyces cerevisiae* has already been successfully used in DGT as a binding agent instead of Chelex-100 resin, but there have been no previous attempts to incorporate live microorganisms into DGT and exploit their interactions with chemical substrates (Menegário et al., 2010). Recently, a bioluminescent biosensor has been described containing *Escherichia coli* reporter strains for the detection and toxicity of Cd and As (Charrier et al., 2011). However, these and other biosensors cannot determine the effects of multianalyte detection and knowledge about protein transportation of metals across the cell membrane is limited (Eltzov and Marks, 2011). By incorporating naturally occurring microorganisms into DGT it should be possible to determine directly their effect on bioavailable metals. 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.

Previous studies of dissimilatory metal reducing bacteria, such as *Shewanella oneidensis* MR-1, showed that insoluble ferric compounds, including hematite and goethite, are reduced by outer membrane bound cytochromes, but only under anaerobic conditions (Meitl et al., 2009 and Ruebush et al., 2006). Other studies showed that (hydr)-oxide ferric deposits within glass beads were indirectly reduced by *S. oneidensis* MR-1 cells growing outside the beads (Lies et al., 2005). 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. *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). Other metals such as chelated cobalt were reduced from $[Co(III)\text{-}EDTA]^-$ to soluble, albeit toxic, $[Co(II)\text{-}EDTA]^{2-}$ by *S. oneidensis* MR-1 (Hau et al., 2008). The above evidence suggests that *S. oneidensis* could possibly reduce oxidized forms of Fe and Co at the surface of a gel in which it is encapsulated. Trace metals that are released from iron oxides as they reductively dissolve should be measured by the DGT device.

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

2. Methods

2.1. Growth of Shewanella oneidensis MR-1

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

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

A growth curve, defined by culture, of *S. oneidensis* was determined by growing the microorganism in 25 ml tubes containing 20 ml minimal medium or 10 ml LB. Minimal medium and LB were inoculated to a density of 1.2×10^6 and 1.4×10^6 cells per ml. Both media contained 100 µg L^{-1} cobalt and 100 µg L^{-1} cadmium that would be found in extremely polluted environments only (Howari et al., 2004 and Yang and Rose, 2005). This experiment determined the attachment of metal to the cells during the growth cycle in both types of media after they had been separated from the media. 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. At each sampling time, triplicate tubes were removed for destructive analysis. From each tube, 1 ml was removed, shaken for 1 min and a serial dilution was made in ¼ strength Ringers solution.

111 The serial dilutions (100 µl) 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 µl amino acids were
127 added [final concentration of 20 µg/L L-arginine, 20 µg/L L-glutamate and 20 µg/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% HNO_3 acid
131 for several hours and then thoroughly rinsed in ultrapure water (milli Q at 18.2 MΩ·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

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

The agarose disks without cells, heat killed cells and live cells were incorporated into the DGTs devices to form cell free DGTs, heat killed BMDGTs and BMDGTs that were used throughout this study. 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. Polycarbonate filter membranes were placed behind the Chelex-100 resin gel during the assembly of the DGTs where the thickness of the agarose disks was insufficient to fill the space within the DGT device. In the classical DGT, a polycarbonate membrane is overlaid onto the diffusive gel but this membrane was not used in the BMDGT to enable direct contact between bacterial cells and metal colloids. Classical cell free DGTs containing agarose were prepared using the standard procedure as previously described (Zhang et al., 1995).

Each DGT was deployed in a separate pot in triplicate in a stationary solution of 0.01 M $NaNO_3$ containing metals at known concentrations and incubated at 30ºC. 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. Simple metal solutions were prepared using $CoNO_3$ and $CdNO_3$ in 0.01 M $NaNO_3$. The

156 BMDGTs were dissembled after they had been deployed. The Chelex-100 resin gel was placed
157 into 1 ml of 1 M HNO_3 and shaken, while the agarose disk was placed into ¼ strength Ringers
158 solution for staining using LIVE/ DEAD *Bac*Light, 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 HNO_3 that is measured by
171 ICP-MS, V_1 is the volume of 1M HNO_3 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 $$M = C_e(V_1 + V_2)/f_e \qquad \text{(eqn. 1)}$$

175 $$C_{DGT} = M\Delta g/(DtA) \qquad \text{(eqn. 2)}$$

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 cm^2 for solution DGT devices and 2.54 cm^2 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 *Bac*Light 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*

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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 *Bac*Light 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

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

3. Results

3.1. Absorption of metals to cells

Growth of *S. oneidensis* in minimal medium and LB containing 100 μg L^{-1} Co and Cd showed that the microorganism was unaffected by high metal concentrations (Figure 1). As expected, growth of *S. oneidensis* in minimal medium was slower than in LB, although final cells numbers were similar. The association of Cd to *S. oneidensis* grown in minimal medium was determined at discrete times when samples were taken for culturable counts. The association with Cd was highest at 5 h, corresponding to late lag phase at 423×10^{-21} ($\pm170\times10^{-21}$) g per cell, and lowest at 14 h, corresponding to initial log phase at 63×10^{-21} ($\pm24.9\times10^{-21}$) g per cell. The association difference between late and initial log phases were significantly different using Student *t*-test ($p = 0.029$), but at other times during the growth phases, there were no significant differences in the association of Cd. Concentrations of Co and Cd measured in solution when *S. oneidensis* had reached stationary phase had decreased by less than 1% showing that cells during the stationary phase would be exposed to relatively equal metal concentrations to those concentrations at the beginning of the experiment.

