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

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Section/Category: Environmental Toxicology and Risk Assessment

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

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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

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

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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.

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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 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

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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

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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

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393

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395

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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.

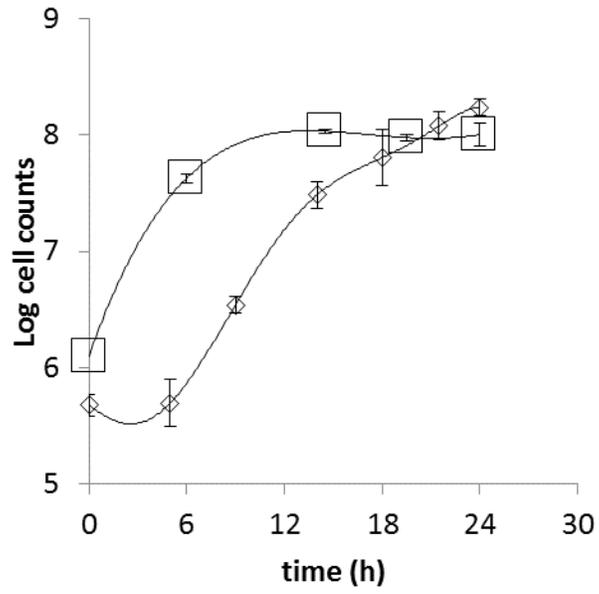


Fig. 1

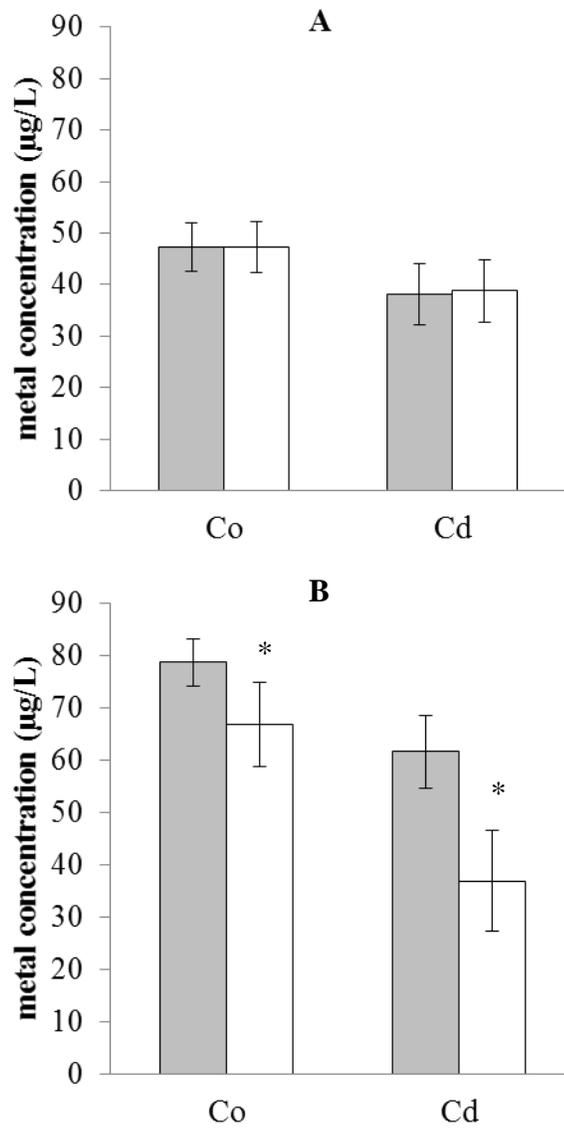


Fig. 2

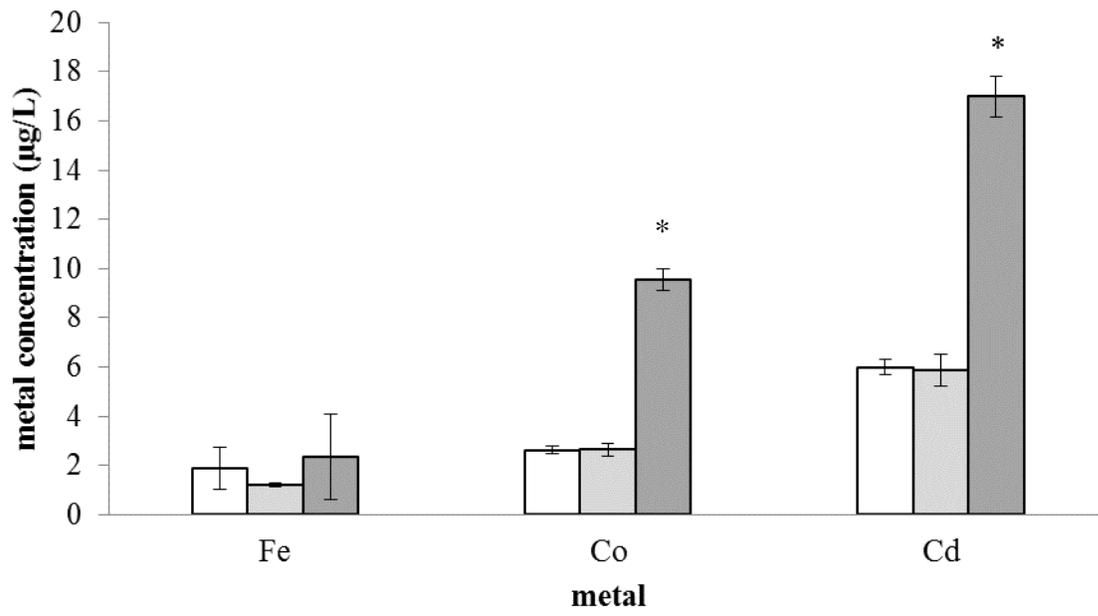


Fig. 3

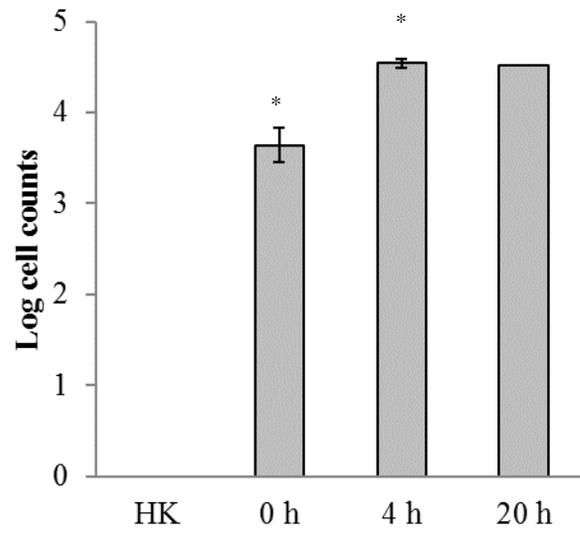


Fig. 4

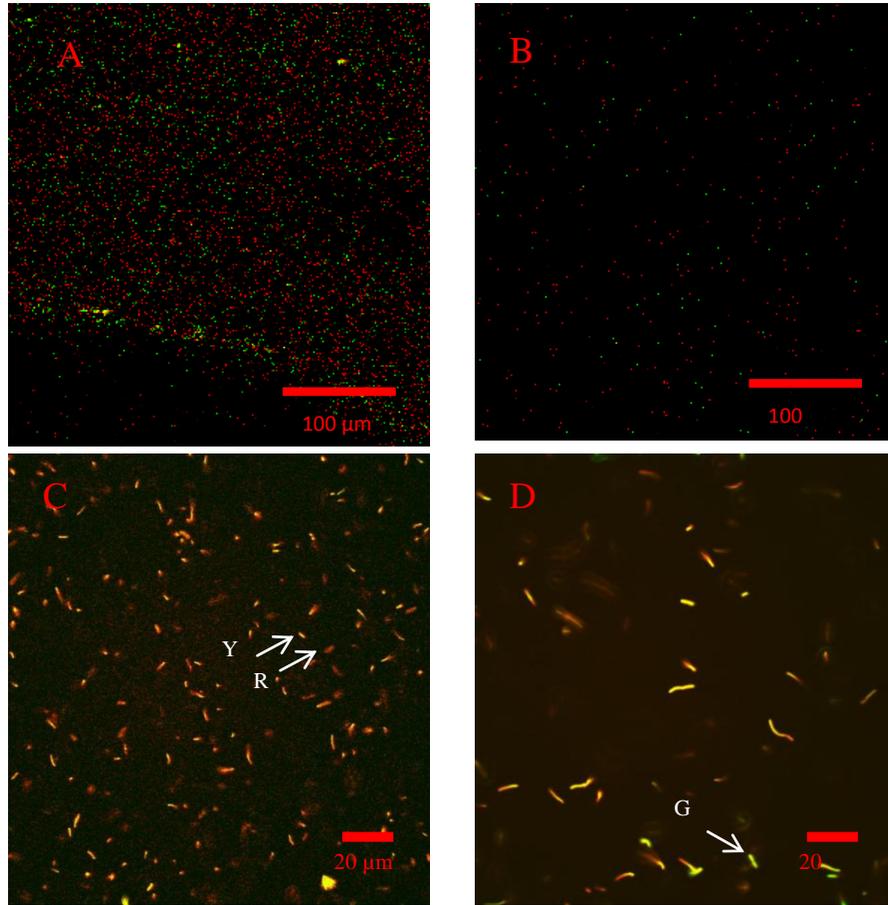


Fig. 5

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

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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*

23 **1. Introduction**

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

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393

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395

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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.

