

1 **Effects of rhamnolipids from *Pseudomonas aeruginosa* DS10-129 on luminescent**
2 **bacteria: toxicity and modulation of cadmium bioavailability**

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4 Olesja Bondarenko ^a, Pattanathu K S M Rahman ^b, Thahira J Rahman ^c, Anne Kahru ^a,
5 Angela Ivask ^{a*}

6

7 ^aLaboratory of Molecular Genetics, National Institute of Chemical Physics and
8 Biophysics, Tallinn, Estonia

9 ^bChemical and Bioprocess Engineering Group, School of Science and Technology,
10 Teesside University, Middlesbrough, United Kingdom

11 ^cInstitute of Human Genetics, Newcastle University, Newcastle upon Tyne, United
12 Kingdom

13 * corresponding author

14

15 **Correspondance to:**

16 Angela Ivask

17 Akadeemia tee 23

18 12618 Tallinn, Estonia

19 angela.ivask@kbfi.ee

20

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23 **Running head:** Rhamnolipids toxicity and modulation of cadmium bioavailability

24

25 **Abstract**

26 In this study the mixture of mono- and di-rhamnolipids produced by *Pseudomonas*
27 *aeruginosa* DS10-129 was characterized for its toxicity and modulatory effects on Cd
28 availability to different bacteria. Gram-negative naturally bioluminescent *Vibrio*
29 *fischeri* and recombinant bioluminescent *Pseudomonas fluorescens*, *Pseudomonas*
30 *aeruginosa*, *Escherichia coli* and Gram-positive *Bacillus subtilis* were used as model
31 organisms. Rhamnolipids reduced the bioluminescence of these bacteria in less than a
32 second of exposure even in relatively low concentrations (30-min EC₅₀ 45–167 mg l⁻¹)
33 which might be due to the enhancement in permeability of bacterial membranes as
34 confirmed by the hydrophobic 1-N-phenyl-naphthylamine dye.

35 The 30-min EC₅₀ value for Cd was 0.16 mg l⁻¹ (*E. coli*), 0.49 mg l⁻¹ (*B. subtilis*), 0.96
36 mg l⁻¹ (*P. fluorescens*) and 4.4 mg l⁻¹ (*V. fischeri*). The sub-toxic concentrations of
37 rhamnolipids (50 mg l⁻¹) remarkably (up to 10-fold) reduced the toxic effect of Cd to
38 Gram-negative bacteria but not to Gram-positive *B. subtilis*, which was probably due
39 to the differences in bioavailability. The reason for that could be difference in
40 bioavailability of Cd in bacteria with different cell wall structures in the presence of
41 rhamnolipids. As Cd-selective electrode analysis revealed significant complexation of
42 Cd by rhamnolipids in aqueous environment, the modulation of Cd toxicity (i.e. net
43 uptake of Cd) by rhamnolipids seems to be an interplay between the complexation of
44 Cd and alterations in bacterial membranes.

45 The analysis of the concentration of free Cd²⁺ in Cd-spiked soils showed that only a
46 minor fraction (0.13% of the total Cd in soil containing 1.5 mg of Cd kg⁻¹) was
47 desorbed from soil if rhamnolipids were not added and as maximum two-fold higher
48 Cd desorption was measured in the presence of 40 mg l⁻¹ rhamnolipids. However,
49 even this small fraction of desorbed Cd remained not available to Cd-sensing
50 recombinant bacteria. Since the concentrations of rhamnolipids showing effects on
51 bacterial membranes (>10 mg l⁻¹ of rhamnolipids) and bioluminescence inhibition
52 (>45 mg l⁻¹ of rhamnolipids) did not influence the viability of Gram-negative and
53 Gram-positive test bacteria, the rhamnolipids could be considered harmless also to
54 soil bacteria. Hence, rhamnolipids could be applied for the remediation of polluted
55 areas, provided the range of concentrations will be carefully chosen.

56

57 **Keywords:** rhamnolipid surfactant, luminescent sensor bacteria, Gram-positive
58 bacteria, Gram-negative bacteria, soil remediation

59

60 **Introduction**

61 Rhamnolipids are surface active molecules produced by *Pseudomonas aeruginosa* –
62 *and* are important biotechnological products with a wide range of applications in
63 many areas, e.g. cosmetics (emulsifiers), food industry (food formulation ingredients)
64 [25], biomedicine (due to their antiadhesive and antimicrobial properties) [31],
65 agriculture (due to their antimicrobial and antifungal effects) and bioremediation
66 (removal of toxic heavy metals from soils) [14, 24, 29].

67 Soil washing with rhamnolipids is one of the most often proposed potential strategy
68 for reducing heavy metal toxicity to indigenous soil bacteria (for review see [24]), and
69 it is postulated that removal of metal toxicity includes complexation of heavy metals
70 by rhamnolipids [26]. Although for remediation of sites polluted with heavy metals
71 both the potential of rhamnolipids to remove heavy metals from soils as well as the
72 possible toxic effects of rhamnolipids to soil microorganisms should be taken into
73 account. Existing data about the interactions between rhamnolipids–metal complexes
74 and soil (micro)organisms are relatively rare and controversial. Stacey et al. [35]
75 postulated that rhamnolipids form neutral lipophilic complexes with cationic metal
76 ions and enhance the absorption of zinc by plant roots. Conversely, Al-Tahhan et al.
77 [1] showed that rhamnolipids reduced the cell surface charge of Gram-negative
78 bacteria resulting in increased hydrophobicity and thus, reduced cadmium uptake.
79 Similarly, several studies have shown reduction in heavy metal toxicity to bacteria in
80 the presence of rhamnolipids [20, 33]. On the other hand, Shin et al. [34] showed that
81 addition of 240 mg l⁻¹ rhamnolipids for an *in situ* remediation inhibited the
82 phenanthrene degrading bacteria referring to the potential toxic effect of
83 biosurfactants. Indeed, rhamnolipids have been shown to exhibit powerful
84 antibacterial, antifungal and algicidal activities [8, 37]. Thus, to be used for *in situ*
85 bioremediation, preparations of rhamnolipids should be thoroughly characterized not
86 only concerning their remediation efficiency but also for potential toxic effects on soil
87 (micro)organisms.

88

89 **Objectives of the study**

90 The main objective of this study was to characterise the rhamnolipids produced by
91 *Pseudomonas aeruginosa* DS10-129 [28] for i) their inherent toxicity to Gram-
92 negative (*Vibrio fischeri*, *Pseudomonas fluorescens*, *P. aeruginosa*, *Escherichia coli*)

93 and Gram-positive (*Bacillus subtilis*) bacteria and ii) their potential to decrease Cd
94 bioavailability and remove Cd- toxicity in aqueous media and soils.

95

96 **Materials and Methods**

97 **1. Materials**

98 CdCl₂ (>98%) was obtained from Sigma, Tween 80 from Serva, components of
99 growth media were either from LabM or Sigma, L-rhamnose, orcinol and 1-N-
100 phenyl-naphthylamine (1-NPN) were from Sigma-Aldrich. Rhamnolipids were
101 purified from the culture broth of *Pseudomonas aeruginosa* DS10-129 as described
102 below. A sandy soil (initial concentration of Cd 0.17 mg kg⁻¹) spiked with CdCl₂ (1.5,
103 15, 150, 1500 or 15 000 mg of Cd kg⁻¹) as previously reported [11] was used for the
104 bioavailability studies. Before spiking, the soil was characterised in a certified
105 laboratory and had the following properties: 10.6% of clay, 10.6% of silt, 72.8% of
106 sand, 5.7% of organic matter; 39 g·kg⁻¹ of CaCO₃, 3.59 g·kg⁻¹ of N, 0.62 g·kg⁻¹ of P,
107 0.17 mg kg⁻¹ of Cd; with 2.3 cmol⁺kg⁻¹ of CEC (cation exchange capacity) and pH of
108 7.3. Deionised water was used throughout the study.

109

110 **2. Characterization of rhamnolipids-producing bacterium *Pseudomonas*** 111 ***aeruginosa***

112 In the current study, the 16S rRNA gene of *P. aeruginosa* DS10-129 was sequenced
113 by DSMZ (German Collection of Microorganisms and Cell Cultures;
114 <http://www.dsmz.de/>) and the partial sequence of the 16S rRNA gene was deposited
115 in the EMBL nucleotide sequence database (<http://www.ebi.ac.uk/embl/>) with
116 accession number AM419153. The sequence was compared with those available in
117 EMBL and NCBI databases using the program BLAST to find its closest homologues.
118 The similarity matrix was constructed by pairwise analysis of validated (most
119 completed) sequences using the corrections computed by the Kimura's 2-parameter
120 model [18]. A phylogenetic tree was constructed with the partial 16S rRNA gene
121 sequences using a multiple sequence alignment software CLUSTALW [10] by the
122 Neighbour-Joining method [32] and illustrated with the TreeView program. The
123 resulting hierarchical clustering tree was "pruned" to save space and the closest
124 relatives were retained.