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 $NaNO_3$ 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

viability of *S. oneidensis* within the BMDGTs was assessed before and after deployment. LB grown cells embedded in agarose that were stained with SYTO-9 and PI revealed that 35% of the cells were still viable (Figure 5A). In contrast only 16% of cells that were grown in minimal medium and embedded into the agarose disks were stained with SYTO-9 (Figure 5B). There were a higher proportion of red (dead) stained cells with PI whether the cells were stained with SYTO-9 beforehand (Figure 5A) or when the cells were stained with SYTO-9 and PI after the cells had been embedded in the agarose disk (Figure 5C). 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. The appearance of the yellow stained cells indicated that the integrity of the cell membranes may have been affected and perhaps more accurately reflected the actual state of the "live" cells (Figure 5C). The phenomenon of yellow stained cells using *Bac*Light LIVE/ DEAD staining has been observed previously (Boulos et al., 1999). After deployment of the agarose disks, LIVE/ DEAD *Bac*Light staining revealed that the cells stained green with SYTO-9, suggesting that the integrity of the cell membranes had improved, and cells stained red with PI were no longer present (Figure 5D). After 20 h incubation at 10°C, the state of the cells remained unchanged (data not shown). These results indicated that a significant proportion of *S. oneidensis* cells remained viable when grown in LB. 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.

Discussion

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

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

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

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

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

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

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

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REFERENCES

Beliaev A. S., Thompson, D. K., Khare, T., Lim, H., Brandt, C. C., Li, G., Murray, A. E., Heidelberg, J. F., Giometti, C. S., Yates, J. 3rd, Nealson, K. H., Tiedje, J. M., Zhoui, J., 2002. Gene and protein expression profiles of *Shewanella oneidensis* during anaerobic growth with different electron acceptors. OMICS. 6, 39-60.

Boulos, L., Pre´vost, M., Barbeau, B., Coallier, J., Desjardins, R., 1999. LIVE/DEAD® *Bac*Light™: application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water. J. Microbiol. Meth. 37, 77–86.

Charrier, T., Chapeau, C., Bendria, L., Picart, P., Daniel, P., Thouand, G., 2011. A multi-channel bioluminescent bacterial biosensor for the on-line detection of metals and toxicity. Part II: technical development and proof of concept of the biosensor. Anal. Bioanal. Chem. 400, 1061–1070.

Chubar, N., Behrends, T., Van Cappellen, P., 2008. Biosorption of metals (Cu^{2+}, Zn^{2+}) and anions (F^{-}, $H_2PO_4^{-}$) by viable and autoclaved cells of the Gram-negative bacterium *Shewanella putrefaciens*. Colloids Surf. B 65, 126–133.

Degryse, F., Smolders, E., Zhang, H., Davison, W., 2009. Predicting availability of mineral elements to plants with the DGT technique: a review of experimental data and interpretation by modeling. Environ. Chem. 6 (3), 198-218.

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 *Bac*Light bacterial viability kit". Biotechnol. Biotec. Eq. 24,
440 567-570.

441

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

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

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

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

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

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

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

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

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

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

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

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.

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

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

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

521 **List of figures**

522 **Fig. 1** Growth curve of *S. oneidensis* in LB □ and minimal medium specifically used for
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537 indicate standard deviations.

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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.