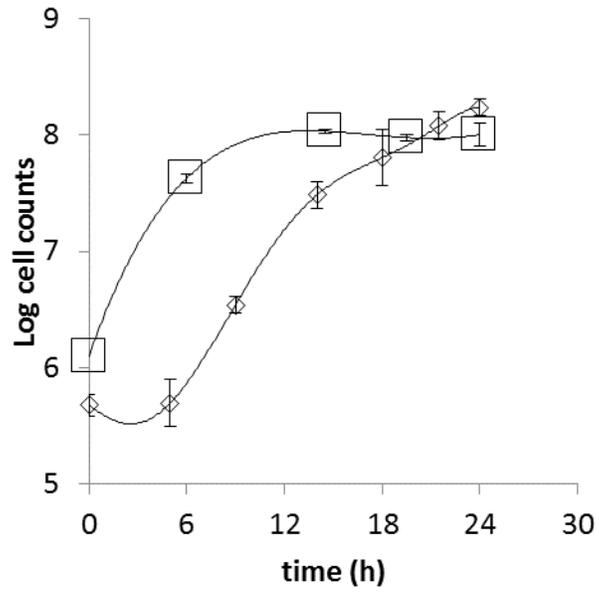


Fig. 1

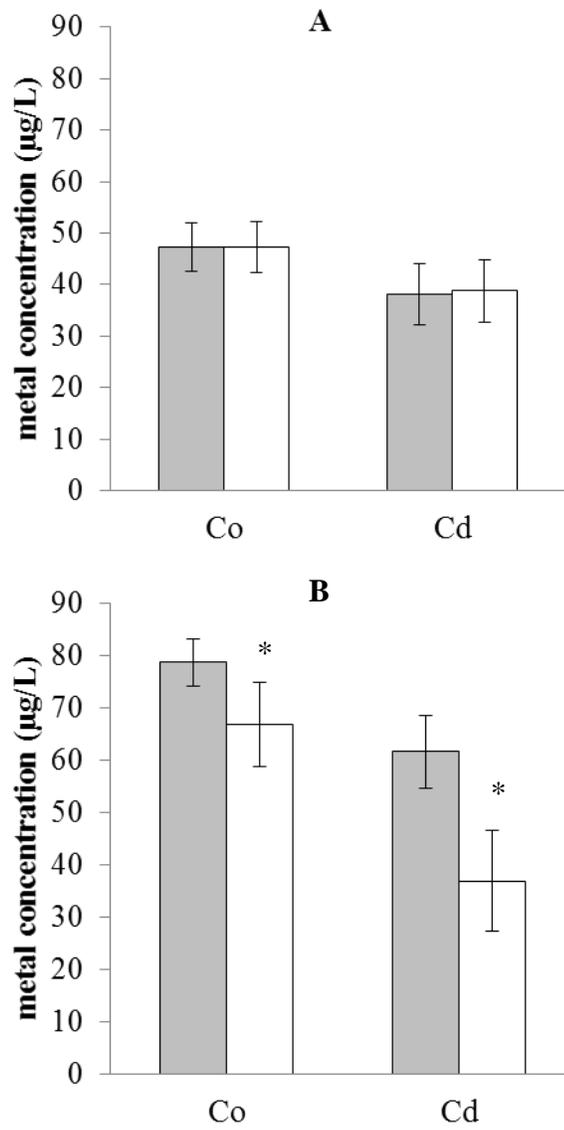


Fig. 2

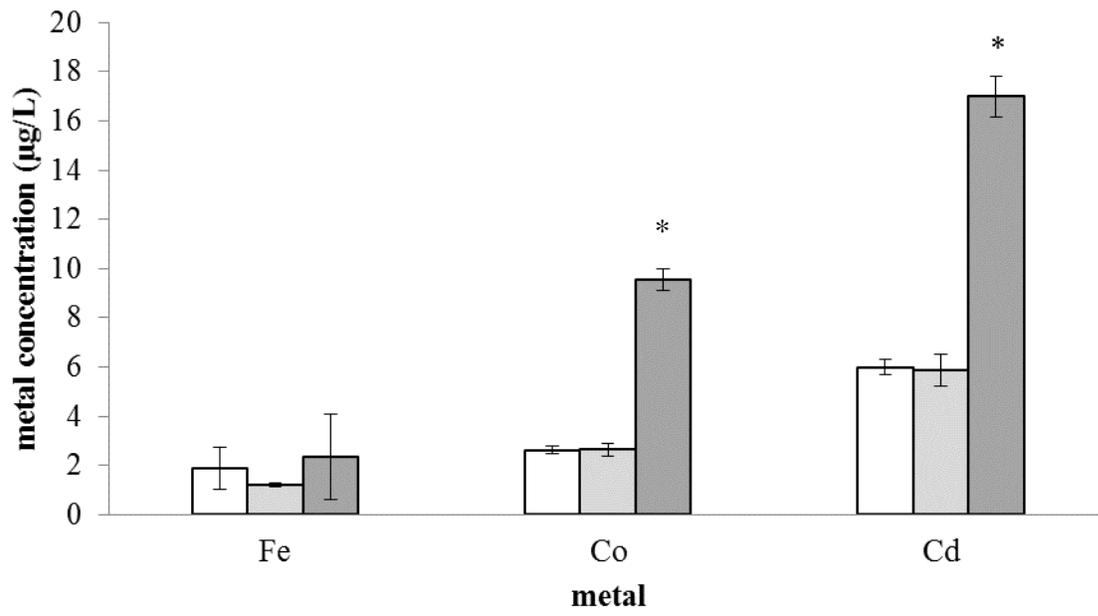