125

126 **3. Isolation, purification and characterization of rhamnolipids**

127 Rhamnolipids were isolated from *P. aeruginosa* DS10-129 cell-free supernatant. *P.*
128 *aeruginosa* was grown on the mineral medium containing (per l water) 20 g of
129 glycerol, 0.7 g of KH_2PO_4 , 2 g of Na_2HPO_4 , 0.4 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g of CaCl_2
130 and 0.001 g of $\text{FeSO}_4 \cdot \text{H}_2\text{O}$ and 1% of Yeast Extract. The presence of the
131 rhamnolipids was verified by thin layer chromatography on silica gel 60 F₂₅₄
132 according to Matsufuji et al., [21]. Rhamnolipids were extracted from the cell-free
133 supernatant of the 4-day bacterial culture by centrifugation at 8000 g for 20 minutes at
134 4°C and the subsequent filtration through a sterile filter (pore size 2 µm). The cell-free
135 culture was acidified to pH 2 with 2 M H_2SO_4 and the precipitated rhamnolipids were
136 extracted with an equal volume of 2:1 dichloromethane/methanol (liquid-liquid
137 extraction) [38]. The organic phase was dried with anhydrous Na_2SO_4 to remove
138 excess water and evaporated on a rotary evaporator (Buchi) at 60–70 °C to yield
139 rhamnolipids. The rhamnolipids were dissolved in 0.05 M NaHCO_3 .
140 The concentration of rhamnolipids was determined using the orcinol assay [6] by
141 mixing 100 µl of diluted solution of rhamnolipids (purified with liquid-liquid
142 extraction) with 900 µl of freshly prepared 0.19% orcinol solution in 53% H_2SO_4 . The
143 mixture was heated at 80°C for 30 min and its absorbance was measured
144 spectrophotometrically at 421 nm. The concentration of rhamnolipids was calculated
145 according to L-rhamnose standard curve (0 to 50 mg l⁻¹) and by multiplying the result
146 with a coefficient of 3.4 obtained from the correlation of pure rhamnolipids/rhamnose
147 [3]. The critical micelle concentration (CMC) was determined by measuring the
148 surface tension of serial dilutions of the rhamnolipids [17]. Fourier Transform
149 Infrared spectrophotometer (FTIR) Perkin Elmer 100 series was used to determine the
150 molecular structure of the rhamnolipids. The cell-free supernatant was acidified to pH
151 2 by adding drops of 2M Sulphuric acid to precipitate the rhamnolipids. The
152 precipitated rhamnolipids were extracted with an equal volume of 2:1
153 dichloromethane/methanol. The organic phase was dried with anhydrous Sodium
154 Sulphate (Na_2SO_4) and evaporated on a rotary evaporator (Buchi, Rota vapour R-200

155 Germany) set at 60-70°C. Approximately 2-5mg of the concentrated rhamnolipids
156 were analysed with the FTIR spectrophotometer.

157

158

159 **4. Luminescent bacterial strains for toxicity and bioavailability assays**

160 The luminescent bacterial strains used for toxicity and bioavailability studies are listed
161 in Table 1. Constitutively luminescent bacterial strains, both natural (*Vibrio fischeri*)
162 and recombinant strains were used to evaluate the toxicity of the rhamnolipids using
163 bioluminescence inhibition as a toxicity endpoint. Recombinant luminescent Cd-
164 inducible sensor bacteria were used to study the modulatory effect of rhamnolipids on
165 availability of Cd to bacteria. All recombinant bioluminescent bacterial strains, except
166 *Pseudomonas aeruginosa* DS10-129 (pDNcadRPcadAlux) were constructed
167 previously (Table 1). *P. aeruginosa* DS10-129 (pDNcadRPcadAlux) was initially
168 constructed as Cd-inducible strain by electroporating a 14,525 bp plasmid
169 pDNcadRPcadAlux [13], which contains bioluminescence-encoding *luxCDABE* genes
170 under the control of Cd response elements: Cd-regulated promoter (promoter of *cadA*)
171 and a Cd-binding regulatory protein (CadR), into *P. aeruginosa* DS10-129 competent
172 cells [3]. Bacteria were plated onto LB agar (10 g of tryptone, 5 g of yeast extract and
173 5 g of NaCl per 1 l of deionised / distilled? water) containing 50 mg l⁻¹ of tetracycline
174 and the plasmid-containing colonies were selected by luminescence. During further
175 experiments *P. aeruginosa* DS10-129 (pDNcadRPcadAlux) failed to be induced with
176 Cd (maximum induction below the limit of detection), mostly due to its high
177 background luminescence. However, the high bioluminescence level favoured the use
178 of this strain as a model organism for general toxicity testing.

179

180 **5. Analysis of toxicity and Cd bioavailability**

181 Luminescent bacterial strains were either rehydrated from lyophilised culture (*Vibrio*
182 *fischeri*) obtained from Aboatox, Turku, Finland or cultivated. *V. fischeri* was
183 reconstituted in heavy metal MOPS medium (HMM medium) supplemented with 2%
184 NaCl at room temperature for 1 h. The HMM medium contained (per l of deionised /
185 distilled? water): 8.4 g of MOPS buffer, 0.4 g of glucose, 0.22 g of glycerol-2-
186 phosphate, 3.7 g of KCl, 0.54 g of NH₄Cl, 0.06g of MgSO₄, 0.162 mg of FeCl₃ [23].
187 All recombinant bacteria were cultivated freshly by growing the cultures overnight in

188 3 ml of LB medium [44] supplemented with appropriate antibiotics as in Table 1. The
189 overnight culture was diluted 1:50 with 10–50 ml of HMM medium, grown until
190 OD₆₀₀ of 0.3 and then diluted to OD₆₀₀ of ~0.1 prior to test.

191

192 **5.1. Toxicity testing**

193 To measure toxicity CdCl₂ or CdCl₂-rhamnolipids mixtures were analyzed by
194 measuring the inhibition of bioluminescence of constitutively luminescent bacterial
195 strains (Table 1). In addition, *Pseudomonas aeruginosa* DS10-
196 129(pDNcadRPadAlux) was used to assess the inhibitory effect of rhamnolipids. The
197 effect of rhamnolipids on viability of bacteria was evaluated by plating the treated
198 bacteria on solidified growth medium (see below). Dilutions of CdCl₂ (0.1 – 100 mg l⁻¹
199 as final concentrations), rhamnolipids (5 – 200 mg l⁻¹ as final concentrations) or Cd-
200 rhamnolipids mixture (the final concentration of rhamnolipids was 50 mg l⁻¹ or a
201 concentration reducing the light output of bacteria by 20%, indicated below) were
202 prepared in HMM medium or HMM medium supplemented with 2% NaCl (in case of
203 *V. fischeri*). For toxicity assessment, luminescence inhibition assay was performed in
204 96-well microplates essentially as in Mortimer et al. [23]. Briefly, 100 µl of the
205 diluted test compound(s) was pipetted into 96-well microplate and 100 µl of the
206 bacterial suspension was automatically dispensed into the wells. Bacteria were
207 incubated at 20°C (*V. fischeri*) or 30°C (recombinant luminescent bacteria) and the
208 luminescence was continuously recorded during the first 30 seconds of exposure and
209 once after 30 minutes of incubation using Fluoroskan Ascent FL plate luminometer
210 (ThermoLabsystems). Inhibition of bacterial bioluminescence by the tested
211 compounds/mixtures was calculated as percentage of the unaffected control (HMM
212 medium or HMM supplemented with 2% NaCl, respectively). 30-s and 30- min EC₅₀
213 and EC₂₀ values (the concentration of chemical which reduces the light output of
214 bacteria by 50 or 20% after the respective exposure times) were calculated by linear
215 regression from dose-response curves of the studied compounds. Measurements were
216 performed in three independent assays.

217 Viability of bacteria was assessed after their exposure to 100 mg l⁻¹ of rhamnolipids
218 by plating the bacteria onto LB agar plates containing appropriate antibiotics. Plates
219 were incubated for 24 h at 30°C after which colony forming units (CFU) were
220 counted.