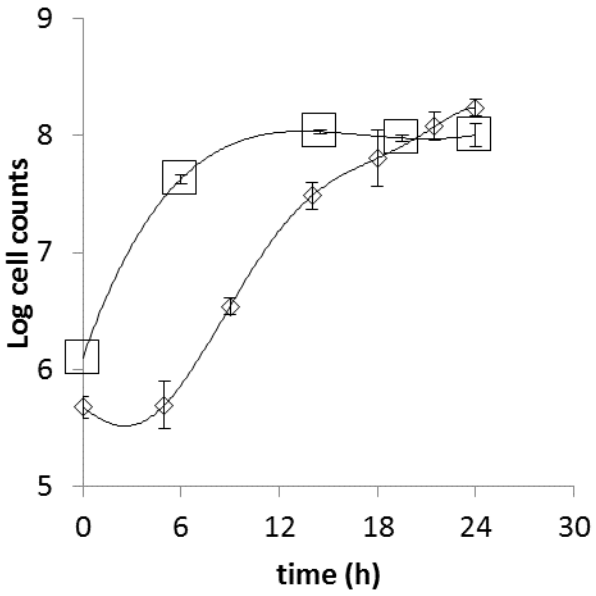
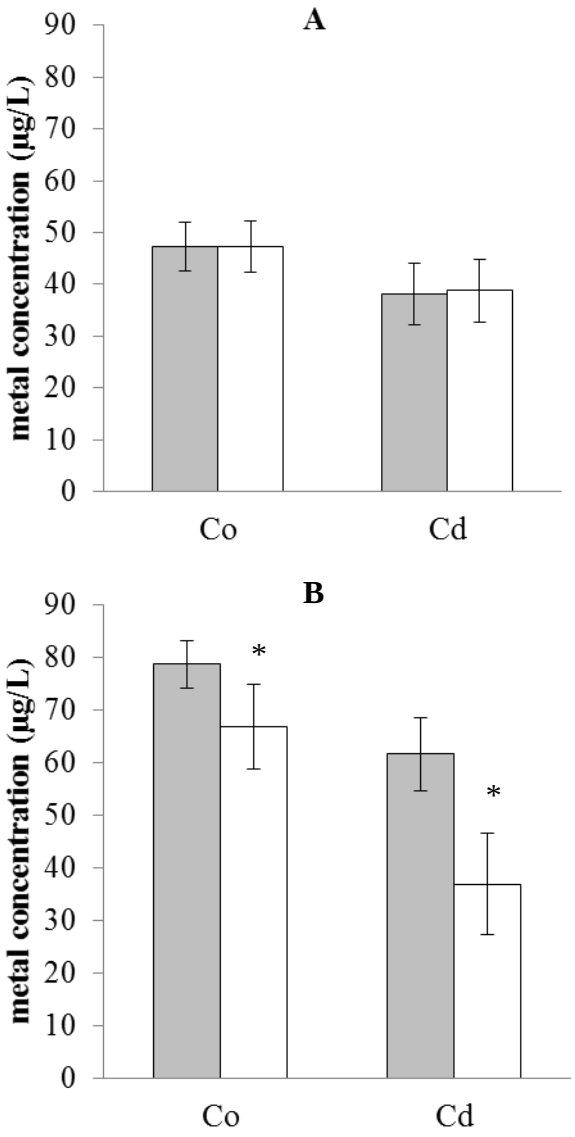


Fig. 1


A
metal concentration (µg/L)
90
80
70
60
50
40
30
20
10
0
Co
Cd
B
metal concentration (µg/L)
90
80
70
60
50
40
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*
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Co
Cd