Fig. 3

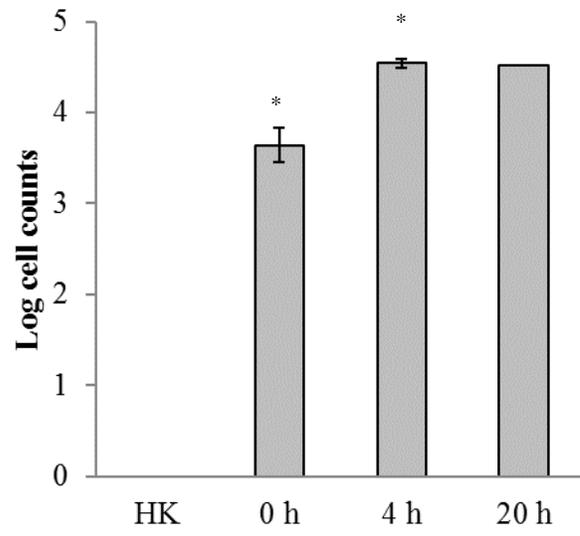


Fig. 4

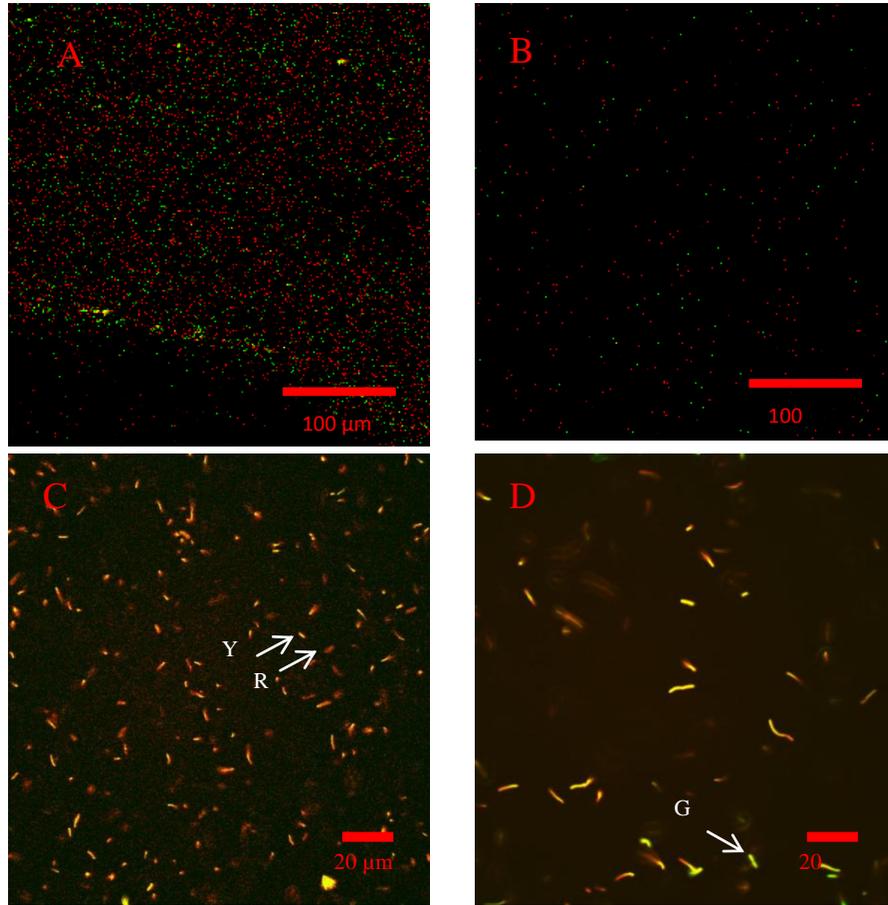


Fig. 5