221

222 **5.2. Bioavailability testing**

223 Availability of Cd (with or without rhamnolipids) to Cd-sensor bacteria (Table 1) both
224 in aqueous environment and in soil-water suspension was analysed as described by
225 Bondarenko et al. [4]. CdCl₂ dilutions at final concentrations of 0.01-10 mg l⁻¹ were
226 prepared by rotating soil:water (1:10) aqueous suspensions of Cd-spiked soils at room
227 temperature for 24 h. Non-spiked soil was used as a control for soil assays and
228 deionised / distilled? water served as a control for CdCl₂ dilutions. Rhamnolipids
229 were added to CdCl₂ dilutions or Cd-spiked soil suspensions to the final
230 concentrations of 10, 20 and 40 mg l⁻¹. Samples (100 µl) were added to 100 µl of the
231 sensor bacterial culture in HMM medium in 96-well microplates and incubated at
232 30°C for 2 h. Luminescence was measured with Fluoroskan Ascent FL plate
233 luminometer. Induction of luminescence of sensor bacteria by Cd was calculated as
234 follows:

$$235 \text{ Induction} = L_S/L_B,$$

236 where L_S is luminescence in the sample (CdCl₂, CdCl₂-rhamnolipids mixture, soil-
237 water suspension or its mixture with rhamnolipids) and L_B is the background
238 luminescence (bacteria in HMM medium added to water or unspiked soil). The
239 concentration of Cd in the sample causing induction of bioluminescence twice above
240 the background value was defined as minimal inducing concentration (defined also as
241 limit of determination (LOD) by Ivask et al. [13]). Bioavailability analyses were
242 performed in three independent assays.

243

244 **6. Analysis of membrane permeability**

245 The enhancement in permeability of *Escherichia coli* MC1061(pDNlux) cell
246 membranes by rhamnolipids was measured by the uptake of a hydrophobic probe 1-
247 N-phenyl-naphthylamine (1-NPN) as described by Helander et al. [12]. As compared
248 to hydrophilic environments, the fluorescence of 1-NPN is significantly enhanced in
249 hydrophobic environments (e.g. membrane phospholipids), rendering it a suitable dye
250 to probe outer membrane integrity of Gram-negative bacteria [9]. Briefly, 50 µl of 40
251 µM 1-NPN dye and 50 µl of the surfactants (rhamnolipids or non-ionic chemical
252 surfactant Tween 80 serving as a positive control) in 5 mM HEPES buffer (pH 7.2)
253 were pipetted into black microplates. 5 mM HEPES buffer was used as a negative
254 control. 100 µl of bacterial suspension in 5 mM HEPES buffer were automatically

255 dispensed into each well and the fluorescence was immediately measured (Fluoroskan
256 Ascent FL plate luminometer; excitation/emission filters 350/460 nm). The final
257 concentrations of both surfactants in the test were 10, 40 and 100 mg l⁻¹. The 1-NPN
258 cell uptake factor was calculated as a ratio of fluorescence values of the bacterial
259 suspension in the presence and absence of surfactants.

260

261 **7. Analysis of free Cd²⁺ with ion-selective electrode**

262 A Cd-selective electrode (Thermo Orion 96-48 and Thermo Orion 4-star meter;
263 ThermoOrion) was used to measure the free Cd²⁺ in the aqueous environment and in
264 soil-water suspensions. Before use, the electrode was polished with alumina strips.
265 The inner filling solution of the electrode was replaced at least weekly. CdCl₂
266 solutions at final concentrations of 11.2-1120 mg l⁻¹ were prepared in deionised /
267 distilled? water; 1:10 water suspensions of Cd-spiked soils were rotated for 24 h prior
268 to the test. Rhamnolipids were added to these solutions at final concentrations of 10,
269 20 and 40 mg l⁻¹ and incubated at 30°C for 2 hours. The concentrations of free Cd²⁺
270 were calculated by comparing the results from different test conditions with the
271 response of the electrode to CdCl₂ solutions in distilled / deionised? water.

272

273 **8. Analysis of Cd desorption from soil**

274 The effect of rhamnolipids on desorption of Cd in Cd-spiked soil was analyzed by
275 Atomic Absorption Spectroscopy (Shimadzu, Kyoto, Japan). Rhamnolipids are added
276 at final concentrations of 10, 20 and 40 mg l⁻¹ to 1:10 water suspensions of Cd-spiked
277 soil, incubated for 2 hours and centrifuged at 13,000×g for 5 minutes. The resulting
278 soil-water extracts were acidified with 1% HNO₃ and the concentration of Cd in the
279 extracts was analysed.

280

281 **9. Analysis of rhamnolipids sorption to soil**

282 The sorption of rhamnolipids to soil was determined by comparing the surface tension
283 of rhamnolipids (10, 20 and 40 mg l⁻¹) in distilled / deionised? water and in soil:water
284 (1:10) suspension. Surface tension was measured by the drop weight method [29].

285

286 **Results**

287 **1. Characterization of rhamnolipids-producing strain *Pseudomonas aeruginosa***
288 **DS10-129**

289 The *P. aeruginosa* DS10-129 strain used in this work has been isolated previously and
290 characterised for the synthesis of rhamnolipids [28]. Based on the 16S rRNA gene
291 sequences evolutionary relationships of *P. aeruginosa* DS10-129 were determined.
292 Neighbour-joining analysis showed that 14 compared isolates of *Pseudomonas*
293 formed four distinct clusters of highly related members (Figure 1) and *P. aeruginosa*
294 DS10-129 belonged to cluster I where the similarity in 16S rRNA gene sequence was
295 more than 90% and the difference in nucleotides was 2–43. Homologies between the
296 16S rRNA gene sequence of *P. aeruginosa* DS10-129 and the other 13 strains of
297 *Pseudomonas* compared ranged between 89-99%. Among the strains, the isolate *P.*
298 *aeruginosa* DS10-129 showed the highest (99%) sequence similarity with *P.*
299 *aeruginosa* B2, a strain capable of degrading lubricant base oil consisting of
300 trimethylolpropaneoleate, and the lowest (89%) with *P. anguillispectica* FTB-40 and
301 *P. frederiksbergensis* AJ28. The sequence similarity of *P. aeruginosa* DS10-129
302 isolate with the out-group *Escherichia coli* (accession number X80724) was 83%.

303

304 **2. Characterization of rhamnolipids produced by *Pseudomonas aeruginosa***
305 **DS10-129**

306 The presence of rhamnolipids in the *P. aeruginosa* DS10-129 culture broth was
307 confirmed by thin layer chromatography where two anise aldehyde positive spots (Rf
308 0.32 and Rf 0.52, corresponding to di- and mono-rhamnolipids) were detected. The
309 concentration of rhamnolipids extracted from the bacterial culture broth was 174 mg l⁻¹
310 and the critical micelle concentration (CMC) of the rhamnolipids was 22 mg l⁻¹.
311 FTIR spectroscopy was used to determine the molecular structure of the rhamnolipids
312 (Figure 2). Strong and broad bands of the hydroxyl group (-OH) free stretch due to
313 hydrogen bonding were observed in the region A (3368 cm⁻¹). The presence of
314 carboxylic acid functional group in the molecule was confirmed by the bending of the
315 hydroxyl (O-H) of medium intensity bands in the region D (1455-1380cm⁻¹). The
316 aliphatic bonds CH₃, CH₂ and C-H stretching with strong bands are shown in regions
317 B and D (2925-2856 and 1455-1380 cm⁻¹). The carbonyl (C=O) stretching was found
318 in the region C (1737cm⁻¹) with strong intensity bands. Two other strong peaks
319 between 1300 cm⁻¹ and 1033 cm⁻¹ in region E due to C-O stretch are characteristic of
320 an ester functional group. The peaks in the range of 1121–1033 cm⁻¹ were also

321 reported as C-O-C stretching in rhamnose by Pornsunthorntawee et al. [27]. These
322 characteristic adsorption bands together demonstrate the presence of rhamnose rings
323 and long hydrocarbon chains, which are characteristic for rhamnolipids according to
324 Guo et al. [7]. Besides, the comparative study of mono- and di-rhamnolipids has
325 shown the presence of the shoulder around 3006 cm^{-1} in the spectrum of mono- but
326 not di-rhamnolipid [7]. In the FTIR spectrum of rhamnolipids produced by *P.*
327 *aeruginosa* DS10-129 we could observe only a minor shoulder in this region, likely
328 because of the dominance of the di-rhamnolipid in the mixture [29]. Moreover, we
329 noticed stronger bands of pyranyl I sorption band in region F at $918\text{-}940\text{ cm}^{-1}$ and α -
330 pyranyl II sorption band in region G at $838\text{-}844\text{ cm}^{-1}$ that according to Guo et al. [7]
331 also suggested the dominance of the di-rhamnolipid in the mixture.