Fig. 2

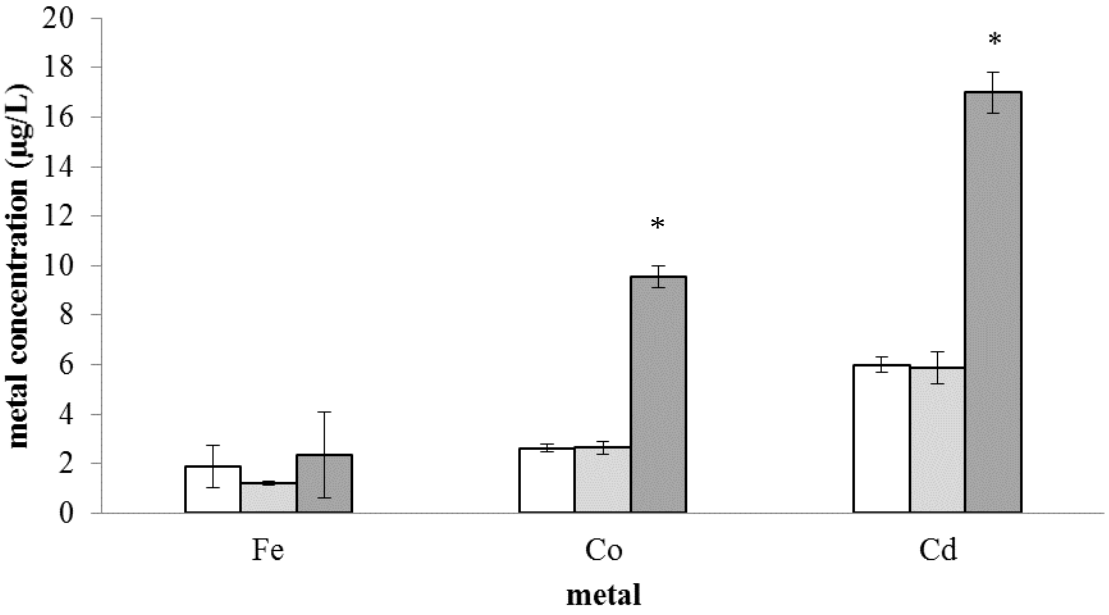

Fig. 3

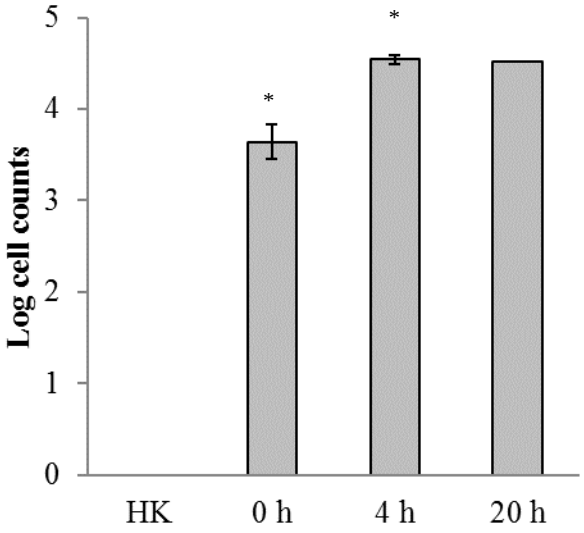

Fig. 4

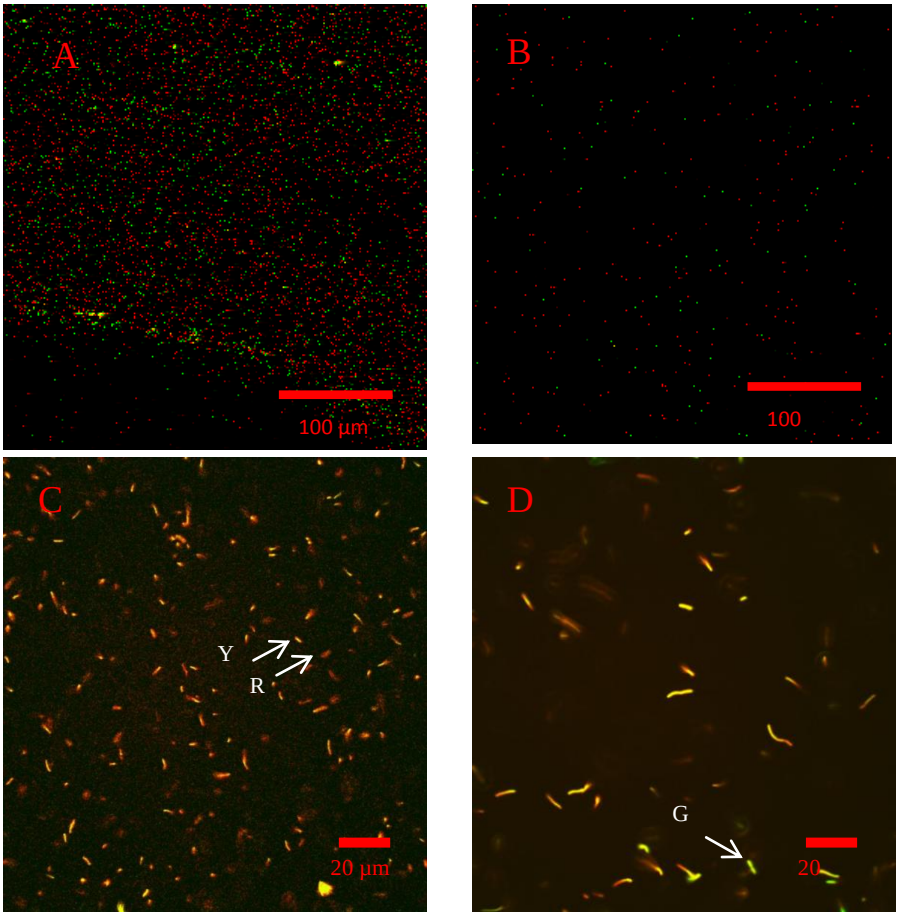

Fig. 5

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

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Abstract

Assessing metal bioavailability in soil is important in modelling the effects of metal toxicity on the surrounding ecosystem. Current methods based on diffusive gradient thin films (DGTs) and Gel-Integrated Microelectrode are limited in their availability and sensitivity. To address this, *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). Viability analysis revealed that 16-35% of the cells remained viable within the BMDGTs depending on the culturing conditions over a 20 h period with/without metals. Deployment of BMDGTs in standardized metal solutions showed significant differences to cell-free BMDGTs when cells grown in Luria Broth (LB) were incorporated into BMDGTs and deployed under anaerobic conditions. Deployment of these BMDGTs in hematite revealed no significant differences between BMDGTs and BMDGTs containing heat killed cells. Whether heat killed cells retain the ability to affect bioavailability is uncertain. This is the first study to investigate how a microorganism that was incorporated into a DGT device such as the metal reducing bacteria, *S. oneidensis*, may affect the mobility of metals.