332

333 **3. Toxicity of rhamnolipids to bacteria**

334 The short-term inhibitory effect of rhamnolipids on bacteria was analysed by
335 measuring the kinetics of bioluminescence of five bacterial strains (both, Gram-
336 negative and Gram-positive bacteria) upon their exposure to rhamnolipids. Naturally
337 luminescent *Vibrio fischeri* and recombinant luminescent *Escherichia coli*,
338 *Pseudomonas aeruginosa*, *Pseudomonas fluorescens* and *Bacillus subtilis* (Table 1)
339 were tested. Among the tested bacterial strains and in the conditions used, the
340 rhamnolipids were least inhibitory to both *Pseudomonas* species (30-s and 30-min
341 EC_{50} values 204 and $138\text{-}164\text{ mg l}^{-1}$, respectively) and most inhibitory to *V. fischeri*
342 (30-s and 30-min EC_{50} values 89 and 45 mg l^{-1} , respectively) (Table 2). The
343 rhamnolipids inhibited the bacterial bioluminescence from the very first second of
344 exposure (Figure 3) and only slight (up to 2-fold) increase in toxicity was observed
345 when the exposure time was extended to 30 minutes (Table 2). Despite the inhibition
346 of bioluminescence, the tested concentrations of rhamnolipids did not decrease the
347 viability of bacteria: the number of viable cells (analysis of colony forming units on
348 LB agar plates—data not shown) was not decreased after 30 minutes of exposure to
349 100 mg l^{-1} rhamnolipids (causing over 50% bioluminescence inhibition in three tested
350 bacteria, Figure 3). It was subsequently shown, that the toxic effect of rhamnolipids
351 was due to the increase in permeability of cell membranes of Gram-negative bacteria.
352 As recorded for the non-ionic chemical surfactant Tween 80, rhamnolipids similarly
353 facilitated the entrance of hydrophobic fluorescent dye 1-N-phenylnaphthylamine (1-
354 NPN) into the cells of Gram-negative *E. coli* MC1061(pDNlux) (Table 3).

355

356 **4. Effect of rhamnolipids on toxicity, bioavailability and concentration of free** 357 **Cd²⁺ in aqueous environment**

358 **4.1. The effect of rhamnolipids on Cd toxicity**

359 Acute effects of CdCl₂ solutions on the bioluminescence of four different bacterial
360 strains were analysed. In contrast to the rhamnolipids, whose effects on bacterial
361 bioluminescence occurred during the first seconds of exposure (Figure 3), the toxic
362 effect of Cd on bacteria was observed only after 30 minutes of exposure. The 30-min
363 EC₅₀ of CdCl₂ solutions were (mg Cd l⁻¹): 0.16 for *E. coli*, 0.49 for *B. subtilis*, 0.96 for
364 *P. fluorescens* and 4.4 for *V. fischeri*. Addition of sub-toxic concentrations of
365 rhamnolipids (50 mg l⁻¹ and EC₂₀ level: 20 mg l⁻¹ for *B. subtilis* and *V. fischeri*, 35 mg
366 l⁻¹ for *E. coli* and 70 mg l⁻¹ for *P. fluorescens*) significantly mitigated the toxic effect
367 of cadmium for all the used Gram-negative strains (Figure 4 A, C, D; Table 4). The
368 most remarkable reduction of Cd toxicity was observed in case of *E. coli* followed by
369 *V. fischeri* and *P. fluorescens* (50 mg l⁻¹ of rhamnolipids reduced the toxic effect of
370 Cd by 10, 4.8 and 2.3-fold respectively; Table 4 and Figure 4). Surprisingly, no
371 mitigating effect of rhamnolipids on Cd toxicity was observed in case of Gram-
372 positive bacterium *B. subtilis* (Figure 4 B), indicating that the effect of rhamnolipids
373 may be related to the structure of the bacterial cell wall.

374

375 **4.2. The effect of rhamnolipids on Cd bioavailability**

376 Similarly to Cd toxicity, Cd bioavailability as measured with Cd-inducible bacterial
377 strains, clearly decreased for the Gram-negative *E. coli* in the presence of
378 rhamnolipids (Figure 5 A). The minimal inducing concentration of Cd for *E. coli*
379 MC1061(pSLzntR/ pDNPzntAlux) decreased by 3 and 5.6-fold in the presence of 20
380 and 40 mg l⁻¹ of rhamnolipids, respectively (Table 4). However, conversely to Gram-
381 negative *E. coli*, the apparent availability of Cd to Gram-positive sensor *B. subtilis*
382 BR151(pcadCPcadAlux) even increased (Figure 5 B, Table 4) showing again the
383 dissimilar effect of rhamnolipids on the availability of Cd to bacterial cells with
384 different cell wall structure.

385

386 **4.3. The effect of rhamnolipids on free Cd ion concentration**

387 A Cd-selective electrode was used to analyse the effect of rhamnolipids on the
388 concentration of free Cd ions. Unfortunately, this electrode does not allow the

389 measurement of low concentrations of Cd ($0.001\text{--}0.03\text{ mg l}^{-1}$) that were inducing the
390 sensor bacteria. However, significant (about 95%) reduction in the amount of free
391 cadmium by rhamnolipids (40 mg l^{-1}) in aqueous environment containing 11.2 mg
392 $\text{Cd}^{2+}\text{ l}^{-1}$ was observed (Table 4) further proving the strong metal complexing ability of
393 rhamnolipids. At the same time, 70% and 9% reduction in free Cd^{2+} was observed in
394 solutions containing 112 mg and 1120 mg of Cd^{2+} per l^{-1} (Table 4) showing the clear
395 concentration-dependent saturation of metal complexing by rhamnolipids.

396

397 **5. Effect of rhamnolipids on desorption, bioavailability and concentration of free** 398 **Cd^{2+} in soil**

399 As one of the potential applications of rhamnolipids is to bind heavy metals from
400 polluted soils, we investigated the effect of rhamnolipids on the bioavailability of Cd
401 in soil-water suspensions. In the absence of rhamnolipids, 1.5% of the total Cd was
402 available to *E. coli* sensor bacteria in the studied soil (calculation based on Tables 4
403 and 5) being in accordance with our previous studies, where median available fraction
404 of Cd to recombinant sensor bacteria was around 1% [12,13,16]. Upon addition of
405 subtoxic concentrations of rhamnolipids ($10\text{--}40\text{ mg l}^{-1}$) to the Cd-spiked soil
406 suspensions, the analysis using the Cd-sensor bacteria showed that the bioavailable
407 fraction of Cd in the soil was up to 2.5-fold decreased, i.e., up to 2.5-fold higher
408 concentrations of Cd were required for the induction of sensor bacteria in the presence
409 of rhamnolipids (Table 5) whereas the highest tested concentration of rhamnolipids
410 (40 mg l^{-1}) was most efficiently decreasing the bioavailable fraction of Cd.

411 Surprisingly, the results were different from those observed in aqueous media as upon
412 addition of rhamnolipids the bioavailability of Cd in soil suspensions was decreased
413 for both sensors, Gram-negative *E. coli* MC1061(pSLzntR/ pDNPzntAlux) and Gram-
414 positive *B. subtilis* BR151(pcadCPcadAlux).

415 The effect of rhamnolipids on the mobility of Cd in soil was even more complex. In
416 general, the Cd added to the soils remained very strongly bound and for example, only
417 0.13% of the total Cd was desorbed from soil containing 1.5 mg Cd kg^{-1} in the current
418 leaching conditions (Table 5). Addition of rhamnolipids facilitated the desorption of
419 Cd in less contaminated soils: Cd desorption in 1.5 mg Cd kg^{-1} spiked soil was two-
420 fold increased in the presence of 40 mg l^{-1} of rhamnolipids (Table 5). However, no
421 increase in Cd desorption upon addition of rhamnolipids was observed in soils with
422 environmentally not relevant high Cd content ($15\ 000\text{ mg kg}^{-1}$) showing again that the

423 effect of rhamnolipids on complexation of Cd is concentration dependent. Due to the
424 detection limit of the Cd-selective electrode, the amount of free Cd ions was not
425 possible to measure in water extracts of less polluted soils (1.5-150 mg Cd kg⁻¹),
426 where desorption of Cd by rhamnolipids was detected by AAS (Table 5). The
427 concentration of both, desorbed and free Cd in soil polluted at 15 000 mg Cd kg⁻¹ was
428 about 300 mg Cd kg⁻¹ (2% of the total) and practically not dependent on the amount
429 of rhamnolipids added (Table 5).