Keywords

diffusive gradient thin films (DGT)

biological mobilizing DGT

Shewanella oneidensis

*Bac*Light

1. Introduction

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

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

(Williams et al., 2011). Some microorganisms in the environment solubilise solid phases and release metals by siderophores, organic acids and metabolites (Gadd, 2010). If such organisms can be incorporated into DGT there is the possibility that the modified DGT will provide a surrogate measurement for environmental metals that have the potential to be mobilized. Dried *Saccharomyces cerevisiae* has already been successfully used in DGT as a binding agent instead of Chelex-100 resin, but there have been no previous attempts to incorporate live microorganisms into DGT and exploit their interactions with chemical substrates (Menegário et al., 2010). Recently, a bioluminescent biosensor has been described containing *Escherichia coli* reporter strains for the detection and toxicity of Cd and As (Charrier et al., 2011). However, these and other biosensors cannot determine the effects of multianalyte detection and knowledge about protein transportation of metals across the cell membrane is limited (Eltzov and Marks, 2011). By incorporating naturally occurring microorganisms into DGT it should be possible to determine directly their effect on bioavailable metals. 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.

Previous studies of dissimilatory metal reducing bacteria, such as *Shewanella oneidensis* MR-1, showed that insoluble ferric compounds, including hematite and goethite, are reduced by outer membrane bound cytochromes, but only under anaerobic conditions (Meitl et al., 2009 and Ruebush et al., 2006). Other studies showed that (hydr)-oxide ferric deposits within glass beads were indirectly reduced by *S. oneidensis* MR-1 cells growing outside the beads (Lies et al., 2005). 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. *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). Other metals such as chelated cobalt were reduced from $[Co(III)\text{-}EDTA]^-$ to soluble, albeit toxic, $[Co(II)\text{-}EDTA]^{2-}$ by *S. oneidensis* MR-1 (Hau et al., 2008). The above evidence suggests that *S. oneidensis* could possibly reduce oxidized forms of Fe and Co at the surface of a gel in which it is encapsulated. Trace metals that are released from iron oxides as they reductively dissolve should be measured by the DGT device.

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

2. Methods

2.1. Growth of Shewanella oneidensis MR-1

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

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

A growth curve, defined by culture, of *S. oneidensis* was determined by growing the microorganism in 25 ml tubes containing 20 ml minimal medium or 10 ml LB. Minimal medium and LB were inoculated to a density of 1.2×10^6 and 1.4×10^6 cells per ml. Both media contained 100 µg L^{-1} cobalt and 100 µg L^{-1} cadmium that would be found in extremely polluted environments only (Howari et al., 2004 and Yang and Rose, 2005). This experiment determined the attachment of metal to the cells during the growth cycle in both types of media after they had been separated from the media. 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. At each sampling time, triplicate tubes were removed for destructive analysis. From each tube, 1 ml was removed, shaken for 1 min and a serial dilution was made in ¼ strength Ringers solution.

111 The serial dilutions (100 µl) 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 µl amino acids were
127 added [final concentration of 20 µg/L L-arginine, 20 µg/L L-glutamate and 20 µg/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% HNO_3 acid
131 for several hours and then thoroughly rinsed in ultrapure water (milli Q at 18.2 MΩ·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

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

The agarose disks without cells, heat killed cells and live cells were incorporated into the DGTs devices to form cell free DGTs, heat killed BMDGTs and BMDGTs that were used throughout this study. 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. Polycarbonate filter membranes were placed behind the Chelex-100 resin gel during the assembly of the DGTs where the thickness of the agarose disks was insufficient to fill the space within the DGT device. In the classical DGT, a polycarbonate membrane is overlaid onto the diffusive gel but this membrane was not used in the BMDGT to enable direct contact between bacterial cells and metal colloids. Classical cell free DGTs containing agarose were prepared using the standard procedure as previously described (Zhang et al., 1995).

Each DGT was deployed in a separate pot in triplicate in a stationary solution of 0.01 M $NaNO_3$ containing metals at known concentrations and incubated at 30ºC. 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. Simple metal solutions were prepared using $CoNO_3$ and $CdNO_3$ in 0.01 M $NaNO_3$. The

BMDGTs were dissembled after they had been deployed. The Chelex-100 resin gel was placed into 1 ml of 1 M HNO_3 and shaken, while the agarose disk was placed into ¼ strength Ringers solution for staining using LIVE/ DEAD *Bac*Light, or culturing onto LB plates containing agar. The eluates from the Chelex-100 resin gels were diluted and metals measured using ICP-MS (Thermo Scientific, X7) ensuring that the percentage of rhodium remained consistent throughout all the samples. They were also compared with metal standards ranging from 1-20 µg L^{-1}.

2.3. Calculation of metal associated with DGT

Previous studies have shown that there is a gradient of metal concentration within the diffusive gel (1% (w/v) agarose disk in this case, an assumption is made that cells and media have no effect on the diffusion coefficient) and the concentration within the gel close to the Chelex-100 resin gel is negligible, as metals are constantly being absorbed by the Chelex-100 resin. The mass of metal, M, accumulated in the resin is given by equation 1 where C_e is the concentration of metals eluted from the Chelex-100 resin into the 1 M HNO_3 that is measured by ICP-MS, V_1 is the volume of 1M HNO_3 immersing the Chelex-100 resin gel, V_2 is the volume of the diffusive gel and f_e is the elution factor. The values for V_2 and f_e are typically 0.15 ml and 0.8, respectively.

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

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

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 cm^2 for solution DGT devices and 2.54 cm^2 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 *Bac*Light 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 *Bac*Light 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

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

3. Results

3.1. Absorption of metals to cells

Growth of *S. oneidensis* in minimal medium and LB containing 100 μg L^{-1} Co and Cd showed that the microorganism was unaffected by high metal concentrations (Figure 1). As expected, growth of *S. oneidensis* in minimal medium was slower than in LB, although final cells numbers were similar. The association of Cd to *S. oneidensis* grown in minimal medium was determined at discrete times when samples were taken for culturable counts. The association with Cd was highest at 5 h, corresponding to late lag phase at 423×10^{-21} ($\pm170\times10^{-21}$) g per cell, and lowest at 14 h, corresponding to initial log phase at 63×10^{-21} ($\pm24.9\times10^{-21}$) g per cell. The association difference between late and initial log phases were significantly different using Student *t*-test ($p = 0.029$), but at other times during the growth phases, there were no significant differences in the association of Cd. Concentrations of Co and Cd measured in solution when *S. oneidensis* had reached stationary phase had decreased by less than 1% showing that cells during the stationary phase would be exposed to relatively equal metal concentrations to those concentrations at the beginning of the experiment.

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 $NaNO_3$ 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

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

3.3. Deployment of BMDGTs in hematite and metal solutions

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

3.4. Viability of cells in BMDGTs

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

viability of *S. oneidensis* within the BMDGTs was assessed before and after deployment. LB grown cells embedded in agarose that were stained with SYTO-9 and PI revealed that 35% of the cells were still viable (Figure 5A). In contrast only 16% of cells that were grown in minimal medium and embedded into the agarose disks were stained with SYTO-9 (Figure 5B). There were a higher proportion of red (dead) stained cells with PI whether the cells were stained with SYTO-9 beforehand (Figure 5A) or when the cells were stained with SYTO-9 and PI after the cells had been embedded in the agarose disk (Figure 5C). 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. The appearance of the yellow stained cells indicated that the integrity of the cell membranes may have been affected and perhaps more accurately reflected the actual state of the "live" cells (Figure 5C). The phenomenon of yellow stained cells using *Bac*Light LIVE/ DEAD staining has been observed previously (Boulos et al., 1999). After deployment of the agarose disks, LIVE/ DEAD *Bac*Light staining revealed that the cells stained green with SYTO-9, suggesting that the integrity of the cell membranes had improved, and cells stained red with PI were no longer present (Figure 5D). After 20 h incubation at 10°C, the state of the cells remained unchanged (data not shown). These results indicated that a significant proportion of *S. oneidensis* cells remained viable when grown in LB. 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.

Discussion

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

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

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

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

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

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REFERENCES

Beliaev A. S., Thompson, D. K., Khare, T., Lim, H., Brandt, C. C., Li, G., Murray, A. E., Heidelberg, J. F., Giometti, C. S., Yates, J. 3rd, Nealson, K. H., Tiedje, J. M., Zhoui, J., 2002. Gene and protein expression profiles of *Shewanella oneidensis* during anaerobic growth with different electron acceptors. OMICS. 6, 39-60.

Boulos, L., Pre´vost, M., Barbeau, B., Coallier, J., Desjardins, R., 1999. LIVE/DEAD® *Bac*Light™: application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water. J. Microbiol. Meth. 37, 77–86.

Charrier, T., Chapeau, C., Bendria, L., Picart, P., Daniel, P., Thouand, G., 2011. A multi-channel bioluminescent bacterial biosensor for the on-line detection of metals and toxicity. Part II: technical development and proof of concept of the biosensor. Anal. Bioanal. Chem. 400, 1061–1070.

Chubar, N., Behrends, T., Van Cappellen, P., 2008. Biosorption of metals (Cu^{2+}, Zn^{2+}) and anions (F^{-}, $H_2PO_4^{-}$) by viable and autoclaved cells of the Gram-negative bacterium *Shewanella putrefaciens*. Colloids Surf. B 65, 126–133.

Degryse, F., Smolders, E., Zhang, H., Davison, W., 2009. Predicting availability of mineral elements to plants with the DGT technique: a review of experimental data and interpretation by modeling. Environ. Chem. 6 (3), 198-218.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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 *Bac*Light 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.