430

431 **Discussion**

432 The potential of rhamnolipids to be used for soil washing due to their ability to
433 decrease the toxicity of heavy metals to soil microbes has been widely acknowledged
434 [20, 33]. However, to be used for soil remediation, the addition of rhamnolipids
435 should not adversely affect soil microorganisms, as microbes play the key role in the
436 mineralization of biological components and in biogeochemical cycles. Previous
437 studies have shown that the properties of rhamnolipids are often determined by their
438 structure (for example the number of hydrophilic carboxyl groups being primary sites
439 for complex formation with metal ions) and proportion of different types of
440 rhamnolipids in the mixture [26, 35]. In order to determine these parameters, FTIR
441 analysis was performed, which showed that *P. aeruginosa* DS10-129 produced a
442 mixture of mono- and di-rhamnolipids where the latter was the predominant species.

443

444 ***The toxicity of rhamnolipids to luminescent Gram-negative and Gram-positive*** 445 ***bacteria***

446 The reduction of light output of naturally luminescent bacteria *Vibrio fischeri* is a
447 reflection of inhibition in bacterial metabolic activity and proportional to the toxicity
448 of the test sample [5]. The photobacterial luminescence inhibition test for the toxicity
449 evaluation (the standard protocol applies 5-30 min exposure times) has been used in
450 our laboratory for the characterisation of various types of chemicals and
451 environmental samples since 1993 [15]. In the current study, however, we observed
452 that the effect of rhamnolipids on bacterial bioluminescence was evident already from
453 the very first seconds of exposure (Figure 3) suggesting a disturbance of cellular
454 energetic metabolism [22] and showing that rhamnolipids were most probably
455 interfering with the normal function of bacterial cell membranes. The membrane
456 permeabilising effect of rhamnolipids was confirmed by fluorescent 1-NPN dye. In

457 the bioluminescence inhibition assay with five bacterial strains, the 30-min EC₅₀
458 values of rhamnolipids ranged from 45 to 167 mg l⁻¹ (Table 2), which exceeded the
459 CMC of the rhamnolipid mixture (22 mg l⁻¹). It is interesting to note, that the EC₅₀ of
460 the rhamnolipids for *P. aeruginosa* DS10-129 (EC₅₀=138 mg l⁻¹; Table 2) was
461 comparable to the highest concentration of rhamnolipids in the culture broth of this
462 strain (174 mg l⁻¹). However, even if inhibiting the bacterial bioluminescence, the
463 tested concentrations of rhamnolipids were not bactericidal. On the contrary, the
464 concentration of rhamnolipids, which inhibited the bioluminescence of the
465 rhamnolipid-producer strain *P. aeruginosa* DS10-129, even remarkably stimulated the
466 bioluminescence of this strain after 30-min exposure (Figure 3) and may show the
467 adaptation of this strain to permeabilising effects of rhamnolipids. This emphasises
468 the action of rhamnolipids *via* reversible modulation of bacterial membranes.

469

470 ***Modulatory effect of rhamnolipids on the toxicity and bioavailability of Cd to***
471 ***luminescent Gram-negative and Gram-positive bacteria in aqueous media***

472 Cadmium inhibited the luminescence of bacteria at sub mg l⁻¹ level in case of all
473 recombinant luminescent bacterial strains and was somewhat less toxic to naturally
474 luminescent *V. fischeri* (30 min EC₅₀ 4.4 mg l⁻¹). The latter effect could be related to
475 different cadmium speciation in the test solution containing 2% NaCl. Indeed,
476 Villaescusa et al. [36] have shown that the toxicity of Cd to *V. fischeri* was
477 remarkably increased at lower NaCl concentrations in the test medium.

478 Addition of the subtoxic concentrations of rhamnolipids remarkably (up to 10-fold)
479 reduced the toxic effect of Cd to Gram-negative bacterial strains. The remarkable (up
480 to 95%) decrease in amount of free Cd ions in the presence of rhamnolipids was also
481 shown by Cd-selective electrode (Table 4). Indeed, the complexing effect of
482 rhamnolipids on heavy metal ions has been shown previously [26]. According to
483 Nitschke and Costa [25], the optimal value of the rhamnolipids-Cd complexation ratio
484 is 2 mol of rhamnolipids per mol of Cd. Thus, as the concentrations of rhamnolipids
485 (10-40 mg l⁻¹) in the test greatly exceeded the bioavailable and toxic concentrations of
486 Cd (0.0014-4.4 mg l⁻¹), the decrease in cadmium availability and toxicity to Gram-
487 negative bacteria was at least partly caused by the reduction of free Cd-ions resulting
488 from Cd complexation by rhamnolipids. On the other hand, despite the complexation,
489 the availability of Cd to Gram-positive *B. subtilis* increased, which resulted in the
490 equal toxicity of Cd in the presence or absence of rhamnolipids (Figure 4, Table 4). A

491 similar trend was demonstrated with Cd sensor strains: Gram-negative *E. coli* and
492 Gram-positive *B. subtilis*. Rhamnolipids decreased the Cd availability to *E. coli* but
493 even increased to *B. subtilis* (Table 4).
494 The modulatory effect of rhamnolipids on membranes of Gram-negative bacteria in
495 the presence of low concentrations of rhamnolipids have been previously shown and
496 include the release of negatively charged lipopolysaccharides (LPS) resulting in the
497 reduction of the overall cell charge and increase in cell surface hydrophobicity leading
498 to better protection from toxic cationic compounds due to their decreased uptake [1,
499 30, 33]. As Gram-positive bacteria differ from Gram-negative bacteria in having only
500 one membrane not containing LPS in their cell envelope, these remarkable changes in
501 cell surface hydrophobicity are theoretically not possible and could explain the
502 differences in Cd availability and toxicity to Gram-negative and Gram-positive
503 bacteria. Thus, the modulation of Cd toxicity by rhamnolipids is obviously not only
504 due to the complexation of Cd and reduction in its bioavailability but rather due to the
505 combination of the interplay between the direct complexation of Cd and the effects on
506 bacterial membranes that may modulate the net uptake of Cd.

507

508 ***Modulatory effect of rhamnolipids on the mobility and availability of Cd in soils***

509 In soils, the effect of rhamnolipids on the mobility of Cd was even more complex:
510 rhamnolipids (10 - 40 mg l⁻¹) caused additional desorption of Cd from Cd-polluted
511 (1.5 -150 mg Cd kg⁻¹) soils (Table 5). However, emphasize must be made that only a
512 minor fraction of Cd (e.g., 0.13% of the total Cd in soil containing 1.5 mg kg⁻¹ Cd) was
513 desorbed from soil in the absence of rhamnolipids. This is in agreement with our
514 previous studies on 60 heavy metal polluted agricultural soils where median water
515 extractability of Cd was 0.2% [16]. The addition of 40 mg l⁻¹ rhamnolipids increased
516 the desorption twice. Our further experiments showed that this additionally desorbed
517 fraction of Cd remained complexed with rhamnolipids and was not available to Cd-
518 sensor bacteria. Interestingly, availability of Cd in soil was decreased to both, Gram-
519 negative Cd sensor *E. coli* MC1061(pSLzntR/ pDNPzntAlux) and Gram-positive
520 sensor *B. subtilis* BR151(pcadCPcadAlux) suggesting that in this environment, the
521 biological effect (possible alterations of bacterial membranes) of rhamnolipids was
522 not significant and had no influence on the bioavailability of cadmium. One
523 explanation for dissimilar behaviour of Cd in aqueous solution and in soil could be the
524 less effective concentration of rhamnolipids in soil-water suspension compared to that

525 of the aqueous environment due to the sorption of rhamnolipids to soil particles (57,
526 34 and 14% of the rhamnolipids at concentrations 10, 20 and 40 mg rhamnolipids l⁻¹
527 in soil-water suspension were sorbed to soil, respectively).

528

529 **Conclusion**

530 In this paper we showed that recombinant luminescent strains of various Gram-
531 negative and Gram-positive bacteria can be very useful in mechanistic analysis of
532 complex environmental problems, especially if bioavailability of heavy metals in
533 various types of soils is concerned. Indeed, after combining the constitutively
534 luminescent and Cd –sensing recombinant bacteria this paper is the first to report on
535 dissimilar effects of rhamnolipids on heavy metal toxicity and bioavailability to
536 Gram-positive and Gram-negative bacteria (due to their different outer cell wall
537 structure) and indicates that rhamnolipids may modulate the bioavailability and
538 toxicity of Cd to bacteria either by complexation of Cd or by effects on bacterial cell
539 membranes.