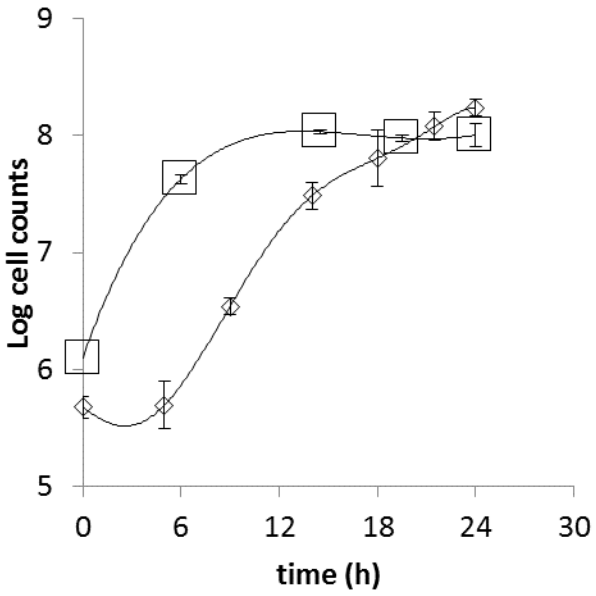
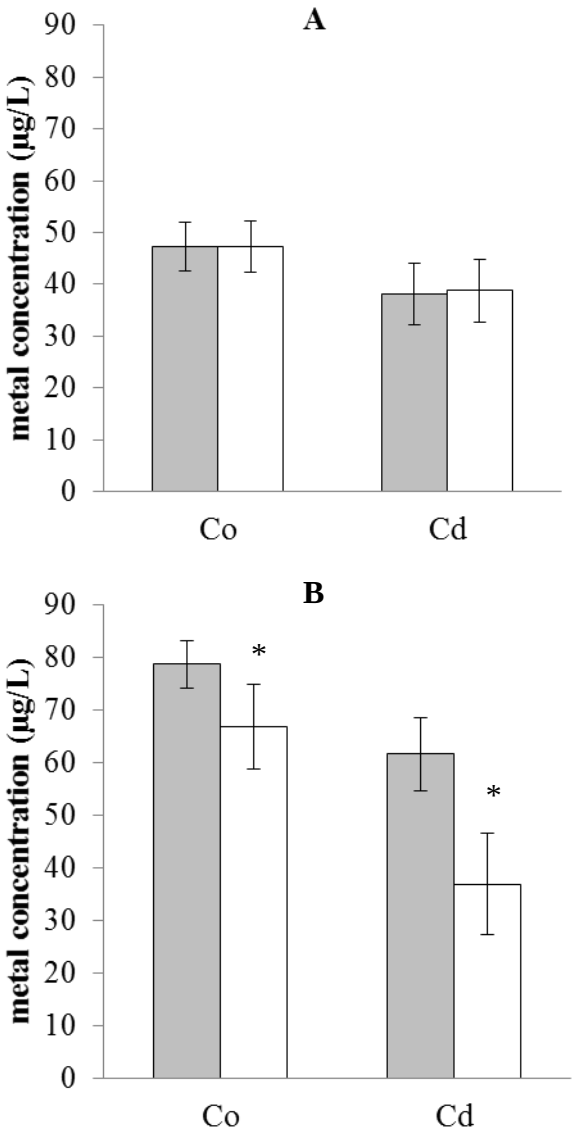


Fig. 1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80
70
60
50
40
30
20
10
0
Co
Cd
B
metal concentration (µg/L)
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80
70
60
50
40
30
20
10
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*
*
Co
Cd

Fig. 2

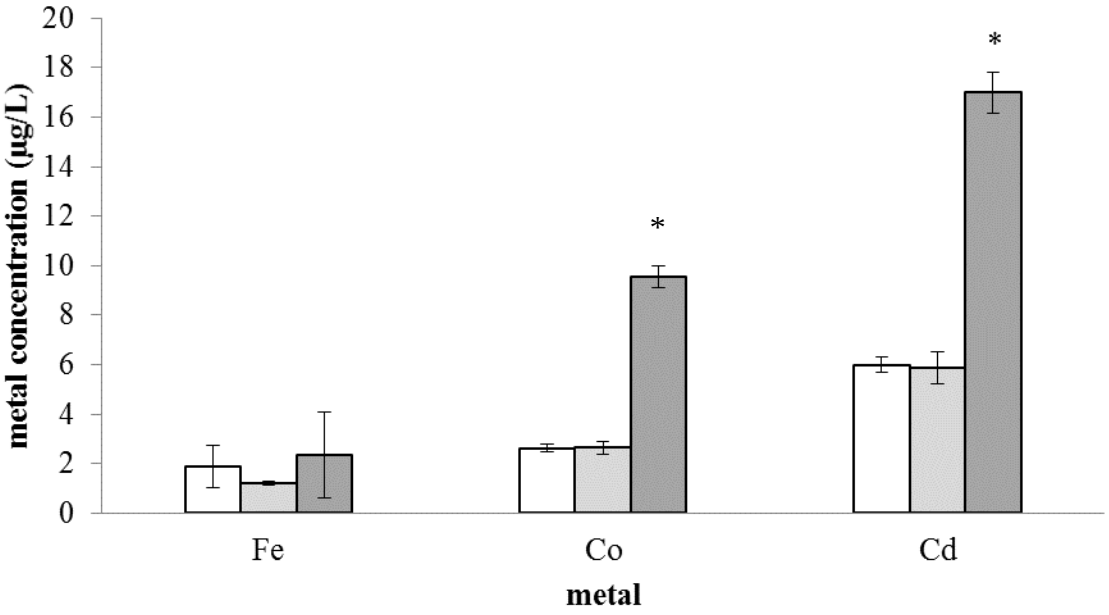

Fig. 3

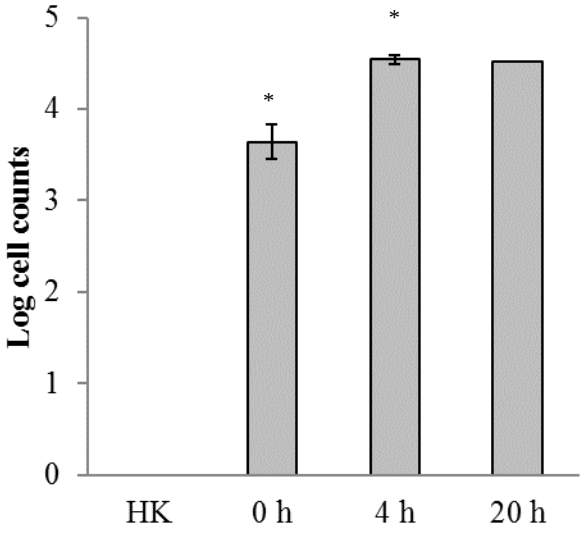

Fig. 4

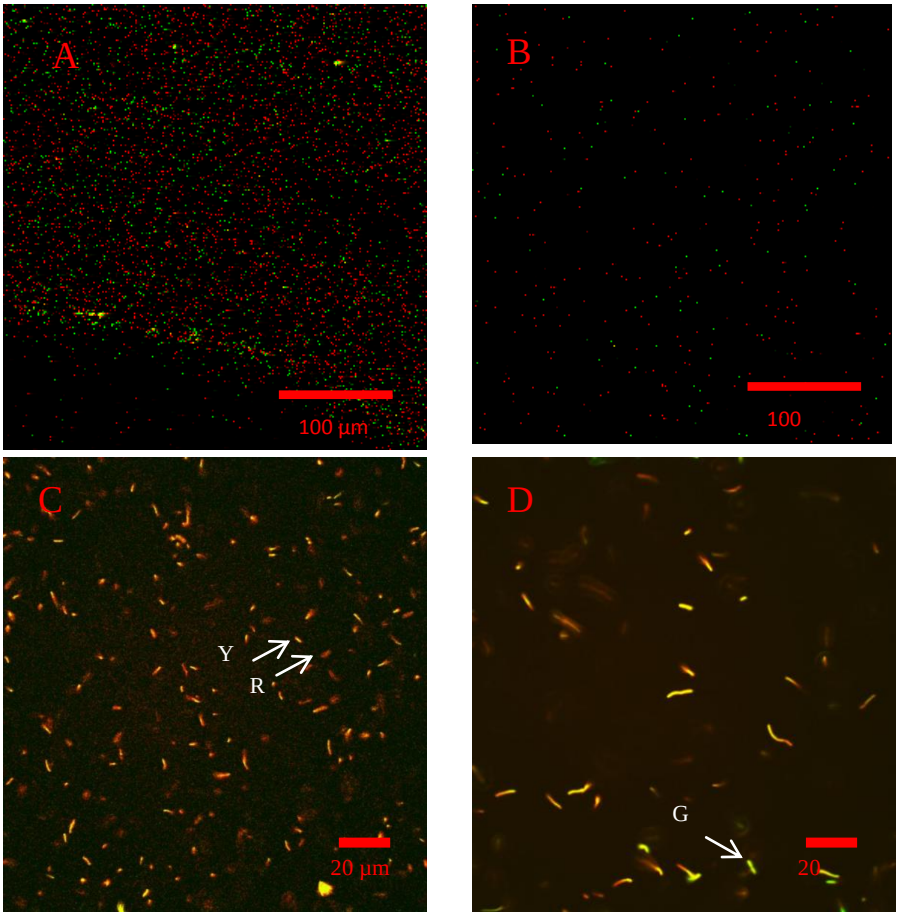

Fig. 5

Graphical Abstract (for review)