540

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692 **Legends for Figures**

693 **Figure 1** Phylogenetic analysis of rhamnolipids-producing *Pseudomonas aeruginosa*
694 DS10-129. The tree topology is based on Neighbor-Joining analysis of 16S rRNA
695 gene sequences. Database sequence accession numbers are given in parentheses. The
696 scale bar at the bottom indicates a length of 0.1 nucleotide substitutions per site.
697 Bootstrap values greater than 35% are indicated at nodes (values are based on 1000
698 bootstrap resampling). Clusters are indicated by numbers I-IV. *Escherichia coli* was
699 used as an out-group bacterium

700
701 **Figure 2** Fourier transform infrared spectra of rhamnolipids produced by
702 *Pseudomonas aeruginosa* DS10-129

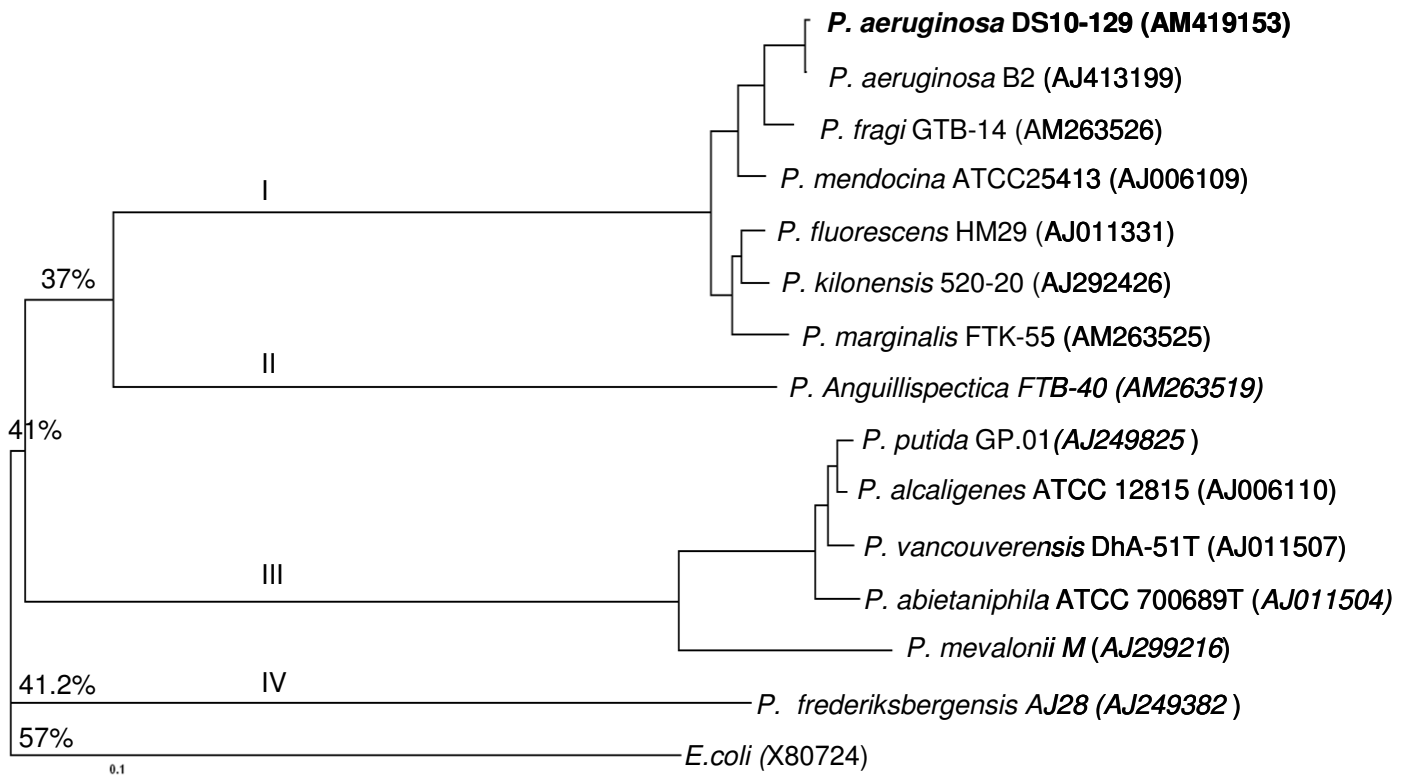
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704 **Figure 3** Inhibition of luminescence (compared to the control) of bioluminescent
705 bacteria at different exposure times (0-30 minutes) to 100 mg l⁻¹ rhamnolipids in
706 heavy metal MOPS (HMM) medium. *Vibrio fischeri* (◆), *Escherichia coli*
707 MC1061(pDNlux) (●), *Pseudomonas fluorescens* OS8(pDNux) (■), *Pseudomonas*
708 *aeruginosa* DS10-129(pDNcadR_PcadAlux) (▲), *Bacillus subtilis*
709 BR151(pBL1/p602/22lux) (*)

710
711 **Figure 4** Inhibition of luminescence in bioluminescent bacteria after 30 minutes of
712 exposure to Cd or Cd-rhamnolipids mixture in heavy metal MOPS (HMM) medium.
713 CdCl₂ without rhamnolipids (◆), CdCl₂ with rhamnolipids at EC₂₀ level (20 mg l⁻¹ for
714 *B. subtilis* and *V. fischeri*, 35 mg l⁻¹ for *E. coli* and 70 mg l⁻¹ for *P. fluorescens*) (▲)
715 and CdCl₂ with 50 mg l⁻¹ rhamnolipids (Δ). Dashed horizontal line indicates 50%
716 inhibition of bioluminescence (respective EC₅₀ values).

717

718 **Figure 5** The effect of rhamnolipids on Cd bioavailability to luminescent sensor
719 bacteria in heavy metal MOPS (HMM) medium after 2 hours exposure to CdCl₂
720 without rhamnolipids (◆), CdCl₂ with 10 mg l⁻¹ rhamnolipids (□), CdCl₂ with 20 mg l⁻¹
721 rhamnolipids (○) and CdCl₂ with 40 mg l⁻¹ rhamnolipids (Δ). Dashed horizontal line
722 indicates significant (2-fold) increase above the background luminescence of sensor
723 bacteria (corresponding to minimal inducing concentrations in x-axis)
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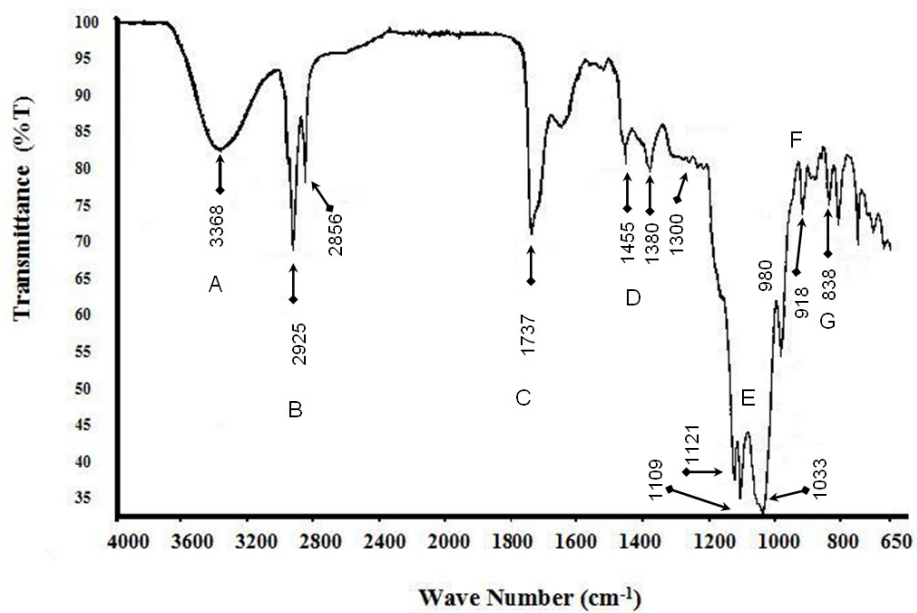
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Figure 1

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789 Figure 2

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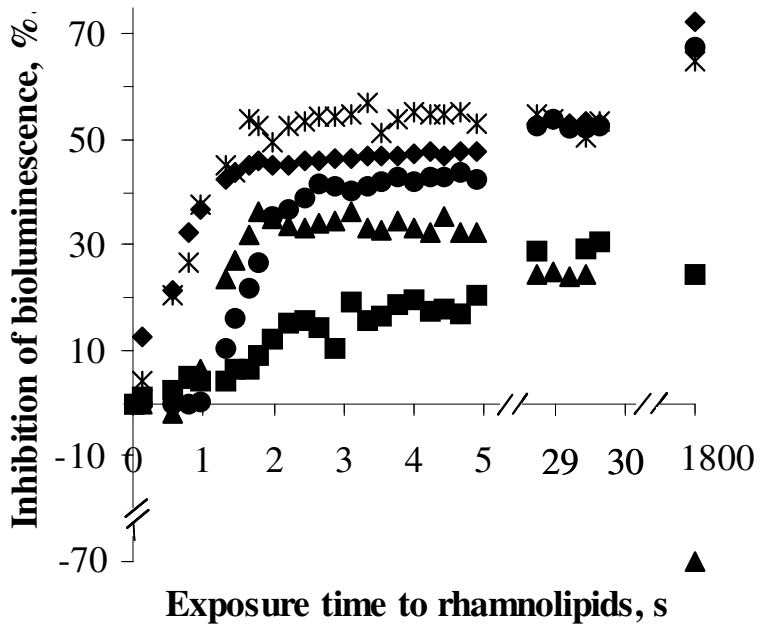
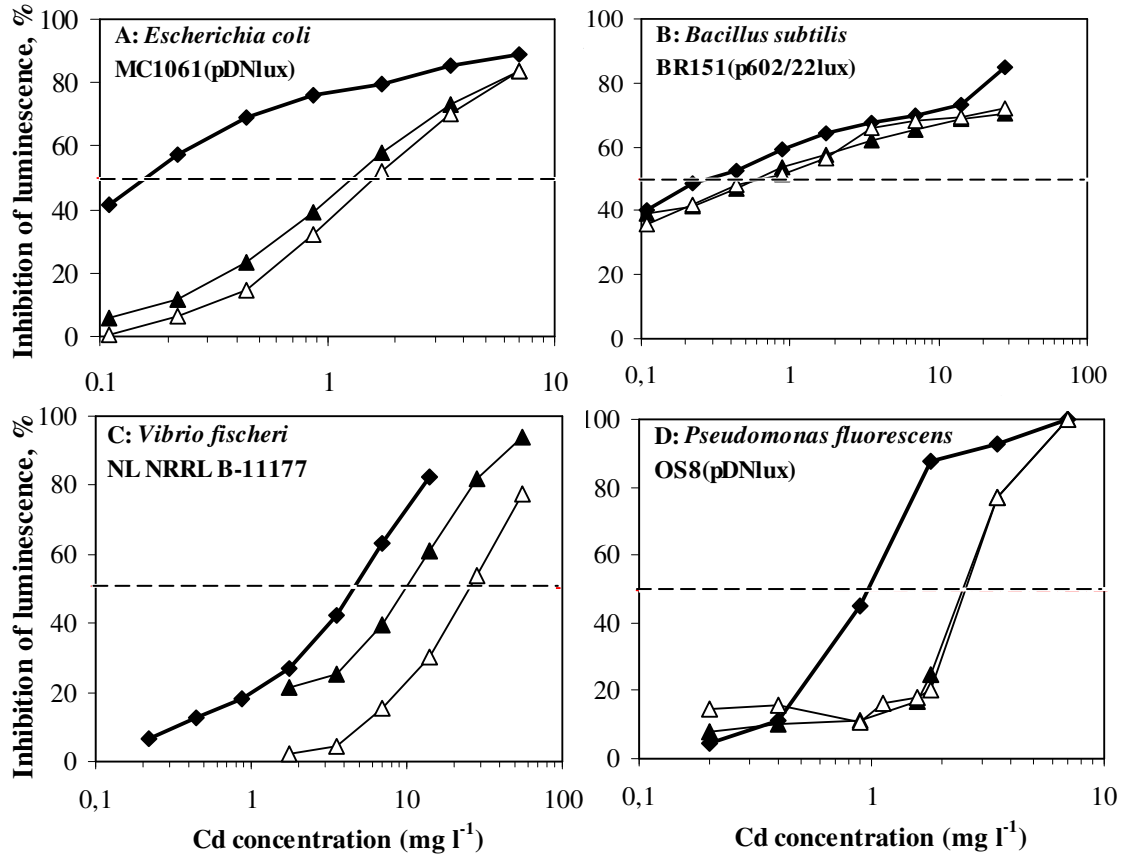


Figure 3



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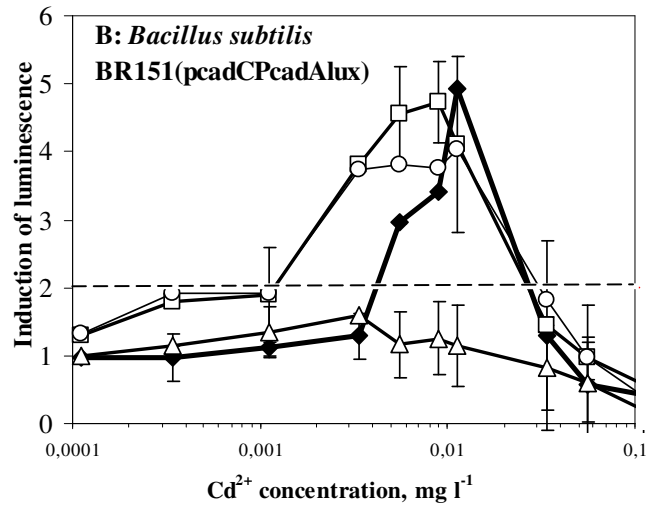
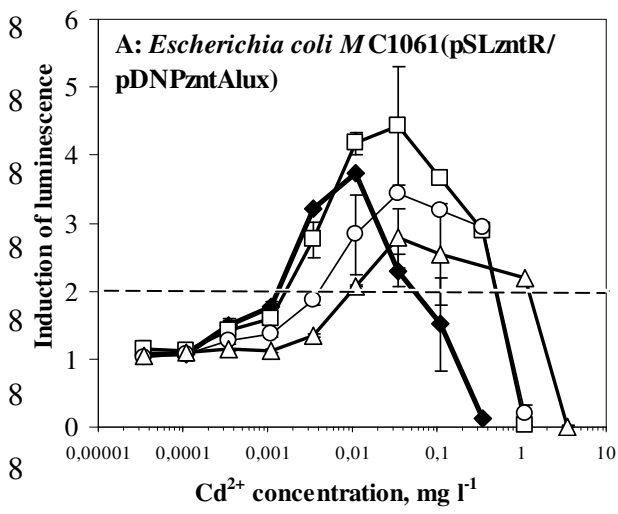
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Table 1 Luminescent bacterial strains used in this study

	Description	Antibiotic resistance (concentration in medium, mg l ⁻¹)	Source or reference
<u>Strains used for toxicity testing</u>			
<i>Gram-negative</i>			
<i>Vibrio fischeri</i> NRRL B-11177	Naturally luminescent strain	no	Aboatox (Turku, Finland)
<i>Pseudomonas fluorescens</i> OS8 ^a (pDNux)	Recombinant luminescent strain	tetracycline (20)	[17]
<i>Escherichia coli</i> MC1061 ^b (pDNlux)	Recombinant luminescent strain	tetracycline (10)	[24]
<i>Pseudomonas aeruginosa</i> DS10-129 ^c (pDNCadR _P cadAlux) ^d	Recombinant luminescent strain, metal-response elements: CadR/ promoter of <i>cadA</i> ^e	tetracycline (50)	This study
<i>Gram-positive</i>			
<i>Bacillus subtilis</i> BR151 ^f (p602/22lux)	Recombinant luminescent strain	kanamycin (50)	[17]
<u>Strains used as Cd-sensors</u>			
<i>Gram-negative</i>			
<i>Escherichia coli</i> MC1061 ^b (pSLzntR/pDNPzntAlux)	Recombinant luminescent strain, metal-response elements: ZntR/ promoter of <i>zntA</i> ^e	tetracycline (10), ampicillin (100)	[17]
<i>Gram-positive</i>			
<i>Bacillus subtilis</i> BR151 ^c (pcadCPcadAlux)	Recombinant luminescent strain, metal-response elements: CadC/ promoter of <i>cadA</i> ^e	kanamycin (50)	[17]

857 ^a Rif^r, isolated from soil polluted with toluates [~~46~~]

858 ^b (*araD139 Δ(ara, leu)7697 ΔlacX74 galU galK hsdR2 strA mcrA mcrB1*) [~~6~~]

859 ^c previously sampled from diesel contaminated sites [~~35~~] and genetically modified

860 ^d not used for the toxicity testing of Cd and Cd-rhamnolipid mixtures due to minor
861 inducibility with Cd

862 ^e regulatory protein binding heavy metal/promoter regulated by that protein

863 ^f (*trpC2 lys-3 metB10*) [~~52~~]

864

865 **Table 2** Toxicity (EC₅₀) of rhamnolipids to bioluminescent bacteria as calculated by
 866 inhibition of bacterial luminescence

Test bacterium	EC ₅₀ , mg l ⁻¹ rhamnolipids ± SD	
	30 s exposure	30 min exposure
<i>Vibrio fischeri</i> NRRL B-11177 ^a	89 ± 8	45 ± 2
<i>Escherichia coli</i> MC1061(pDNlux) ^b	99 ± 14	64 ± 28
<i>Pseudomonas fluorescens</i> OS8(pDNlux) ^b	204 ± 14	167 ± 20
<i>Pseudomonas aeruginosa</i> DS10- 129(pDNcadlux) ^b	204 ± 4	138 ± 7
<i>Bacillus subtilis</i> BR151(p602/22lux) ^b	99 ± 17	83 ± 33

867 ^aTested at 20°C in heavy metal MOPS medium (HMM) supplemented with 2% NaCl

868 ^bTested at 30°C in heavy metal MOPS medium (HMM)

869

870 **Table 3** Permeabilizing effect of rhamnolipids on *Escherichia coli* MC1061(pDNlux)
 871 cells as measured by hydrophobic fluorescent dye 1-N-phenylnaphthylamine (1-NPN)

	Fluorescence value, RFU^a ± SD	1-NPN uptake factor^b
Cells	13.5 ± 0.1	1
Cells + 10 mg l ⁻¹ rhamnolipids	20.2 ± 0.4	1.5
Cells + 40 mg l ⁻¹ rhamnolipids	48.7 ± 0.9	3.6
Cells + 100 mg l ⁻¹ rhamnolipids	57.5 ± 1.6	4.3
Cells + 10 mg l ⁻¹ Tween 80	29.6 ± 0.8	2.2
Cells + 40 mg l ⁻¹ Tween 80	71.4 ± 0.8	5.3
Cells + 100 mg l ⁻¹ Tween 80	91.7 ± 1.4	6.8

872 ^a Relative fluorescence units

873 ^b The 1-NPN uptake factor was calculated as a ratio of fluorescence values of the
 874 bacterial cells in the presence and absence of surfactants

875

876 **Table 4** Effect of the rhamnolipids on toxicity (inhibition of bioluminescence of test
 877 bacteria), bioavailability and concentration of free Cd²⁺ ions in aqueous environment

Toxicity: bioluminescence inhibition test^a; 30-min EC₅₀, mg Cd l⁻¹ ± SD				
	<i>E. coli</i>	<i>B. subtilis</i>	<i>V. fischeri</i>	<i>P. fluorescens</i>
	Gram-negative	Gram-positive	Gram-negative	Gram-negative
Cd only	0.16 ± 0.06	0.49 ± 0.3	4.4 ± 0.1	0.96 ± 0.4
Cd and rhamnolipids EC ₂₀ ^b	1.34 ± 0.07	0.59 ± 0.3	10.7 ± 2.1	2.26 ± 0.7
Cd and rhamnolipids 50 mg l ⁻¹ ^c	1.7 ± 0.02	0.71 ± 0.4	20.9 ± 5.3	2.24 ± 0.9

Bioavailability: induction of Cd-induced bacteria^d; minimal inducing concentration, mg Cd l⁻¹ ±

SD		
	<i>E. coli</i>	<i>B. subtilis</i>
	Gram-negative	Gram-positive
Cd only	0.0014 ± 0.0006	0.0043 ± 0.0005
Cd and rhamnolipids 10 mg l ⁻¹	0.0025 ± 0.0013	0.0013 ± 0.0009
Cd and rhamnolipids 20 mg l ⁻¹	0.0042 ± 0.001	0.0008 ± 0.0003
Cd and rhamnolipids 40 mg l ⁻¹	0.0079 ± 0.032	not induced

Concentration of free Cd²⁺ ions: Cd selective electrode; mg Cd l⁻¹ ± SD

	11.2 mg l ⁻¹ Cd	112 mg l ⁻¹ Cd	1120 mg l ⁻¹ Cd
Cd only	12.3 ± 1.9	119 ± 10	1186 ± 42
Cd and rhamnolipids 10 mg l ⁻¹	3.6 ± 0.8	111 ± 4	1241 ± 174
Cd and rhamnolipids 20 mg l ⁻¹	1.5 ± 1.1	78 ± 2	1216 ± 68
Cd and rhamnolipids 40 mg l ⁻¹	0.6 ± 0.1	36 ± 4	1074 ± 8

878 ^a analysed with constitutively luminescent bacteria *Vibrio fischeri*, *Pseudomonas*

879 *fluorescens* OS8(pDNux), *Escherichia coli* MC1061(pDNlux), *Bacillus subtilis*

880 BR151(p602/22lux). EC₅₀ values were calculated from Figure 4

881 ^b 20 mg l⁻¹ for *B. subtilis* and *V. fischeri*, 35 mg l⁻¹ for *E. coli* and 70 mg l⁻¹ for *P.*

882 *fluorescens*

883 ^c 50 mg l⁻¹ of rhamnolipids was causing inhibition of 50% to *V. fischeri*, 37% to *E.*

884 *coli*, 23% to *B. subtilis* and 17% to *P. fluorescens*

885 ^d analysed with Cd-induced luminescent bacteria *E. coli* MC1061(pSLzntR/

886 pDNPzntAlux and *B. subtilis* BR151(pcadCPcadAlux). Minimal inducing

887 concentrations are calculated from Figure 5

888

889 **Table 5** Effect of rhamnolipids on Cd bioavailability, desorption from soil and free
 890 Cd²⁺ concentration in soil

<u>Bioavailability: induction of Cd-induced bacteria^a, minimal inducing concentration, mg</u>			
Cd l⁻¹ of soil-water suspension ± SD			
	<i>E. coli</i>	<i>B. subtilis</i>	
	Gram-negative	Gram-positive	
Cd only	0.092 ± 0.021	0.036 ± 0.01	
Cd and rhamnolipids 10 mg l ⁻¹	0.123 ± 0.008	0.39 ± 0.016	
Cd and rhamnolipids 20 mg l ⁻¹	0.189 ± 0.002	0.39 ± 0.013	
Cd and rhamnolipids 40 mg l ⁻¹	0.246 ± 0.006	0.84 ± 0.009	
<u>Concentration of free Cd²⁺ ions: Cd selective electrode^b, mg Cd²⁺ l⁻¹ of soil-water extract ±</u>			
	SD		
	Soil containing 1.5 mg kg ⁻¹ Cd	Soil containing 150 mg kg ⁻¹ Cd	Soil containing 15 000 mg kg ⁻¹ Cd
Cd only	below detection	below detection	312 ± 10
Cd and rhamnolipids 10 mg l ⁻¹	below detection	below detection	308 ± 2
Cd and rhamnolipids 20 mg l ⁻¹	below detection	below detection	270 ± 16
Cd and rhamnolipids 40 mg l ⁻¹	below detection	below detection	242 ± 38
<u>Desorption of Cd from soil: AAS^c, mg Cd l⁻¹ of soil-water extract</u>			
	Soil containing 1.5 mg kg ⁻¹ Cd	Soil containing 150 mg kg ⁻¹ Cd	Soil containing 15 000 mg kg ⁻¹ Cd
Cd only	0.0020	0.034	372
Cd and rhamnolipids 10 mg l ⁻¹	0.0025	0.037	373
Cd and rhamnolipids 20 mg l ⁻¹	0.0039	0.048	374
Cd and rhamnolipids 40 mg l ⁻¹	0.0040	0.090	374

891 ^a measured in 1:10 soil-water suspension with Cd-induced luminescent bacteria

892 *Escherichia coli* MC1061(pSLzntR/ pDNPzntAlux) and *Bacillus subtilis*

893 BR151(pcadCPcadAlux)

894 ^b measured in 1:10 soil-water extracts

895 ^c atomic absorption spectroscopy, measured in 1:10 soil-water extracts. The standard

896 deviation was less than 4%

897

898

899